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Peptide-Mediated Targeting of Angiogenesis for Molecular Imaging and Treatment of Cancer

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Graduate Program in Medical Biophysics

A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

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PEPTIDE-MEDIATED TARGETING OF ANGIOGENESIS FOR MOLECULAR IMAGING AND TREATMENT OF CANCER

(Thesis format: Integrated Article)

by

Choi-Fong Cho

Graduate Program in Medical Biophysics

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Science

The School of Graduate and Postdoctoral Studies
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London, Ontario, Canada

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THE UNIVERSITY OF WESTERN ONTARIO
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Abstract

The development of screening approaches to identify novel affinity ligands has paved the way for a new generation of molecular targeted nanomedicines. To identify novel targeting ligands, several studies have demonstrated the advantages in screening one-bead-one-compound (OBOC) libraries. Conventional methods typically bias the display of the target protein to ligands during the screening process. We have developed an unbiased multiplex ‘beads on a bead’ strategy to isolate, characterize, and validate high affinity ligands from OBOC libraries. In addition, due to the advantages associated with screening OBOC libraries directly against living cells, we sought to combine cell-based screen methods with automated high-throughput technologies to facilitate the identification of novel affinity ligands. We have shown that bound cells can be reversibly cross-linked onto the beads, and then easily sorted through automated means using the Complex Object Parametric Analyzer and Sorter (COPAS) large format flow cytometer (purchased from Union Biometrica) without affecting the sequence deconvolution of peptides using matrix-assisted laser desorption/ionization-time of flight MALDI-TOF mass spectrometry (MS). This high throughput strategy can accelerate the discovery and generation of new targeting agents.

Using the ‘beads on a bead’ approach, we have discovered novel peptides that do not contain the Arg-Gly-Asp (RGD) motif that bind αvβ3 integrin without affecting the biology of cancer or endothelial cells. The peptides identified here represent novel targeting agents for integrins that can be applied to cancer imaging without the risk of increased tumor invasion and metastasis. In order to target angiogenesis, we used the ‘beads on a bead’ strategy again to screen an OBOC library to isolate novel high-affinity peptides against EGFL7. A high-affinity peptide ligand, E7-p72 was shown to target cancer cells and endothelial cells in an EGFL7-dependent manner. This lead candidate could provide a basis for a new generation of sensitive angiogenesis targeting agents for imaging early cancers or delivery of therapeutics to disease sites.

The expansion of our understanding of EGFL7 has also led us to identify and design bioactive peptides from the sequence of EGFL7 that could interfere with its function and serve as angiogenesis inhibitors. Particularly, one peptide, E7-C13 derived from the C-terminus of
EGFL7 inhibits angiogenesis. This peptide could provide a basis for a new generation of therapeutic agents for locally advanced cancers.

**Keywords:**
One-bead-one-compound (OBOC) library, peptide, beads on a bead, $\alpha_\text{v}\beta_3$ integrin, EGFL7, angiogenesis, angiogenesis inhibitor


Co-Authorship Statement

Discovery of novel integrin ligands from combinatorial libraries using a multiplex “beads on a bead” approach

The chapter with this title is an adaptation of a manuscript that has been published in Nano Letters (ACS Publication). I designed the ‘beads on a bead’ screening approach, and performed all the proof of principle experiments to establish the strategy. I then used this method to screen an OBOC library against $\alpha_v\beta_3$ integrin. I also performed all the experiments to validate the affinity of the isolated hits with $\alpha_v\beta_3$-expressing cells. I then isolated the high-binding hits and validated the affinity of each peptide to $\alpha_v\beta_3$ integrin through surface plasmon resonance (SPR). I also analyzed the sequences for conserved motifs, and performed all the cellular uptake experiments. I also conducted all the experiments to show that our novel $\alpha_v\beta_3$-targeting peptides do not alter the biology of cells. I wrote the manuscript with the help of Dr. John Lewis. Dr. John Lewis was also heavily involved in the study design and data analysis. Dr. Giulio Amadei synthesized the OBOC peptide library and sequenced all the peptides that were isolated from the screen. Dan Breadner synthesized and purified the peptides for further characterization. Dr. Leonard Luyt was involved in the study design and manuscript revision.

A rapid and efficient cell-based approach to screen a one-bead-one-compound library for novel ligands against cell surface receptors

This chapter is an adaptation of a manuscript that we intend to submit to ACS Combinatorial Science. I performed the cell-based screen and sorted the hits using the COPAS large format biosorter. Dr. Babak Azad performed all the sequence deconvolution. Dr. John Lewis and Dr. Leonard Luyt was involved in the study design and manuscript revision.
Novel peptide ligand against EGFL7, E7-p72 as a tool for imaging early cancers and angiogenesis

This chapter is an adaptation of a manuscript that we intend to submit to Nature Nanotechnology. I performed all the experiments to express and purify EGFL7 from the baculovirus expression system. I then screened an OBOC library against EGFL7 using the ‘beads on a bead’ method and isolated the high-binding hits using a magnet. I performed all the experiments to validate the affinity of the isolated hits with EGFL7-expressing cells. After that, I isolated the high-binding hits and validated the affinity of each peptide to purified EGFL7 using SPR. I performed all the cellular uptake and flow cytometry experiments. I wrote this manuscript. Dr. John Lewis was also heavily involved in the study design and data analysis. Dr. Giulio Amadei sequenced the peptides that were isolated from the screen. Dr. Tienabe Nsiama and Andre St. Amant synthesized and purified the peptides for this project. Dr. Leonard Luyt was involved in the study design.

EGFL7 peptide, E7-C13 for modulating angiogenesis

This chapter is an adaptation of a manuscript that we intend to submit to Cancer Research. Dr. John Lewis and I designed the peptides from the EGFL7 sequence. I performed the in vitro angiogenesis assay by monitoring HUVECs morphogenesis on matrigel. I also used the avian chorioallantoic membrane (CAM) on-plant assay to evaluate angiogenesis level in vivo. I performed the serum stability study and the cell adhesion assay. I am also conducting all the experiments to elucidate the mechanism in which E7-C13 undertakes, which includes monitoring the changes of an array of receptor tyrosine kinase (RTK) activity upon peptide treatment. I performed all the data analysis and wrote this manuscript. Dr. John Lewis contributed to study design and data analysis. Dr. Daniela Quail and Dr. Lynne Postovit performed the directed in vivo angiogenesis assay in mice. Dr. Tienabe Nsiama and Andre St. Amant synthesized and purified the peptides for this project.
Acknowledgement

I am genuinely grateful to my supervisor Dr. John Lewis for providing me with this wonderful opportunity to work in his laboratory that is driven by such a high level of research. I was provided with an excellent source of multidisciplinary training, and state-of-the-art facility at the London Regional Cancer Program (LRCP), and at the University of Alberta. Over the years, you have been a valuable mentor that has taught me many important lessons in the laboratory and in life. With all your support, guidance and encouragement, I have learned to become not only a better researcher, but also a better person. I would also like to thank my committee members, Dr. Ann Chambers and Dr. Paula Foster for their continuous help, constant support and invaluable advice through my entire PhD program. I also thank the Luyt laboratory (Dr. Leonard Luyt, Dr. Tienabe Nsiama, Dr. Babak Azad, Dr. Giulio Amadei and Andre St. Amant), as well as Dr. Daniela Quail and Dr. Lynne Postovit for their invaluable help and contribution to my research.

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Finally, I would like to thank my friends for their never-ending encouragement. Also, to Tracy and Dietmar Schlodder, thank you for providing me with such warm care in your lovely home while I am in Edmonton. Most importantly, I would like to thank my family: Mom and Dad, my amazing sisters Mun Cho and Yan Cho, and the love of my life, Francis Wong, for always believing in me and never giving up on me. I would never have made it without you.

Thank you
This dissertation is dedicated to my
Mother and Father

Yueh Lean Chow and Heng Yuen Cho
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<th>Definition</th>
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<tbody>
<tr>
<td>Ac₂O</td>
<td>Anhydride acetic</td>
</tr>
<tr>
<td>AF647</td>
<td>Alexa Fluor 647</td>
</tr>
<tr>
<td>AGD</td>
<td>Alanine-glycine-aspartic acid</td>
</tr>
<tr>
<td>Ahx</td>
<td>Amino-hexanoic acid</td>
</tr>
<tr>
<td>Ala</td>
<td>Alanine</td>
</tr>
<tr>
<td>ALK</td>
<td>Anaplastic lymphoma kinase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>Asn</td>
<td>Asparagine</td>
</tr>
<tr>
<td>Asp</td>
<td>Aspartic acid</td>
</tr>
<tr>
<td>αvβ3</td>
<td>Alpha v beta 3</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>BME</td>
<td>Basement membrane extract</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>Calcium chloride</td>
</tr>
<tr>
<td>CAM</td>
<td>Chorioallantoic membrane</td>
</tr>
<tr>
<td>CPMV</td>
<td>Cowpea mosaic virus</td>
</tr>
<tr>
<td>Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>Daa</td>
<td>D-amino acid</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DIPEA</td>
<td>N,N-Diisopropylethylamine</td>
</tr>
<tr>
<td>DIVAA</td>
<td>Directed in vivo angiogenesis assay</td>
</tr>
<tr>
<td>Dll-4</td>
<td>Delta-like 4</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
</tbody>
</table>
LOX        Lysyl oxidase
Lys        Lysine
MALDI      Matrix-assisted laser desorption/ionization
Met        Methionine
MgCl2      Magnesium chloride
MRI        Magnetic resonance imaging
MS         Mass spectrometry
MTBE       Methyl tert-butyl ether
Na2HPO4    Disodium hydrogen phosphate
NHS        N-Hydroxysuccinimide
NP40       Nonidet P-40
OBOC       One-bead-one-compound
PBS        Phosphate buffer saline
PEG        Polyethylene glycol
PET        Positron emission tomography
Phe        Phenylalanine
Pro        Proline
PVDF       Polyvinylidene fluoride
RES        Reticuloendothelial system
RGD        Arginine-glycine-aspartic acid
RHAMM      Receptor for hyaluronic acid mediated motility
RNA        Ribonucleic acid
RTK        Receptor tyrosine kinase
SDS PAGE   Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser        Serine
siRNA      Small interfering RNA
SPECT      Single-photon emission computed tomography
SPR        Surface plasmon resonance
tdT        td-Tomato
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>TEV</td>
<td>Tobacco etch virus</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>Thr</td>
<td>Threonine</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TOF</td>
<td>Time of flight</td>
</tr>
<tr>
<td>Trp</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>Tyr</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>Val</td>
<td>Valine</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>WGA</td>
<td>Wheat germ agglutinin</td>
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</table>
Chapter 1

Introduction

1.1 Advancement of nanotechnology

Although there has been a tremendous progress in our understanding of the basics of cancer biology, this advancement has not translated comparably in the clinic. This discrepancy may be due to the current inadequacies in the ability to selectively delivery diagnostic probes or cancer therapeutics to desired disease sites without affecting surrounding healthy tissues or organs. Ligand-mediated targeting has advanced the field of nanotechnology by enabling the delivery of high concentration of contrast agents to disease sites for molecular imaging, leading to the generation of high-contrast images with reduced noise levels to facilitate early diagnosis and monitoring of disease progression (as reviewed in (1)). Similarly, ligand-mediated targeting of nanomedicines has driven exciting new applications in cancer therapy. A major challenge that is facing cancer therapy is that common cancer chemotherapeutics currently used in the clinic including doxorubicin, cyclophosphamide and paclitaxel have little selectivity for cancer cells. As these drugs attack cells with high proliferation rates, treatment can lead to increased toxicities against normal tissues with high proliferation rates, such as the bone marrow and hair follicles (as reviewed in (2)). Ligand-targeted therapeutics are developed to delivery of high concentration of drugs directly to cancer cells that uniquely express specific antigens or receptors, thereby increasing the efficacy of therapy with reduced adverse side effects. The advancement in nanotechnology has also given rise to the development of nanotheranostics, multifunctional platforms that were designed to simultaneously diagnose and treat cancer (as reviewed in (3)). Nanocarriers usually comprise of three constitutions – a core material, an imaging agent and/or therapeutic payload, and a biological surface modifiers (Figure 1.1). Permeation enhancers can also be incorporated into the nanocarrier to transiently increase the penetration characteristics of tissues using substances such as surfactants and calcium chelators to enhance delivery of imaging or therapeutic agents into the target cells (4) (Figure 1.1).
Nanocarriers also protect small peptides from enzymatic degradation, leading to prolonged circulatory time.

**Figure 1.1 Multifunctional nanocarriers.** A nanoparticle has the ability perform the following function: 1) Carry more than one therapeutic agent, 2) biomolecular targeting through antibodies or other recognition agents, 3) imaging by conjugation or co-encapsulation of contrast agents, and 4) biobarrier avoidance through permeation enhancers (to open endothelial tight junctions) and polyethylene glycol (PEG) (to avoid macrophage uptake). (This figure was reproduced from (I)).
1.2 Ligand mediated targeting

1.2.1 Peptide-mediated targeting

The development of affinity probes for ligand-mediated targeting has advanced the field of molecular imaging (5-9), and directed therapy of disease (as reviewed in (10)). Affinity probes that have been approved for clinical use include several anti-tumor monoclonal antibodies (or antibody fragments), and non-antibody ligands (as reviewed in (2)). Antibody engineering and phage display technologies have led to the generation of monoclonal antibodies or antibody fragments with high degree of specificity for the target tissue (11)(12). Despite the fact that monoclonal antibodies have demonstrated high success in the clinic for targeting tumors (13), their major limitations, including immunogenicity (14), large sizes, and their non-specific uptake by the reticuloendothelial system (RES) (ie. liver or spleen) make them less compelling for inpatient use. Peptide-based affinity probes serve as an attractive alternative, as they are significantly smaller, easy to synthesize, have better diffusion and tissue penetration, demonstrate improved pharmacokinetics, and reduced interaction with the RES (15).

1.2.2 One-bead-one-compound (OBOC) combinatorial library

Despite several studies demonstrating the success of isolating therapeutic peptides from phage display peptide libraries (as reviewed in (15)), we chose to exploit the synthetic nature of one-bead-one-compound (OBOC) libraries for their ability to incorporate D-amino acids and other non-natural amino acids, as well as specific secondary structures and other macromolecular components (including nucleotides, lipids) that may enhance biological activity or confer enzymatic degradation (as reviewed in (6)). OBOC combinatorial peptide libraries comprise of 80–100 µm beads. Amino-derivatized beads (such as TentaGel S resin) are used as the solid support and underwent several rounds of “split-mix” synthesis (as described in Figure 1.2), resulting in the display of only one chemical entity on each bead (16). This synthesis is extremely easy and efficient, since thousands to billions of peptides or small molecules can be prepared in less than a week. The library is screened against a biological target for the isolation
of affinity probes or bioactive ligands using a number of different assays (as reviewed in (17)). Positive hit beads containing high affinity ligand for the target protein can then be isolated for sequencing.

Figure 1.2. Schematic of the “split-mix” synthesis strategy. Generation a one-bead-one-compound (OBOC) combinatorial peptide library. (This figure was adapted from (18)).

This dissertation describes the strategies that we have designed and utilized to rapidly and accurately discover novel peptides for the development of new molecular imaging agents. As metastasis contributes to greater than 90% of cancer mortalities, non-invasive detection of angiogenic factors expressed during the earliest stages of tumor development can aid in the diagnosis of neoplasms before they metastasize. My research goals involve screening OBOC
combinatorial peptide libraries against different cell surface proteins that are expressed during the earliest stages of cancer development.

Most high-throughput strategies to screen OBOC libraries utilize tagged or fluorescently labeled soluble biological targets (19-21). Although these techniques have been proven successful, soluble target proteins need to be purified and derivatized prior to screening, which could potentially impair the conformation and function of the native target protein (22). In addition, proteins displayed on cellular surface, or proteins that formed complexes with other cell surface proteins may have several unexposed epitopes, allowing only certain sites of the protein to be available for targeting. These limitations highlight the attractiveness of using cell-based assays to screen OBOC libraries (as reviewed in (6)). Several studies have demonstrated much success in using whole cell binding assays to screen OBOC libraries to isolate ligands specific to cell surface receptors of numerous human cancer cell lines (23), i.e. Jurkat T-leukemia cells (24), T-lymphoma cell (25), and breast cancer cells (26). Nonetheless, conventional methods for isolating few positive hits from billions of OBOC library beads through manual techniques remain impractical and challenging. The COPAS (Complex Object Parametric Analyzer and Sorter) large particle biosorter (purchased from Union Biometrica) carries great potential for high-throughput and rapid isolation of surface binding ligands from a random OBOC library. However, the biggest challenge associated with this strategy is the dissociation of cells from the beads upon flowing them through the tubes of the instrument. Currently, there have been no studies to combine on-bead cell-based screening approaches with automated sorting.

1.2.3 Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS)

Peptide sequences that are isolated from OBOC libraries can be determined through either Edman degradation (27) or mass spectrometry (MS) techniques (ESI (28) and MALDI (29, 30)). Edman degradation is a sequencing method that involves the labeling of the amino-terminal residue with phenylisothiocyanate under mildly basic conditions. Under acidic conditions, the amino-terminal residue is cleaved as a thiazolinone derivative, which can be extracted, stabilized and identified using chromatography or electrophoresis approaches (27). While Edman
degradation is a widely used sequencing approach, the high cost per sample of this technique hinders its application in sequencing large amount of samples from a library screen. MS is a sequencing technique that measures the mass-to-charge ratio of an ionized molecule (as reviewed in (31). MALDI-MS is typically utilized for analysis of relatively simple peptides, while ESI is more commonly used for analysis of more complex samples. MALDI is often coupled to TOF to measure the mass of intact peptides by measuring the time it takes for an ionized fragment to reach a detector at a known distance (as reviewed in (31, 32). A major disadvantage associated with MS approaches is that they are intrinsically non high-throughput techniques due to the need of a two-step process: cleavage of the peptide from the resin, followed by MS analysis. We have previously reported a high-throughput, inexpensive and reproducible peptide sequencing approach using a MALDI-TOF/TOF instrument to combine photochemical cleavage of peptide from its solid support, its desorption-ionization, and generation of its fragmentation patterns for full sequence deconvolution all in one step (33). This analytical tool that we have established aided in the determination of small peptides without the need for complex derivatization or labeling, and is continuously utilized to facilitate the discovery of novel affinity peptide ligands for targeting cancers (34).

1.3 Overview of angiogenesis

Blood vessels form very extensive and complex networks that are essential for the transport of gasses, fluid, nutrients and circulating cells between tissues and organs. In cancer, capillaries sprout and branch into larger and more complex networks to increase blood transport into growing tissues through a process known as angiogenesis (as reviewed in (35)). This remodeling process is most common during early embryogenesis, and is also important for the growth of blood vessels during physiological angiogenesis (ie. during wound healing) and pathological angiogenesis (ie. during tumor development) in adults.

Angiogenesis is tightly regulated by several signaling molecules including the vascular endothelial growth factor (VEGF) (as reviewed in (36)) (Figure 1.3). The composition of the extracellular matrix (ECM) around the vasculature also plays a role in regulating angiogenesis through the release of VEGF isoforms, as well as the modulation of physical contact between
endothelial cells and pericytes (Figure 1.3) (37). During angiogenesis, some endothelial cells that are selected for sprouting, known as tip cells, lead the growing sprout towards gradients of VEGFA (Figure 1.3). Sprout extension can be promoted via the migration of endothelial cells behind the tip, or proliferation of local endothelial cells (38) (39). Inhibition of Notch signaling, or inactivation of its ligand delta-like 4 (Dll-4) promote increased the formation of tip cells, leading to enhanced sprouting and branching of endothelial tubes (40)(41)(42)(43). The expression of Dll-4 is induced in response to VEGFA, while Notch activation suppresses the expression of VEGFR-2, indicating that Dll-4 acts as a negative feedback regulator to prevent overexuberant angiogenic sprouting (41)(43, 44). It is also possible that Notch activation in neighboring endothelial cells inhibit pro-angiogenic responses (Figure 1.3).

Endothelial sprouts undergo many further steps to transform into functional blood vessels. In order to establish blood flow, vascular lumen is required to form. This process could take place in growing endothelial cells before or after merge with other vessels. Although the exact mechanism of this process is still unclear, there is strong evidence suggesting that the lumen of endothelial cells is formed by intracellular, and subsequently intercellular fusion of large vacuoles within the vessels (45). The generation of a lumen permits blood flow and improves oxygen delivery, thereby reducing the level of hypoxia-inducible factors (HIFs), leading to a lower of local VEGFA concentration. This process, along with the recruitment of pericyte and reduced endothelial cell proliferation promotes vessel maturation and quiescence (Figure 1.3).
Figure 1.3. Molecular regulation of angiogenesis. (a) Angiogenic sprouting is modulated by the balance of pro-angiogenesis signals (such as vascular endothelial growth factor (VEGF)), and factors that promote quiescence (such as tight pericyte contact, specific extracellular matrix (ECM) molecules or VEGF inhibitors). Under favorable conditions, some endothelial cells sprout (green), while others do not respond (grey). (b) Endothelial cells sprout toward VEGF gradients. Other signals include attractive or repulsive matrix cues, as well as cells in the environment controls the growth of the sprout. (c) Fusion of adjacent sprouts through adhesive interactions when tip cells encounter each other. (d) Fusion of endothelial cells to establish a continuous lumen. Hypoxia-induced pro-angiogenic signals are reduced as a result of improved oxygen delivery. Maturation processes such as junction stabilization, matrix deposition and pericyte attachment also take place. (This figure was adapted from (27)).

1.3.1 Tumor angiogenesis

During pathological situations such as cancer, angiogenesis signaling pathways are induced to provide the growing tumor with blood (46). In order to stimulate angiogenesis, tumor
cells tilt the balance towards pro-angiogenic factors to induce vascular sprouting by attracting cells within the tumor microenvironment (as reviewed in (47). For endothelial cells to adopt an activated phenotype, a variety of angiogenesis factors induce the detachment of endothelial cells, followed by sprouting and tube formation toward gradients of pro-angiogenic factors, leading to perivascular cells recruitment to provide vessel maturation, and ultimately, vascular remodeling to form a fully functional network (as reviewed in (48)).

In cancer, multiple pathways are involved in regulating angiogenesis in order to promote disease progression. Once tumor growth exceeds a few millimeters in size, oxygen and nutrient deprivation induces the ‘angiogenic switch’ to enable the tumor to progress. Tumor cells release growth factors (such as VEGF) into the microenvironment to activate quiescent cells around them to become angiogenic. Although this process initially supports the growth of the tumor, the continuously remodeled tumor vessels are leaky and tortuous, leading to irregular blood flow and ultimately, poor tumor response (as reviewed in (49). The sustained release of platelet-derived growth factor (PDGF) contributes to the recruitment of perivascular cells. Although perivascular coverage plays an important role in vascular maturation and stability, it does not lead to the production of fully functional vessels, as these vessels are constantly exposed to ceaseless stimulatory signals within the tumor microenvironment (as reviewed in (50)).

In response to hypoxia, the tumor releases a variety of factors that affect their microenvironment through ECM remodeling in order to accommodate tumor growth and progression. Integrins and receptors that are present on the surface of endothelial cells recognize and bind these factors to initiate signaling pathways that lead to migration, invasion, and proliferation that are important for sprouting. Integrins are the main cell-adhesion molecules that are responsible for transducing information from the ECM by triggering intracellular signaling pathways (51). Upon binding to a specific matrix protein, signaling mechanisms are used to determine whether a particular microenvironment is suitable for survival, migration and invasion (as reviewed in (47)).
1.4 The role of $\alpha_v\beta_3$ integrin in tumor angiogenesis

$\alpha_v\beta_3$ integrin consists of a 145 kDa $\alpha$ and a 92 kDa $\beta_3$ subunit. $\alpha_v\beta_3$ integrin interacts specifically with numerous ECM proteins (including vitronectin, fibronectin and fibrinogen) through an Arg-Gly-Asp (RGD) tri-peptide motif (52, 53). $\alpha_v\beta_3$ integrin is most highly expressed on angiogenic blood vessels and in remodeling and pathological tissues (47, 54) (Figure 1.4). This expression pattern allows for the development of multiple strategies to detect, monitor and treat angiogenesis-related pathologies. Multiple strategies have used the RGD-targeted nanoparticles to specifically deliver imaging and therapeutic agents to tumor-associated blood vessels (55). Targeted delivery of anti-angiogenic agents to tumor-associated vessels leads to regression of the endothelial cells and reduction of established primary and metastatic tumors (56). The inhibition of $\alpha_v\beta_3$ integrin function using monoclonal antibodies, RGD peptides and peptidomimetics has been shown to hamper angiogenesis and promote endothelial cell apoptosis (as reviewed in (57). Due to the fact that $\alpha_v\beta_3$ integrin expression is a general feature of endothelial cell activation, and not exclusive to angiogenic vessels associated with cancer, treatments that exploit $\alpha_v\beta_3$ integrin inhibition also affect other processes such as wound repair (58) and arthritis (59).
Figure 1.4. The expression of $\alpha_v$ integrins on various cell types and their contribution to angiogenesis and tumor progression. Endothelial cells undergoing sprouting angiogenesis express unique integrin profiles, which can be targeted for imaging and drug delivery. Tumor cells alter their integrin expression profile to facilitate migration, invasion, metastasis and survival in hostile environments. Integrin signaling on fibroblast cells leads to the production of ECM proteins and growth factors that fills the tumor stroma. Proteolytic degradation of ECM proteins leads to the generation of fragments that can bind and inhibit the function of integrins that are present on angiogenic endothelial cells. (This figure was adapted from (47)).
1.5 Epidermal growth factor-like domain 7 (EGFL7)

1.5.1 Structure of EGFL7

My research also focuses on the epidermal growth factor-like domain 7 (EGFL7), a ~30 kDa protein that is expressed at very low levels in quiescent endothelium, but is dramatically up-regulated upon onset of physiological angiogenesis (ie. during embryogenesis, wound healing and in the uterus during pregnancy) (60) and pathological angiogenesis upon arterial injury (60), hypoxia (61), and in solid tumors (62). EGFL7 is also expressed in neurons of adult mice and primordial germ cells (63) (64). EGFL7 expression correlates with poor prognosis in malignant glioma, hepatocellular carcinoma and non-small cell lung cancer (65)(66)(67). EGFL7 contains an N-terminal signal secretion peptide, followed by an Emilin (EMI) domain and two EGF-like repeats (68)(62) (as depicted in Figure 1.5). The presence of these domains indicates that EGFL7 is a secreted protein that forms multimeric complexes and incorporates into the ECM through protein-protein interactions (69)(70). EGFL7 is found to mainly localize to the ECM of cells (71), making it an ideal imaging biomarker for clinically relevant neoplasms and metastases.

Figure 1.5. Structural organization of EGFL7. EGFL7 contains an N-terminal signal secretion peptide, an EMI domain, two EGF-like repeats and a C-terminal coiled-coil structure. (This figure was adapted from (72)).
1.5.2 Function of EGFL7

Several recent studies reveal that EGFL7 controls vasculogenesis (embryonic formation and differentiation of the vascular system) and angiogenesis (formation of new blood vessels from pre-existing vessels) by creating a permissive environment for angiogenesis (62)(60)(71)(73). Although the exact mechanism of EGFL7 is not known, it is postulated that EGFL7 functions to promote the proliferation, sprouting, migration and invasion of endothelial cell (74) (Figure 1.6a). EGFL7 knockdown in human primary endothelial cells leads to the inhibition of endothelial cell proliferation, migration and sprouting (75). Additionally, EGFL7 serves as a chemoattractant for endothelial cells, and promotes endothelial cell adhesion (62)(60). It may also be possible that EGFL7 remodels the ECM to promote endothelial cell migration and invasion. Because the expression of EGFL7 is localized to nascent endothelial sprouts, it is possible that EGFL7 is secreted by sprouting vessels and deposited in the ECM, thereby modulating its rigidity through the inhibition of lysyl oxidase (LOX) activity (Figure 1.6b). EGFL7 has also been implicated to modulate angiogenesis through inhibition of the Notch signalling pathway (64)(75). Altogether, it appears that EGFL7 plays a crucial role in angiogenic growth by affecting the property of endothelial cells and signaling to the ECM environment. Altered levels of EGFL7 leads to abnormal blood vessel patterning and remodeling (as reviewed in (74)).
Figure 1.6. Models showing the role of EGFL7 in angiogenesis. EGFL7 is a protein that is secreted by sprouting vessels, and then deposited onto the ECM. (a) EGFL7 regulates endothelial cell sprouting, migration and proliferation. (b) EGFL7 modulates the rigidity of ECM by inhibiting LOX-mediated conversion of tropoelastin into elastin. (This figure was adapted from (74)).
1.6 Hypotheses

1. A rapid and unbiased approach to screen OBOC libraries should facilitate the isolation of novel non-canonical peptides against key targets in cancer. We hypothesize that non-RGD peptides that target the αvβ3 integrin without affecting cancer and endothelial cell biology will facilitate the development of nanomedicines without risking increased tumor invasion or metastasis.

2. Combining cell-based screening of OBOC libraries with high-throughput automated sorting of high-affinity ligands can accelerate the discovery novel targeting probes. We hypothesize that cross-linking of cells onto bead containing high affinity peptide through chemical fixation will prevent cells from detaching during automated sorting using the COPAS large format flow cytometer, and that this cross-linking will not affect the sequence deconvolution of peptides through MALDI-TOF/TOF mass spectrometry.

3. A comprehensive analysis of tumor vasculature in prostate cancers have shown that men with the most irregularly shaped vessels are 17.1 times more likely to develop lethal disease. These vessels specifically express EGFL7. We hypothesize that molecular imaging using peptides targeted to EGFL7 will allow for non-invasive detection of early cancers and distant metastases.

4. EGFL7 is an important regulator of angiogenesis. We hypothesize that bioactive peptides derived from the EGFL7 sequence will interfere with function of EGFL7 and lead to the inhibition of angiogenesis.
References


Chapter 2

The discovery of novel integrin ligands from combinatorial libraries using a multiplex 'beads on a bead' approach

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2.1 Abstract

The development of screening approaches to identify novel affinity ligands has paved the way for a new generation of molecular targeted diagnostics and therapeutics. However, conventional high-throughput methods often involve the immobilization of the target protein in a specific orientation, thus biasing its display and surface exposure to ligands during the screening process. To address this, we have developed an unbiased multiplex ‘beads on a bead’ strategy to isolate, characterize and validate high affinity ligands from one-bead one-compound (OBOC) libraries. This scalable approach enriches for non-canonical ligands and incorporates multiple selection and validation parameters, including on-bead binding validation. We report the discovery of novel peptide ligands that target $\alpha_v\beta_3$ but do not contain the RGD motif. We demonstrate that, in contrast to RGD, they do not affect cancer or endothelial cell biology. This strategy is suitable for both laboratory-based benchtop or large scale high throughput approaches. The peptides identified here represent novel targeting agents for integrins that can be applied to cancer imaging or treatment without the risk of increased tumor invasion and metastasis.
2.2 Introduction

The development of affinity probes for ligand-mediated molecular targeting has driven exciting new applications in molecular imaging (1-6), and directed therapy of disease (7). Recent advances in biomedical nanotechnology combine targeting molecules with therapeutic and/or imaging agents within a single entity, enabling their delivery directly to disease sites while avoiding normal tissues. This can improve imaging performance and potentially reduce the dangerous side effects of treatment. Affinity probes that have been approved for clinical use include several anti-tumor monoclonal antibodies such as trastuzumab (for treatment of breast cancer) (8) and bevacizumab (for inhibition of angiogenesis) (9). Despite success in the clinic, antibodies have several limitations that preclude their broad use, including immunogenicity (10), size-related tissue diffusion characteristics (11), and their non-specific uptake by the reticuloendothelial system (RES). Furthermore, their size and complexity makes their incorporation into chimeric drugs or multifunctional nanomedicines challenging. For this reason, peptide-based affinity probes provide a compelling alternative. Compared to antibodies, peptides are significantly smaller, resulting in improved diffusion and tissue penetration, demonstrate improved pharmacokinetics, and reduced interaction with the RES (12). Furthermore, the synthesis and production of peptides is straightforward and they can be engineered for improved stability and reduced immunogenicity. Peptide-based therapeutics are in extensive clinical use, and affinity peptides such as bombesin (13) and cyclic RGD peptides (14) have been successfully utilized as cancer homing agents.

The discovery of novel peptide affinity probes has been facilitated by combinatorial library screening methods that have been developed in the last 15 years. Phage display libraries are comprised of expressed peptides on the surface of filamentous phage (15, 16). This biological approach, however, allows for the display of only L-amino acid peptides, which are susceptible to proteolytic degradation. Similarly, it is not possible to incorporate cyclic or branched structures using phage display methods (3). In contrast, 'one-bead one-compound' (OBOC) combinatorial peptide libraries are comprised of random peptides anchored to TentaGel resin beads that are generated using chemical synthesis (17). The synthetic nature of OBOC libraries allows for the incorporation of unnatural amino acids such as D-amino acids, and enables the
investigation of cyclic, turned or branched peptides which can confer enhanced resistance to proteolytic degradation, a key requirement for clinical applications.

The OBOC combinatorial library approach has been utilized to discover novel peptide ligands for numerous human cancer targets, yet conventional strategies to manually isolate bead hits from the millions of library beads are impractical, labor intensive and time consuming (18, 19). While a number of recent advancements have partially addressed this limitation (20, 21), typical screening approaches utilize a biased display of the target protein, whereby all surfaces of the protein are not equally exposed to the ligands on the library beads. A scalable and unbiased approach to screen OBOC libraries should facilitate the isolation of novel non-canonical peptides against key targets in cancer and other diseases.

To address these limitations, we developed a multiplex ‘beads on a bead’ combinatorial library screening approach that incorporates several important innovations that facilitates the straightforward identification of peptide affinity ligands for non-dominant epitopes. These include unbiased target protein display, iterative library bead separation by both magnetic and fluorescence sorting and cell-based on-bead binding validation. Incorporation of a photo-cleavable linker into the library design enables on-bead MALDI mass spectrometric sequence determination, making this approach amenable to high throughput or laboratory bench top ligand discovery. To evaluate the effectiveness of this approach, we performed a screen for novel peptides targeting $\alpha_v\beta_3$ integrin.

Integrin $\alpha_v\beta_3$ is a receptor for extracellular matrix proteins such as vitronectin and fibronectin that contain arginine-glycine-aspartic acid (RGD) tripeptide sequences (22). Because $\alpha_v\beta_3$ integrin is over-expressed on angiogenic endothelial cells and tumors, peptides incorporating RGD have been widely used in the design of imaging agents to visualize tumors (14), as well as a targeting moiety for cancer therapeutics (23). Radiolabeled RGD peptide has been successfully implemented in the clinic to detect lesions in cancer patients (24). Nonetheless, several recent studies have raised concerns regarding the use of RGD due to its effect on the biology of tumors (25). Cancer cells treated with RGD-labeled iron oxide nanoparticles lose intercellular contacts and have decreased cell adhesion (25). RGD-mimetic peptide and RGD-mimetic small molecules inhibit angiogenesis (26, 27) and are currently in clinical trials for
cancer therapy. However, recent revelations suggest that the inhibition of angiogenesis is a

driving force in tumor progression, which ultimately leads to increased tumor aggressiveness,

invasiveness (28) and micrometastases (29). Therefore, it would be prudent to identify new non-

RGD peptides for imaging and drug delivery that effectively target \( \alpha_\varepsilon \beta_3 \) without negatively

affecting the biology of the tumor.

We demonstrate here the utility of the multiplex ‘beads on a bead’ screening strategy to

screen an OBOC peptide library for non-canonical \( \alpha_\varepsilon \beta_3 \) targeting peptides. Through the

incorporation of unbiased target protein display, sequential bulk and high-throughput separation,

cell-based validation and on-bead peptide deconvolution, this approach facilitated the isolation of

novel non-RGD peptide ligands that target the \( \alpha_\varepsilon \beta_3 \) integrin without impacting the biology of the

cells that express it.
2.3 Methodology

Synthesis of peptides on TentaGel beads

For proof of principle experiments, H-GRGDS-ANP-TentaGel, H-GKGDS-ANP-TentaGel, and H-GAGDS-ANP-TentaGel pentapeptides were synthesized on a parallel automated synthesizer APEX 396 (AdvancedChemTech) using standard Fmoc chemistry. A photocleavable linker, N-Fmoc-3-amino-3-(2-nitrophenyl) propionic acid [Fmoc-ANP], was first attached onto TentaGel S –NH₂ resin (0.29 mmol/g; 90 µm) as per Brown and co-workers (30). Fmoc amine protecting groups were removed by treatment with 20% piperidine in DMF (2 cycles) prior to coupling cycles. Amino acid couplings were carried out in DMF with 4 eq. Fmoc-protected amino acid, 4 eq. HBTU and 8 eq. DIPEA. Successful coupling was identified by a negative Kaiser test (31), confirming the absence of residual free primary amino groups. Between deprotection and coupling steps, the resin was thoroughly washed with DMF (5 cycles). The removal of all side chain protecting groups from the synthesized peptides was accomplished by treatment with cleavage cocktail: TFA:PhOH:H₂O:TIS (87.5:5.0:5.0:2.5) for 2 h at room temperature (22 °C), under constant shaking (800-1000 rpm, IKA-VIBRAMAX-VXR).

OBOC peptide library synthesis

A one-bead one-compound (OBOC) combinatorial library was synthesized using the Fmoc-ANP loaded TentaGel S –NH₂ resin, as previously described. The resin was divided equally into 19 wells of the automated synthesizer. For each well, Fmoc deprotection was carried out, then a single amino acid was coupled to the resin, following the protocols outlined previously. The following L-amino acids were included in the creation of the OBOC library: Fmoc-Ala-OH; Fmoc-Arg(Pbf)-OH; Fmoc-Asn-OH; Fmoc-Asp(tBu)-OH; Fmoc-Glu(tBu)-OH; Fmoc-Gln-OH; Fmoc-Gly-OH; Fmoc-His(Trt)-OH; Fmoc-Ile-OH; Fmoc-Leu-OH; Fmoc-Lys(Boc)-OH; Fmoc-Met-OH; Fmoc-Phe-OH; Fmoc-Pro-OH; Fmoc-Ser(tBu)-OH; Fmoc-Thr(tBu)-OH; Fmoc-Trp(Boc)-OH; Fmoc-Tyr(tBu)-OH; Fmoc-Val-OH. After the coupling was
complete, the resin was removed from all wells, thoroughly mixed and re-distributed into the 19 wells. Thus, split-and-mix synthesis resulted in a random OBOC octapeptide library. Removal of all permanent protecting groups was accomplished using trifluoroacetic acid and scavengers as previously described. All synthetic steps were carried out in the dark.

**Synthesis of Fluorescein-Labeled Peptides**

Fluorescein-labeled peptides were prepared following the general protocols outlines previously. Rink amide resin was used with Cys as the C-terminal amino acid, followed by the addition of Fmoc-Ahx-OH (amino-hexanoic acid) as a linker, followed by the amino acids for the sequence of either LCE62 or LCE64. Peptides were cleaved using cleavage cocktail comprised of TFA:EDT:TIS (95:2.5:2.5) and precipitated with cold t-butyl methyl ether. Peptides were dye-labeled using maleimide-fluorescein, purified by preparative HPLC (RP-C18) and characterized by ESI+-MS: FITC-LCE62 Calc. [M+2H]^{2+} 808.4, found 808.4, purity >90%; FITC-LCE64 Calc. [M+2H]^{2+} 801.4, found 801.5, purity >95%.

**Generation of α,β₃ integrin-coated magnetic/fluorescent screening beads**

Purified α,β₃ integrin (0.26 µg/µl) were biotinylated using the N-hydroxy succinimide biotin (Pierce, Rockford, USA) via the succinimide ester reaction. Biotinylation was performed using a 100-fold molar excess of biotinylation reagent and incubated on ice for 2 h. Excess biotinylation reagent was removed by gel filtration purification using G25-sepharose columns. The concentration of purified integrin was determined using the Bradford assay and the efficiency of biotinylation was determined via Western blot analysis.

Two-micron streptavidin-coated rhodamine magnetic beads (Spherotech), hereafter referred to as "screening beads", were washed with phosphate buffered saline (PBS) secured using a strong neodymium magnet (K&J Magnetics, Pennsylvania, USA) to remove sodium azide. 50 µg of the magnetic beads (binding capacity 0.95 nmol/mg) were incubated with 2.3 µg of biotinylated α,β₃ integrin for 30 mins at 25 °C. 20 µg of biotin were added into the solution of
α,β3-magnetic beads and incubated for 30 mins to block any remaining streptavidin sites. The α,β3-magnetic beads were washed three times in PBS secured using a magnet. The extent of biotinylated α,β3 incorporation on the magnetic beads was evaluated by Western blot analysis. Magnetic beads coated with α,β3 integrin were added into NuPAGE LDS containing 50 mM DTT and boiled for 10 mins. The sample was loaded onto a NuPAGE Novex Bis-Tris Gel (Invitrogen, Burlington, Canada) and transferred onto a PVDF membrane for Western blot analysis using streptavidin-HRP (Invitrogen, Burlington, Canada).

**Confirmation of binding of α,β3 integrin to TentaGel library beads**

To confirm the binding properties of purified α,β3 integrin to control peptides on library beads, approximately 50 TentaGel beads coated with RGD, KGD or AGD peptides were suspended in 20 µl of binding buffer (25 mM Tris-HCl, 145 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 0.02 % Triton-X 100, pH 7.4). 0.13 µg of free α,β3 integrin was added into the bead suspension and incubated for 1 h at 25 °C. The beads were then washed three times in binding buffer, and blocked in 5 % BSA in binding buffer for 2 h at 37 °C with occasional mixing. Beads were then incubated with anti-α,β3 antibody (Millipore, Toronto, Canada) for 1 hour at 25 °C with gentle shaking, and then washed three times in binding buffer and incubated with the Alexa Fluor 647 secondary antibody (Invitrogen, Burlington, Canada) for 1 hour at 25 °C with gentle shaking. The beads were then washed three times in binding buffer and visualized using an AxioImager Z1 microscope (Zeiss Canada, Toronto, Canada).

**Proof-of-principle "beads on a bead" screen**

Approximately 50 RGD, KGD or AGD TentaGel beads were suspended in 100 µl of binding buffer. 1 µl of α,β3 integrin-coated screening beads were added into each wells containing the RGD, KGD or AGD TentaGel beads and incubated for 1 h at 37 °C with constant shaking at 200 rpm. The bead mixtures were then washed with binding buffer to remove any unbound magnetic beads and visualized using confocal microscopy.
A cell-based binding validation was performed using two cancer cell lines. The expression of \( \alpha, \beta_3 \) integrin by human MDA-MB-435 breast carcinoma cells (generous gift from Ann Chambers) expressing GFP and human HT-1080 fibrosarcoma cells expressing tdTomato (generous gift from Roger Tsien) was analyzed by Western blot using a \( \beta_3 \) primary antibody (Abcam, Cambridge, Canada). Approximately 50 RGD, KGD or AGD TentaGel beads were suspended in serum-free DMEM in each well of a 6-well tissue culture plate. MDA-MB-435GFP and HT-1080tdTomato cells were seeded to a final concentration of \( 1 \times 10^6 \) cells/well, and shaken at 50 rpm at 37 \( ^\circ \)C for 1 h. Beads were visualized using an Olympus IX70 inverted fluorescent microscope (Olympus, Toronto, Canada), and the number of cells attached to each bead was counted. Statistics were performed using a one-way ANOVA and Tukey post hoc test.

\( \beta_3 \) siRNA (sense: 5` GAAGAAUUUCUCCAUCCAAAdTdT3’, antisense: 5’ UUGGAUGGAGAAAUCUUCdGdA3`) (32) (Sigma, Oakville, Canada) was used to knockdown \( \alpha, \beta_3 \) integrin in MDA-MB-435GFP cells. MDA-MB-435GFP cells were seeded onto a 6-well plate and transfected with \( \beta_3 \) siRNA at a final concentration of 20 nM using siLentFect. After 48 h, Western blot analysis of cells transfected with \( \beta_3 \) siRNA or control siRNA was performed to confirm knockdown. MDA-MB-435GFP cells transfected with \( \beta_3 \) siRNA or control siRNA were trypsinized, resuspended in DMEM and directly incubated with the TentaGel library beads as described above.

**Multiplex screen for novel \( \alpha, \beta_3 \) integrin peptide ligands**

The OBOC library beads (~1.0g) were washed twice with ethanol, and three times with binding buffer. A three-step screening process was utilized to obtain library beads that interacted strongly with \( \alpha, \beta_3 \) integrin. First, the TentaGel library beads that bind non-specifically to the screening beads were removed by mixing the OBOC library with unconjugated magnetic beads for 1 h at 37 \( ^\circ \)C with constant shaking at 200 rpm. Beads that strongly associated with the magnetic beads (false positives) were removed by placing a strong neodymium magnet at the side of the vial for several minutes. Beads that did not respond to the magnet were transferred using a micropipette into another vial and washed five times with binding buffer. The second
(α,β3 integrin specific) screen was performed by adding 50 μg of α,β3 integrin-coated screening beads to the OBOC library under the conditions described above. Library bead hits were separated by placing a strong neodymium magnet at the side of the vial for several minutes. A micropipette was used to carefully transfer these library beads into another vial. This was repeated several times until no library beads remained that interacted with the magnet. Bead hits were washed three times with binding buffer. The third screen (cell-based) was performed by incubating the library bead hits with a mixture of MDA-MB-435GFP and HT-1080tdTomato cells in a 10-cm plate in serum-free DMEM for 1 h at 37 °C with constant shaking at 50 rpm. The beads were then washed twice with PBS, and visualized under an Olympus IX70 inverted fluorescent microscope. Beads that associated with the green (α,β3 integrin expressing) MDA-MB-435GFP cells, but not the red (α,β3 integrin null) HT-1080tdTomato cells, were manually isolated using a micropipette. These beads were collected in a microcentrifuge tube, treated with 6 M urea to remove the cells, washed several times with water, and then sequenced on-bead as we have previously described using MALDI-TOF/TOF mass spectrometry (33). Isomers such as isoleucine and leucine, as well as isobars such as lysine and glutamine are indistinguishable by their mass to charge ratios and must be uniquely identified using alternate methods (34).

**Cellular uptake assays**

Human MDA-MB-435 breast cancer cells and HT-1080 fibrosarcoma cells were detached using EDTA and resuspended in DMEM into microcentrifuge tubes. Cells were incubated with 3.3 μM of fluorescein-conjugated LCE62, LCE64, RGD and AGD peptides in suspension for 1 h at 37 °C. Blocking studies were conducted in the presence of 10x excess of non-fluoresceinated peptides. Cells were washed three times with PBS by centrifugation and seeded onto coverslips, washed and mounted. Images were captured using the Zeiss AxioImager Z1 microscope. The boundaries of cells were manually determined and the average fluorescein intensities within the selected region of interest were quantified using Volocity, version 4.0 (PerkinElmer, Massachusetts, USA). All statistics were performed using a one-way ANOVA and Tukey post hoc test.
Cell adhesion assay

MDA-MB-435 breast cancer cells were treated with 170 µM of LCE62, LCE64, RGD or AGD for 1 h in serum free DMEM at 37 °C. Cells were then washed six times with PBS and then permeabilized with 0.2% Triton-X. The cells were stained with DAPI, and imaged under an Olympus IX70 inverted fluorescent microscope. The number of cells that remained adhered to the plate was determined by selecting the DAPI signal and counting the selected regions using by Volocity, version 4.0. All statistics were performed using a one-way ANOVA and Tukey post hoc test.

Endothelial morphogenesis/angiogenesis assay

A 96-well plate was coated with 50 µL per well of Matrigel. After the Matrigel solidified, approximately 2000 human umbilical vein endothelial cells (HUVECs) were seeded onto the surface of the polymerized Matrigel. VEGF (10 ng/ml) and bFGF (10 ng/ml) were added into each well as the angiogenic stimulus. LCE62, LCE64, RGD or AGD peptide was introduced at a concentration of 200 µM and cells were incubated at 37°C in 5% CO₂ for 6 hours. Cells were then visualized under an inverted microscope and endothelial tube formation was quantified by counting the number of branch points from each well (n = 3). All statistics were performed using a one-way ANOVA and Tukey post hoc test.
2.4 Results

An unbiased ‘beads on a bead’ approach to screen OBOC libraries

We developed a multiplex approach to exploit the ability of small (2 μm diameter) screening beads coated with a target protein to reversibly associate with much larger (90 μm diameter) TentaGel beads that comprise random OBOC libraries. In order to promote an unbiased display, the target protein was biotinylated incompletely to average one biotin per molecule. The biotinylated target protein was then adsorbed to 2 μm streptavidin beads that are both magnetic (due to an iron oxide core) and fluorescent (due to the incorporation of rhodamine dyes) (Figure 2.1a). It was expected that the mixing of magnetic/fluorescent screening beads coated with target protein with an OBOC library, the screening beads would associate with the TentaGel beads in an affinity-dependent manner (Figure 2.1a). The ligands with the highest affinity for the target protein could then be isolated using a variety of magnetic separation approaches, including bulk or flow-based methods (Figure 2.1b). Using these magnetic/fluorescent screening beads, peptide library hits can be further stratified using flow-based fluorescence separation approaches. This approach is also compatible with validation using live cells, subsequent to which the isolated hits can be sequenced using a mass spectrometry technique that we have described previously (33).
Figure 2.1. Overview of ‘beads on a bead’ library screening approach. (a) A target protein of interest is chemically biotinylated and is allowed to interact with streptavidin-coated 2 µm magnetic beads (red) in solution. The magnetic beads-protein complex is allowed to react with the 90 µm peptide-coated TentaGel beads (white). High amount of magnetic beads are present on the surfaces of TentaGel beads coated with peptides that have a high affinity for the target protein compared with that coated with low affinity peptides; No magnetic beads are found on the surfaces of TentaGel beads coated with peptides that are non-specific to the target protein. (b) Positive hits can be isolated from the library using a bulk method or a high throughput method. The bulk method involves collecting the positive hits by placing a strong magnet at the side of the tube containing the library, and removing the negative beads by several washes. The high throughput method involves separating the positive hits from the library at a steady flow rate through a microfluidic channel. The magnet is used to divert the positive hits into the positive channel, while the negative beads are led into the negative channel by gravity.

To demonstrate the feasibility of this ‘beads on a bead’ approach, proof-of-principle experiments were designed to exploit the well-characterized binding of Arg-Gly-Asp (RGD)-containing peptides to αvβ3 integrin (22). The IC50 of KGD for αvβ3 integrin is ten times higher than RGD, while the AGD sequence does not bind αvβ3 integrin (35). First, RGD, KGD or AGD peptides were synthesized on TentaGel beads. The association of purified αvβ3 integrin with these beads was then evaluated. Indirect immunofluorescence of the peptide-coated TentaGel
beads using monoclonal antibody to $\alpha_\nu\beta_3$ integrin confirmed that purified $\alpha_\nu\beta_3$ integrin associated most abundantly with RGD beads and less abundantly with KGD beads, while no $\alpha_\nu\beta_3$ integrin was detected on the AGD beads (Figure 2.2a).

Integrin-coated magnetic/fluorescent screening beads were then generated by biotinylating purified $\alpha_\nu\beta_3$ integrin using Sulfo-NHS-LC-biotin, using stoichiometry that favored the addition of a single biotin per integrin. An excess of biotinylated $\alpha_\nu\beta_3$ integrin was then mixed with streptavidin-coated 2 $\mu$m magnetic/fluorescent beads and allowed to bind completely, followed by an excess of free biotin to saturate the streptavidin. Western blot confirmed that purified $\alpha_\nu\beta_3$ integrin was successfully biotinylated and conjugated onto streptavidin-coated magnetic particles (Figure 2.2b). The darker band at 92 kDa indicated that the $\beta_3$ subunit was biotinylated to a greater extent than the 145 kDa $\alpha_\nu$ subunit. As expected, the $\alpha_\nu\beta_3$-integrin coated magnetic/fluorescent beads interacted strongly with the RGD beads as evidenced by large numbers bound to the library bead surface. The integrin-coated screening beads interacted much less strongly with the KGD library beads, and did not detectably interact with AGD beads (Figure 2.2c). The stability and binding affinity of proteins are dependent upon the local salt concentration (36). To further demonstrate that the association of $\alpha_\nu\beta_3$-magnetic beads with the TentaGel beads was affinity-dependent, binding studies were conducted at several salt concentrations. Indeed, the number of $\alpha_\nu\beta_3$-magnetic beads bound to RGD or KGD beads decreased significantly as the salt concentration increased (Figure 2.2d).
Figure 2.2. Proof-of-principle ‘beads on a bead’ screen. (a) Immunofluorescence staining of GRGDS, GKGDS or GAGDS pentamers synthesized on TentaGel beads using αvβ3 monoclonal primary antibody followed by Alexa Fluor 647 secondary antibody (red). Scale bar, 50 µm. (b) Western blot analysis of biotinylated purified αvβ3 integrin and αvβ3-magnetic beads. Blots were probed with streptavidin-HRP. Purified αvβ3 integrin was biotinylated and conjugated onto magnetic beads. The molecular weight of αv subunit (145 kDa) and β3 subunit (92 kDa) are indicated. (c) The level of association of αvβ3-magnetic beads with RGD, KGD or AGD-coated TentaGel beads correlated with the binding of purified αvβ3 integrin to the peptide-coated TentaGel beads seen in immunofluorescence staining (a). Scale bar, 50 µm. (d) Increasing sodium chloride concentration from 0.15 M to 1.0 M decreases number of magnetic beads associated with TentaGel beads. The fluorescence intensity of rhodamine-labeled magnetic beads on each TentaGel population were quantified using the Volocity software (Improvision). All statistics were performed using a one-way ANOVA and Tukey post hoc test. (e) Positive hits coated with magnetic beads (indicated by arrows) in a library consisting of a large population of negative AGD beads (n > 200) and a few positive RGD beads (n < 20). Images captured in the red channel show αvβ3-magnetic beads (indicated by arrows). Scale bar, 300 µm.
Next, the ability of this approach to effectively distinguish positive hits from an OBOC library was assessed by mixing integrin-coated screening beads with a test library comprised of a small amount of RGD beads (n = 20) and a larger population of AGD beads (n = 200). The identification of all of the "positive" RGD-coated library beads was straightforward using fluorescence microscopy (Figure 2.2e). Taken together, these proof of principle data suggest that our ‘beads on a bead’ screening approach could be utilized for efficient screening of OBOC peptide libraries.

**Live cell validation of peptide hits**

The native conformation of protein expressed at the cell surface can differ significantly from that of the purified protein (37). Thus, to ensure that peptide hits identified in a screen would bind target proteins in their native confirmation, a direct on-bead cell-binding assay was employed to further refine the hits before sequencing. In proof of principle experiments, the ability of RGD, KGD or AGD-coated TentaGel library beads to associate with αvβ3-integrin expressing and αvβ3-integrin knockdown MDA-MB-435 breast cancer cells, and αvβ3-integrin negative HT-1080 fibrosarcoma cells was evaluated. The expression of αvβ3 integrin in these cells was confirmed by Western blot (Figure 2.3a). For straightforward identification under a fluorescence microscope, MDA-MB-435 cells expressing cytoplasmic GFP and HT-1080 cells expressing cytoplasmic tdTomato were utilized. The αvβ3-integrin negative red fluorescent HT-1080 fibrosarcoma cells did not bind to any of the peptide-coated library beads (Figure 2.3b). Incubation of αvβ3-integrin expressing MDA-MB-435 cells with RGD-coated library beads resulted in abundant binding of cells that was significantly reduced when αvβ3-integrin expression was knocked down (Figure 2.3b, c). Analogous to what was seen with the αvβ3-integrin screening beads, MDA-MB-435GFP cells exhibited very weak interaction with the KGD beads, and no association with AGD beads (Figures 2.3b and c). These data indicate that cells expressing αvβ3-integrin specifically bind to the TentaGel library beads in a ligand-dependent manner. Furthermore, this suggests that a direct cell-on-bead binding validation step can be useful to refine the peptides to those with high affinity for the target protein in its native conformation.
Figure 2.3. Live cell validation of peptide hits. (a) Western blot analysis using the β3 primary antibody showing the expression of β3 integrin in MDA-MB-435GFP and HT-1080tdTomato cells, as well as the efficacy of αvβ3 knockdown in MDA-MB-435GFP cells using the β3 siRNA. (b) Fluorescent images showing that MDA-MB-435GFP cells had the strongest association with RGD TentaGel beads compared with KGD TentaGel beads. MDA-MB-435GFP cells did not have association with AGD TentaGel beads. Knockdown of αvβ3 integrin significantly decreased the number of MDA-MB-435GFP cells on RGD TentaGel beads. HT-1080tdTomato cells (red) did not bind to any TentaGel beads. Scale bar, 20 µm. (c) Bar graph showing the average number of MDA-MB-435GFP cells that associated with each bead from all peptide groups (n = 20, p < 0.001). All statistics were performed using a one-way ANOVA and Tukey post hoc test.
The identification of non-canonical $\alpha_v\beta_3$-binding peptides LCE62 and LCE64

To isolate novel non-canonical peptides targeting $\alpha_v\beta_3$ integrin, 1 g of an octapeptide OBOC library was screened using 2 $\mu$m $\alpha_v\beta_3$-integrin coated magnetic/fluorescent beads. The initial magnetic separation step isolated roughly 300 hits, which were subsequently refined by incubating them with live cells and sorting. In total, 32 library beads that bound to the surface of $\alpha_v\beta_3$-integrin expressing MDA-MB-435 breast cancer cells but not $\alpha_v\beta_3$-integrin negative HT-1080 fibrosarcoma cells were isolated and sequenced using a previously described mass spectrometry approach (33). This resulted in the successful identification of 11 unique peptide sequences, one of which contained RGD (Figure 2.4a). The remaining peptides had some homology to one another, evidenced by a recurring motif Lys/His-X-Lys/His, where X represents any amino acid, which was observed in 8/10 peptides (Figure 2.4a). Four peptides that did not contain the RGD motif (LCE60, LCE61, LCE62 and LCE64) were selected for synthesis and further characterization. The binding affinity of these peptides for purified $\alpha_v\beta_3$ integrin was determined using surface plasmon resonance (SPR) (Supplementary Figure 2.1), and the $K_D$ values are presented in Table I. Interestingly, the two peptides with the highest affinity for $\alpha_v\beta_3$ integrin, LCE62 and LCE64 (10.8 ± 1.2 nM and 4.7 ± 0.3 nM, respectively), each contained two Lys/His-X-Lys/His motifs, which suggests that this motif may be important for integrin binding. The chemical structures of LCE62 (MAFKHKAH) and LCE64 (KTKKVHSQ) are shown in Figure 2.4b.

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Sequences</th>
<th>$K_D$</th>
</tr>
</thead>
<tbody>
<tr>
<td>RGD</td>
<td>H-GRGS-Ahx-C-NH$_2$</td>
<td>6.98 nM 0.56 nM</td>
</tr>
<tr>
<td>LCE60</td>
<td>H-HESFGKHK-Ahx-C-NH$_2$</td>
<td>16.32 nM</td>
</tr>
<tr>
<td>LCE61</td>
<td>H-VDRKFHDG-Ahx-C-NH$_2$</td>
<td>N/A</td>
</tr>
<tr>
<td>LCE62</td>
<td>H-MAFKHKAH-Ahx-C-NH$_2$</td>
<td>10.80 nM</td>
</tr>
<tr>
<td>LCE64</td>
<td>H-KTKKVHSQ-Ahx-C-NH$_2$</td>
<td>4.68 nM</td>
</tr>
</tbody>
</table>
Figure 2.4. The identification of non-canonical $\alpha_v\beta_3$-binding peptides, LCE62 and LCE64.

(a) Peptide sequences from MALDI-TOF/TOF mass spectrometry. The RGD motif is shown in white, the Lys/His-X-Lys/His motif is indicated by black boxes. Sequences were aligned using the ClustalW multiple sequence alignment tool. LCE62 and LCE64 sequences are shown. (b) Chemical structures and sequences of LCE62 and LCE64. Structures were generated using the ChemDraw software.
LCE62 and LCE64 bind MDA-MB-435 breast cancer cells in a \(\alpha_\beta_3\) integrin-dependent manner

Peptides LCE62 and LCE64 were conjugated with a fluorescent dye (fluorescein) and their ability to selectively bind \(\alpha_\beta_3\)-expressing MDA-MB-435 breast cancer cells was assessed using fluorescence microscopy and flow cytometry. The uptake of FITC-LCE62 and FITC-LCE64 by MDA-MB-435 cells was 3.5 times and 2.7 times higher respectively than that of FITC-AGD (Figure 2.5). Knockdown of \(\alpha_\beta_3\) integrin using \(\beta_3\)-siRNA reduced the binding and uptake FITC-LCE62 and FITC-LCE64 in MDA-MB-435 cells to the same level as FITC-AGD. Furthermore, no significant uptake of FITC-LCE62 and FITC-LCE64 by \(\alpha_\beta_3\)-integrin negative HT-1080 fibrosarcoma cells was observed. Taken together, these data suggest that LCE62 and LCE64 can be utilized to specifically target cells expressing \(\alpha_\beta_3\) integrin.
Figure 2.5. LCE62 and LCE64 are taken up by MDA-MB-435 breast cancer cells in a $\alpha_v\beta_3$ integrin-dependent manner. (a) Fluorescence images showing the uptake of FITC-conjugated LCE62, LCE64, and AGD (control) (final concentration of 3.3 $\mu$M) by MDA-MB-435 cells. Left panels: FITC signal from peptide uptake (green). Right panels: merged images of brightfield image, nuclei staining (blue) and FITC-conjugated peptide (green). Scale bar, 10 $\mu$m. (b) Bar graph showing the mean FITC intensity in each cell. Measurements of the peptide uptake by cells were performed by selecting the perimeter of individual cells and obtaining the mean FITC intensity within selected regions using the Volocity software (Improvision). Data were normalized to AGD. The uptake of LCE62 and LCE64 were significantly higher than that of AGD ($n = 30$, $p < 0.001$). The uptake of these peptides were significantly reduced in $\alpha_v\beta_3$-knockdown cells ($n = 30$, $p < 0.001$). All statistics were performed using a one-way ANOVA and Tukey post hoc test.

LCE62 and LCE64 do not impact the biology of human endothelial or breast cancer cells

RGD peptides or peptidomimetics have been widely used to target sites of abundant $\alpha_v\beta_3$ integrin expression such as those found in angiogenic blood vessels (38) and tumors (39). Cancer cells treated with RGD peptide lose intercellular contacts and exhibit decreased cell adhesion in vitro (25), and peptides incorporating cyclic RGD inhibit angiogenesis in vivo (26). The biological impact of RGD peptides when utilized as cancer seeking agents must therefore be considered, as their use may lead to increased tumor aggressiveness, invasiveness (28) and micrometastases (29). In light of these concerns, the biological impact of LCE62 and LCE64 on human endothelial and cancer cells was assessed.

A comparison was undertaken to determine the relative effect of LCE62, LCE64 and RGD on cellular morphology, cell adhesion and angiogenesis. First, MDA-MB-435 cells were treated with equivalent concentrations of LCE62, LCE64, RGD or AGD (negative control) peptides. Cells treated with RGD peptide adopted a more rounded morphology and exhibited fewer elongated protrusions compared with cells treated with LCE62, LCE64 or AGD (Figure 2.6a). During live imaging experiments, the majority of cells treated with RGD peptide rounded
up and detached from the surface, while cells treated with LCE62, LCE64 or AGD remained adhered and migrated normally. Cell spreading was assessed by measuring the cross sectional area of the adhered cells after 1 hour. While the presence of RGD peptides significantly decreased cell spreading, no significant differences were observed between cells treated with LCE62, LCE64 and AGD (Figure 2.6b). RGD significantly reduced the adhesion of MDA-MB-435 cells to the surface of a 96-well plate compared with AGD, while LCE62 and LCE64 had no effect on cell adhesion (Figure 2.6c). The impact of cyclic RGD, AGD, LCE62 and LCE64 on angiogenesis was investigated using an in vitro angiogenesis assay where human umbilical vein endothelial cells (HUVECs) are allowed to spontaneously sprout and form tubes on Matrigel in the presence of VEGF and bFGF. Cyclic RGD at 200 µM significantly inhibited tube formation compared to the AGD control, while αvβ3-integrin targeting peptides LCE62 and LCE64 had no effect at equivalent concentrations (Figure 2.6d and e). This demonstrates that peptides LCE62 and LCE64 do not appreciably alter the morphology, cell spreading and cell adhesion of αvβ3-expressing MDA-MB-435 breast cancer cells, nor do they affect the tube formation of endothelial cells in vitro. Taken together, these data indicate that peptides LCE62 and LCE64 can be utilized for the targeting of αvβ3-integrin without affecting target cell biology.
Figure 2.6. LCE62 and LCE64 do not impact the biology of human endothelial or MDA-MB-435 breast cancer cells. (a) Phalloidin staining showing F-actin distribution in MDA-MB-435 cells after treatment with 170 µM of LCE62, LCE64, RGD and AGD. Cells treated with RGD have edges that are more rounded and decreased filopodia (indicated by short arrows) compared with treatment with AGD. The elongated filopodia in cells treated with AGD is also seen in cells treated with LCE62 and LCE64 (indicated by long arrows). F-actin distribution in cells treated with LCE62 and LCE64 remained unchanged when compared with cells treated with AGD. Scale bar, 10 µm. (b) Cross sectional area of cells after treatment with each peptide (as in (a)). Cells treated with RGD had significantly decreased cell spreading compared with treatment with AGD (p < 0.05). No difference was observed between cells treated with AGD and LCE62 or LCE64. Quantitation of cross-sectional area occupied by cell was performed using Volocity. (c) Cell adhesion assay showing that treatment of cells with RGD significantly
decreased the attachment of cells to the plate compared with treatment with AGD (p < 0.001), while no difference was observed between cells treated with AGD and LCE62 or LCE64. (d) \textit{In vitro} angiogenesis assay demonstrating cyclic RGD inhibited human umbilical vein endothelial cells (HUVECs) sprouting and tube formation on Matrigel in the presence of VEGF and FGF (p < 0.01), while no difference was observed upon treatment with LCE62 or LCE64 compared with AGD. (e) The level of sprouting was quantified by counting the number of branch points formed by the HUVECs. All statistics were performed using a one-way ANOVA and Tukey post hoc test.
2.5 Discussion and conclusion

We report here a versatile screening strategy for the discovery of novel non-canonical affinity ligands from OBOC libraries, and demonstrate its utility by identifying several high affinity 8-mer peptide ligands for αvβ3 integrin that do not contain RGD. The strength of this "beads on a bead" approach lies in its utilization of small magnetic/fluorescent screening beads, which, at 2 µm, are much smaller than the typical 90 µm TentaGel peptide library beads and can efficiently coat their surface. By incorporating multiple magnetic and/or fluorescence separation steps, this approach can be performed either on the benchtop using bulk separation techniques or in a more high throughput manner using automated sorting technology. An enrichment of non-canonical epitopes can be accomplished using incomplete biotinylation of the target protein before it is adsorbed onto the screening beads, which promotes the display of multiple epitopes and serves to partially mask dominant binding motifs. In contrast to other described approaches that utilize antibodies (20, 21), this approach does not immobilize the protein in a single orientation. This screening method, coupled with recently developed high throughput sequencing approaches (33), provides an accurate yet affordable strategy for the discovery of novel affinity ligands.

A significant challenge facing peptide screening approaches is the reduction or elimination of false positives, which is typically exacerbated by the incorporation of extrinsic biomacromolecules (i.e. chemicals, proteins, antibodies, viruses or cells) into the screening methodology (40). The utility of the biotin-streptavidin interaction to directly conjugate the target protein onto magnetic beads eliminates the use of other macromolecules that do not pertain to the screen. For target proteins that do not contain exposed lysine residues available for biotinylation, alternative conjugation strategies using small adaptor molecules can be employed (i.e. aptamers). A further reduction in false positives can be achieved by performing a library enrichment step. This facilitates the removal of peptide library beads that interact with streptavidin by incubating the library with naked magnetic/fluorescent screening beads, before magnetic/fluorescent screening beads coated with the target protein are used to screen the enriched library. This additional step can be completed in less than an hour using a bulk magnetic separation approach with a minimal impact on workflow. False positives can be further reduced by incorporating an on-bead cell-based assay prior to peptide deconvolution. As
demonstrated here, this step can be performed to validate the binding of isolated hit beads to target proteins that are expressed in their native conformation on the cell surface. The approach can accommodate the use of populations of both target-positive and target-null cells to further increase the confidence of hits. This step can significantly narrow down the number of hits selected for subsequent analysis, and increases the prospect of attaining high affinity ligands that can specifically target the native conformation of the target protein on living cells.

There are several strategies one can take to improve the stringency and throughput of this "beads on a bead" screening approach. A bulk separation approach (Figure 1a) enables a rapid but potentially crude isolation of hits. It can be utilized either as the principle separation method or in stepwise combination with other separation strategies. If bulk separation is the only method used to identify hit beads, several strategies can be employed to improve accuracy. For example, a number of low stringency separations can be conducted iteratively. Furthermore, the strength of the magnetic field can be modulated to either increase or reduce the stringency in order to obtain higher or lower affinity ligands. Increased control over the magnetic separation can be achieved using flow-based methods (Figure 1b). In our hands, this approach can provide exquisite control over the stringency of separation and can improve both the throughput and accuracy of sorting. The use of screening beads that are both magnetic and fluorescent allows for multiparametric separation. High throughput fluorescence separation of OBOC libraries can be accomplished using a large format sorting platform such as the Complex Object Parametric Analyzer and Sorter (COPAS) from Union Biometrica. While the use of this specialized platform for sorting bead libraries has been previously described(20), its considerable cost precludes its general use. Bulk magnetic separation provides a low-cost alternative that can be performed on the benchtop.

We identified several high affinity peptide ligands of $\alpha_v\beta_3$ integrin, the majority of which did not contain RGD. In 8 of the 10 peptides identified that did not contain RGD, recurring motif Lys/His-X-Lys/His was observed. Peptides LCE62 and LCE64 had exceptional affinity for purified $\alpha_v\beta_3$ integrin (Table I) and were taken up by $\alpha_v\beta_3$ integrin-expressing breast cancer cells in an integrin-dependent manner. LCE62 and LCE64 each contain two Lys/His-X-Lys/His motifs, which suggests that sequences comprised of alternating basic amino acids may be important for integrin binding. Fibrinogen has been reported to contain at least one non-RGD
\(\alpha_\beta_3\)-dependent adhesive site that does not play a role in the spreading of human melanoma cells (41). A cluster of basic amino acids in the fibrinogen sequence was found to be the binding site for \(\alpha_{IIb}\beta_3\) integrin (42). It is plausible that the positively charged Lys/His-X-Lys/His-containing peptides LCE62 and LCE64 may recognize an analogous binding site on \(\alpha_\beta_3\) integrin. Peptide candidates that contain this Lys/His-X-Lys/His motif may have potential to be developed and utilized as \(\alpha_\beta_3\)-targeting ligands, and it is possible that these ligands may be further optimized. There were no significant similarities detected between any of the novel \(\alpha_\beta_3\) integrin ligands and extracellular proteins. However, this does not preclude the possibility that LCE62 and LCE64 may adopt structures that mimic those possessed by other proteins that interact with \(\alpha_\beta_3\) integrin. Taken together, our data suggests that peptides LCE62 and LCE64 recognize the extracellular domain of \(\alpha_\beta_3\) integrin at a site that is distinct from the active site.

Since its discovery in 1985 (43) as the component of fibronectin required for cell binding, there has been an explosion in the use of RGD peptides for integrin-targeted imaging and drug delivery efforts in the experimental, pre-clinical and clinical realms. Given the well described anti-angiogenic activity of these peptides, the potential biological impact of their use must be considered. For example, a relatively high concentration (in the millimolar range) is generally required for drug delivery and imaging modalities such as optical or magnetic resonance imaging (MRI) (25, 44, 45). Imaging modalities such as positron emission tomography (PET) and single-photon emission computed tomography (SPECT) typically require very low ligand concentrations (nanomolar to femptomolar) (45), and are unlikely to exert a biological effect. This is complicated, however, by evidence that suggests that low concentrations of RGD peptide can enhance tumor growth in vivo by promoting VEGF-mediated angiogenesis (46). We demonstrate here that LCE62 and LCE64 target cancer cells expressing \(\alpha_\beta_3\) integrin without altering their morphology, their ability to spread, or their adhesion. Furthermore, these peptides, even at high concentration (200 µM), do not inhibit angiogenesis. It will be important going forward to validate the biological impact of these peptides in vivo.

While these novel integrin ligands (LCE62 and LCE64) effectively target breast cancer cells in vitro, the in vivo targeting of these peptides to \(\alpha_\beta_3\) integrin in tumors and angiogenic vasculature need to be evaluated. Cyclic RGD peptides (\(IC_{50} = 0.58\) nM) and peptidomimetics
(IC$_{50}$ = 12 nM) have been successfully utilized for in vivo tumor imaging (47, 48). Since LCE62 and LCE64 have K$_D$ values comparable to that of cyclic RGD, we are optimistic that they can be used to target $\alpha_v\beta_3$-expressing tumors and angiogenic vessels. Thus, these novel ligands are promising tumor homing agents for translational applications in drug delivery, targeted MRI and intraoperative imaging that do not impact tumor biology.
References


Chapter 3

High throughput screening of one-bead-one-compound peptide libraries using intact cells

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Keywords: OBOC library, cell-based screen, MALDI-TOF mass spectrometry, cross-linking, RGD peptide, integrin, COPAS biosorter

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3.1 Abstract

The discovery of peptide ligands from one-bead-one-compound (OBOC) combinatorial libraries has driven advances in molecular imaging and the development of targeted nanomedicines. Recognition of cell surface proteins is optimally achieved using live cells, yet screening intact cell populations is time-consuming and inefficient. Here, we evaluate the COPAS large particle biosorter for high throughput sorting of bead-bound human cell populations for the purpose of screening. By screening a library of RGD-containing peptides against human cancer cells that express $\alpha_v\beta_3$ integrin, we find that bead-associated cells are rapidly dissociated when sorted through the COPAS instrument. However, we demonstrate that when the bound cells are reversibly cross-linked onto the beads, they can then be sorted quickly and accurately. We go on to show that this reversible cross-linking approach is compatible with MALDI-TOF/TOF mass spectrometry-based peptide sequence deconvolution, allowing for rapid screening and identification of peptide ligands from bead-based combinatorial libraries. This approach should facilitate the discovery of improved peptide ligands against cell surface targets in their native conformation.
3.2 Introduction

Recent advancements in nanotechnology have combined targeting molecules with imaging agents and/or therapeutics into a single entity, enabling their delivery to disease sites while avoiding healthy tissues (1-6). Peptides that are isolated from biological systems, such as phage display libraries, have provided many valuable targeting agents (7-10). However, the biological nature of these libraries limits their application to the discovery of natural peptides, which have increased susceptibility to proteolytic degradation under physiological conditions. For this reason, peptides that are discovered using these approaches can be unsuitable for in vivo studies (11). One-bead-one-compound (OBOC) library screening methods are a promising chemistry-based alternative to peptide ligand discovery, and have been used previously to identify novel ligands for molecular imaging (2, 12-14), protein inhibition (15, 16) and directed therapy of diseases (17-19). OBOC combinatorial libraries, are comprised of 90 micron-sized beads each bearing a unique ligand, can be synthesized using straightforward chemistries and screened in parallel against cell surface targets (20). The primary advantage of OBOC peptide libraries is the incorporation of non-natural components, such as D-amino amino acids, or the incorporation of cyclic, turned or branched ligands (12, 21). This facilitates the identification of peptide ligands that are resistant to proteolytic degradation, making them more suitable for in vivo applications.

For the purpose of OBOC library screening, the target protein is chemically tagged or fluorescently labeled (22-24). While this approach is feasible for many targets, proteins must be purified and derivatized prior to screening. This increases the risk that these proteins would adopt an altered conformation and could impair their function (25). In many cases, this approach will not account for changes in conformation due to protein activation (26, 27). For cell surface proteins and/or proteins that typically form complexes with other cell surface proteins, the presence of the plasma membrane and binding partners may be required for proper folding and the display of biologically relevant epitopes. These limitations can be addressed through the development of cell-based assays to screen OBOC libraries. Indeed, screening approaches using living cells have been successfully utilized to discover ligands against human cancer cell lines (28), ie. Jurkat T-leukemia cells (13), T-lymphoma cell (29), and breast cancer cells (14).
Nevertheless, conventional methods for isolating rare positive hits from a large OBOC library through manual techniques remain challenging and impractical.

To increase the throughput of library screening, instruments such as the COPAS bead sorter (Union Biometrica) have been employed to isolate high affinity ligands from OBOC libraries using purified target proteins (30-32). In an initial evaluation of this platform to screen combinatorial libraries using living cells, we have found that associated cells would fall off the beads once they were passed through the COPAS instrument, preventing proper sorting. This is likely caused by a variety of factors, such as turbulence and shear forces within the instrument that exceeded the affinity of the target with its ligands. In this work, we have evaluated a reversible cross-linking method to stabilize the association of cells and the bead they are bound to. We hypothesize that chemical fixing of cells to their associated bead prior to insertion into the COPAS instrument would prevent the cells from detaching during the flow-based sorting process.

An important element of screening throughput is the efficient deconvolution of hit peptides, which we have previously addressed using a MALDI-TOF/TOF mass spectrometry (MS) approach (33). This strategy allows one to perform the photochemical cleavage of peptide from the solid support, peptide desorption-ionization, and generation of fragmentation pattern in a single step. Our concern, however, is that chemical cross-linking might interfere with accurate sequence determination using mass spectrometry. To test the compatibility of reversible cross-linking with this approach, we have performed a limited screen using αvβ3 integrin-expressing fluorescent cancer cells and a focused OBOC peptide library. The library is comprised of RGD sequences fused with a representative mixture of the remaining amino acids. After cross-linking the cancer cells to the library beads, the library is sorted using the COPAS instrument, and the resultant hits are sequenced using the on-bead MS approach. Sequence results are compared between peptides that are not cross-linked, peptides that are cross-linked, and peptides that are first cross-linked and then treated with heat to reverse the cross-links.
3.3 Methodology

**Peptide library synthesis**

Fmoc-based solid-phase peptide synthesis was carried out using an APEX 396 autosynthesizer (AAPPTEC) with 0.05 mequiv of 0.26 mmol/g TentaGel S NH2 (0.27 mmol/g) resin. A threefold excess of Fmoc-ANP and subsequently, the protected amino acids was used in coupling reactions. Fmoc removal was carried out using a solution of 20% piperidine in DMF (N,N-dimethylformamide) over two cycles (10 and 20 min). Amino acid activation was carried out with three equivalents of HBTU and six equivalents of DIPEA (N,N-diisopropylethylamine), which was followed by amino acid coupling over 30 and 120 min cycles. Deprotection of peptide side chains was accomplished using a solution of 88% TFA (v/v) + 5% H2O (v/v) + 5% phenol (m/v) + 2% triisopropylsilane (v/v) over 6 h.

**OBOC library screening using live cancer cells**

αvβ3 integrin-expressing MDA-MB-435 breast cancer cells were labeled with green fluorescent protein. Approximately 1 000 beads containing each peptide were equilibrated with serum-free DMEM in a 12-well plate. MDA-MB-435 cells were detached from the flask by EDTA and resuspended in serum-free DMEM. 200 000 of each MDA-MB-435 cells were added into each well containing the library beads and placed in a shaking incubator (50 rpm) for 1 hr at 37°C. The beads were washed twice with PBS, and then imaged under the Olympus IX70 inverted fluorescent microscope.

The cells were then fixed onto the beads with 4% formaldehyde for 5 minutes at room temperature, and washed twice with PBS.
Sorting of positive hits using COPAS

The beads from each well were inserted into the COPAS large particle flow cytometer (Union Biometrica), and sorted into a 96 well plate. Firstly, the sorting threshold was established using TentaGel beads coated with empty TentaGel beads that have never been previously treated with cells. This step is necessary because TentaGel beads auto-fluoresce, especially in the green (excitation wavelength 488 nm) and red (excitation wavelength 561 nm) channel. The instrument was then gated to only analyze and isolate beads with the highest fluorescence well above the set threshold. This population represents beads that have the strongest association with cells. Any beads with fluorescent intensity higher than the set threshold were sorted into a 96-well plate. Beads that were isolated were imaged under the Olympus IX70 inverted fluorescent microscope. The beads were treated rigorously with ethanol to remove any bound cells, and washed several times with water.

MALDI TOF MS/MS sequence analysis

Cleavage of peptides from TentaGel beads was carried out using UV irradiation. All care was taken to prevent light exposure to synthesized peptides prior to ANP-linker cleavage. For this reaction, approximately 1-3 peptide conjugated TentaGel beads were placed in 200 µL of MilliQ water in an open-top 384 well polypropylene plate. UV irradiation was carried out using a 365 nm UV lamp (UV Products, Upland, CA, model EL25, 8 mWcm−2) over 2.5 hours. Water was added periodically in order to prevent wells from drying, thus reducing possible peptide decomposition. The resulting peptide-containing solution was then used for MALDI-TOF/TOFTM analysis.

In a typical experiment, the exact molecular ion mass [M+H]+ of a peptide is determined using MS analysis. MS/MS spectra are subsequently recorded for the desired molecular ion peak, previously observed by MS. This was then followed by manual deconvolution of all peptide sequences in this study.
3.4 Results

Combining automated cell-based with OBOC peptide library screen

The biggest challenge associated with automated sorting of hits from screening OBOC libraries against living cells was that the cells dissociate from the beads upon insertion into the sorter. Overcoming this issue would enable an easy and high-throughput automated sorting of high-affinity ligands from OBOC libraries. To do this, we tested our hypothesis that bound fluorescent cells can be chemical cross-linked onto the library bead, and that this would prevent the cells from detaching throughout the sorting process. Hit peptides with the strongest affinity for the cells would have the highest fluorescence, and can be sorted and isolated into a 96-well plate (Figure 3.1). Beads that did not associate, or had low interaction with the cells were pooled into a sample recovery unit.

The GRGDS peptide was demonstrated to bind the αvβ3 integrin with high affinity in several studies (34-37). As a proof-of-principle, a biased peptide library incorporating the GRGDS moiety and a photolabile ANP linker was synthesized, via solid phase methodology, on TentaGel resin. The photo-cleavable linker was utilized to enable efficient release of the peptide from the TentaGel bead for sequencing. The two C-terminal amino acids were randomized in order to better mimic a randomized peptide library. The GRGDSYT, GRGDSTW, GRGDSWK, GRGDSVP, GRGDSDL, GRGDSFA, and GRGDSPS peptides were synthesized onto TentaGel beads (Figure 3.2). Peptides were fully deprotected, while still on TentaGel, prior to fluorescent-based analysis by COPAS biosorter. We treated the biased RGD-containing library using live αvβ3-expressing MDA-MB-435 breast cancer cells. These cells were labeled with green fluorescent protein (GFP) so that they can be easily detected or visualized under a microscope. Cells were incubated and allowed to associate with RGD-containing TentaGel beads. High binders were then sorted into a 96-well plate as shown in Figure 3.1.
Figure 3.1. Cell-based screening of OBOC peptide libraries to identify high affinity ligands to cellular targets using the COPAS biosorter. Beads incubated with live fluorescent cells are washed, fixed with 3% formaldehyde and loaded into the sorter. Bead hits with strong interactions with cells (highest fluorescent intensity) are sorted into a 96-well plate, while beads with few bound cells are excluded.
**Figure 3.2. Design of focused library of integrin-binding peptides.** GRGDS-containing heptameric peptides were synthesized onto TentaGel beads via a photo-cleavable linker. Different residues (indicated in blue) represent different amino acids that were tested.

**Proof-of-principle: Cross-linking associating cells to beads facilitates automated large particle sorting**

$\alpha_\text{v}\beta_\text{3}$ integrin-expressing MDA-MB-435 breast cancer cells labeled with GFP were incubated with each species of the RGD-containing beads. Although MDA-MB-435 cells associated with all the beads, the two additional amino acids at the C-terminus affected the affinity of each peptide with the cells (Figure 3.3a and Supplementary Figure 3.1). MDA-MB-435 cells interacted most strongly with the GRGDSWK, GRGDSPS and GRGDSFA peptides, while showing moderate association with the remaining peptides (Figure 3.3a and Supplementary Figure 3.1). The beads were then washed with phosphate buffer saline (PBS) to
remove any unbound cells, and cells that were tightly associated with the beads were fixed with 3% formaldehyde so that they remained attached during and after the sorting process (Figure 3.3a and Supplementary Figure 3.1). Although cross-linking is necessary to maintain the association of MDA-MB-435 cells with the bead throughout the automated sorting process, this step may be omitted when using “stickier” cells, such as fibroblast. To distinguish between the bead and cell populations, TentaGel beads alone or MDA-MB-435 GFP cells were incorporated separately into the COPAS flow cytometer, and their sizes and optical density were evaluated (Figure 3.3b). The bead population appeared in the top right quadrant, while majority of the cell population appeared in the bottom left quadrant (Figure 3.3b). To establish the gate and sorting threshold, TentaGel beads coated with a negative control peptide that has undergone cell treatment, fixation and washes were incorporated and analyzed in the COPAS instrument. As TentaGel beads auto-fluoresce, the sorting gate was set to sort beads with cells bound onto them that have higher fluorescent intensities than the large population of the control beads (Figure 3.3c). Although it was indicated that 12.5% of beads were sorted, microscopy analysis revealed that this population consisted of bare beads with abnormally high autofluorescence that did not have cells bound onto them (data not shown). Approximately 68% of GRGDSPS beads were sorted, and microscopy analysis revealed that these beads were completely coated with cells (Figure 3.3a and c). Using the same procedures, we sorted the remaining test GRGDS-containing TentaGel beads. The GRGDSDK and GRGDSFK peptides also had high affinity for MDA-MB-435 cells, with a sorting rate of 80.3% and 48%, respectively (Supplementary Figure 3.2). The GRGRDSTYT, GRGDHSL, GRGDSTW and GRGDSVP have relatively weaker associations with MDA-MB-435 cells, resulting in sorting rates of less than 30% (Supplementary Figure 3.2).
Figure 3.3. Establishing the sorting parameters for library beads coated with intact cells.

(a) TentaGel beads containing GRGDSPS peptide incubated with MDA-MB-435 GFP cells (left) and fixed with 3% formaldehyde (right). Beads with the highest association for cells were sorted using the COPAS instrument. (b) Dot plot showing the bead or cell population. EXT represents extinction (measurement of total light scatter), TOF stands for time of flight, and FLU1 represents the green fluorescence intensity. (c) Dot plot showing the cell-bead populations and their sorting profiles. The upper panel shows the two distinct bead and cell population (indicated by arrows) and the beads were gated for sorting. The bottom panel shows the green fluorescence intensity of each bead (gated from the upper panel). The sort gate was established using negative control TentaGel beads that has undergone cell treatment, fixation and washes so that only beads with cells attached (higher fluorescent intensities) were sorted. GRGDSPS-TentaGel beads with
MDA-MB-435 GFP were then inserted into the COPAS instrument, and beads with the highest association with cells were sorted into a 96-well plate.

**Confirmation of MALDI-TOF/TOF peptide sequence deconvolution pre- and post-sorting**

Samples of all peptides in the library, at pre- and post-sortal stages, were completely deprotected and cleaved for MALDI-TOF analysis. Similar signal to noise ratios of obtained mass spectra as well as the subsequent complete deconvolution of all peptide sequences, pre- and post-sorting (Figure 3.4 and Supplementary figure 3.3), proves that cross-linking cells onto beads stabilizes their association for automated sorting and does not prevent sequencing through mass spectrometry approaches. Therefore this approach enables a viable and versatile high-throughput screen of peptide libraries.

**Figure 3.4. On-bead MALDI-TOF/TOF MS sequencing of peptide before and after fixation.** MS/MS spectra of H-GRGDSPS-NH$_2$ before fixing with formaldehyde (left), and after fixing plus sorting (right). Peptide sequences from both samples were successfully attained. Fragments labeled b$_j^a$ and y$_j^a$ were calculated by complementarity.
3.5 Discussion and conclusion

Here, we demonstrate the usefulness of a reversible cross-linking method to improve the association of cells and high-affinity ligand-containing beads during an automated sorting process. MDA-MB-435 breast cancer cells expressing GFP are incubated with each type of the RGD-containing library bead, and the cells are then cross-linked onto their associated beads. Positive hit beads with bound cells display much higher fluorescent intensities compared to negative empty beads, and can be sorted individually into each well in a 96-well plate. We also show that the cross-linking step does not prevent us from accurately obtaining the sequences of our test peptides through MS approaches.

The pentameric GRGDS sequence is incorporated into all test peptides. Two common natural amino acids are incorporated at the end of the test sequences to closely mimic a random combinatorial library. The cysteine residue is excluded from the test peptides to avoid the formation of disulfide linkages, while methionine is omitted to avoid potential oxidation. Isoleucine is also excluded because it is isobaric to leucine, rendering them indistinguishable from one another by MALDI MS. Similarly, glutamine is excluded from the test peptides because it is isobaric to lysine. We found that the composition of the last two amino acids significantly affect the binding affinity of the peptides to cells. This observation is consistent with other studies that demonstrated that different residues flanking the RGD motif of proteins can significantly alter their binding specificity and affinity to integrins (38-40).

Even though other studies have demonstrated the success in isolating peptides from screening an OBOC library using cells (14, 41), the process of isolating positive hits manually is a time consuming and challenging. The COPAS large format biosorter enables us to accurately execute high throughput automated sorting at up to 300 beads per second, allowing the entire screening process to be completed in less than a week. Although other studies have demonstrated the benefits of incorporating magnetic isolation in a cell-based screening process using antibodies (42), these methods pose an immense risk of attaining a large number of false positives that bind non-specifically to the extrinsic biomacromolecules that are present on the surface of the magnetic beads. Our study relies solely on the interaction between the cells and peptide-coated library beads, therefore decreasing the risk of isolating false positives caused by
other non-specific interactions. To further optimize the specificity of our screen, one can enrich the library by first using knockout/knockdown cells before screening with cells that express the target protein. This process can eliminate false positives by decreasing the chances of isolating ligands that bind to other surface receptors that are normally present on a particular cell.

We have initially encountered many challenges associated with the cells detaching from their associated bead, most likely due to mechanical forces as it passes through the flow cell. As a result, very few beads displayed cells by the time they were detected for sorting. Cross-linking cells to the bead they are bound to with formaldehyde prior to sorting significantly improves the attachment of cells on the bead throughout the sorting process. Although we have demonstrated that treating the beads with formaldehyde does not impede MALDI MS sequencing, we have nonetheless, attempted to reverse the cross-links by heating the beads to 60°C for 10 minutes, and then 95°C for 15 minutes. However, we have shown that reliable mass spectrometry spectra could not be consistently obtained from beads that have undergone heat treatment.

One other major challenge that we have encountered during the sorting process is the inability of the COPAS instrument to distinguish the fluorescence emitted by bound cells from the auto-fluorescence that are naturally produced by the library beads. Nonetheless, this limitation can be easily overcome by direct visual examination through fluorescence microscopy after sorting has been completed.

This method can accommodate the use of both target-positive and target-null cells to further increase the confidence of isolated hits. Not only can this significantly narrow down the number of hits selected for subsequent analysis, but it can also increase the prospect of obtaining high affinity ligands that specifically target the native conformation of the target protein on living cells. The use of this technique can also be very advantageous for screening for ligands against cell surface receptors that adopt very specific conformations in living cells. Surface receptors typically adopt diverse conformations under various conditions. For example, a decrease in extracellular pH causes conformation changes in integrins, which facilitate their activation (43). Additionally, ligand binding regulates the function of several extracellular surface receptors, i.e. G-protein-coupled receptors (44) through the establishment of new conformational equilibrium. We can further expand the scope of our study to using induced cells
to screen for ligands specific for only activated receptors. Ultimately, there is a vast potential for our findings to be further augmented for applications in personalized medicine by screening for ligands specific for cells (i.e. localized vs. metastatic cancers) collected from patients.

Our results show that we can easily and reliably perform automated screening of very large OBOC combinatorial libraries by fixing live cells onto the bead that they are bound to, and that we are able to isolate and sequence high-affinity ligands in a high-throughput manner within a short period of time. Our findings can help accelerate the rate for isolating targeting ligands that can be applied for the purpose of molecular imaging and drug delivery.
References


Chapter 4

Novel peptide ligand against EGFL7, E7-p72 as a tool for imaging early cancers and angiogenesis

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4.1 Abstract

As a tumour grows beyond 1mm, it generally recruits new blood vessels through the process of angiogenesis. Metastasis is the cause of 90% of cancer mortalities, and targeting areas of active angiogenesis should aid in suppressing tumours before they metastasize. Epidermal growth factor-like domain 7 (EGFL7) is protein that is dramatically up-regulated in the endothelium of tumours. EGFL7 expression correlates with poor prognosis in malignant glioma, hepatocellular carcinoma and non-small cell lung cancer. Although the function and mechanism of action that EGFL7 is involved in currently not well understood, it is an important biomarker for clinically relevant neoplasms and metastases. Here, we describe the identification and characterization of novel anti-EGFL7 peptide ligands from screening a combinatorial peptide library using a novel ‘beads on a bead’ approach. We discovered a novel EGFL7 ligand, E7-p72 that was shown to have high affinity for EGFL7. A strong uptake of fluorescein-conjugated E7-p72 was observed in human EGFL7-expressing cancer cells and endothelial cells. We also demonstrated that CPMV nanoparticles coated with E7-p72 bind to endothelial cells in an EGFL7-dependent manner. This peptide could provide a basis for a new generation of sensitive molecular imaging probes for the non-invasive diagnosis of early cancers.
4.2 Introduction

The development of affinity probes for ligand-mediated targeting has advanced the field of molecular imaging (1-6), and directed therapy of disease (as reviewed in (7)). Advancement in nanotechnology has led to the generation of “smart” nanoparticles, allowing for the combination of targeting ligands with therapeutic and/or imaging agents all within a single entity. The development of targeted nanoparticles has enabled the delivery of high concentrations of imaging agents and/or drugs to disease sites while avoiding normal tissues, leading to the generation of high-contrast images with reduced noise levels, as well as increased efficacy of therapy with reduced adverse side effects. While monoclonal antibodies have demonstrated high success in the clinic for targeting tumors (8), they have major limitations that need to be addressed, including immunogenicity (9), size-related tissue diffusion properties, and their non-specific uptake by the reticuloendothelial system (RES). Peptide-based affinity probes serve as an attractive alternative, as they are significantly smaller, have better diffusion and tissue penetration, demonstrate improved pharmacokinetics, and reduced interaction with the RES (10). Examples of affinity peptides that are in extensive clinical use as cancer homing agents include bombesin (11) and cyclic RGD peptides (12).

Strategies for screening combinatorial peptide library, such as phage display (13) and one-bead-one-compound (OBOC) (14) libraries have facilitated the discovery of novel peptides for molecular targeting. While several studies have demonstrated the success of isolating therapeutic peptides from phage display peptide libraries (as reviewed in (10)), we exploited the synthetic nature of OBOC peptide libraries as it provides several key advantages, such as 1) the ability to incorporate non-natural amino acids, and 2) the potential to construct cyclic, turned or branched peptides, all of which can confer enhanced resistance to proteolytic degradation. We have recently described a multiplex ‘beads on a bead’ combinatorial library screening approach that enables a rapid and straightforward identification of peptide affinity ligands for a specific target protein (4). This strategy incorporates the display of target protein on small screening magnetic/fluorescent beads, followed by iterative library bead separation (either by magnetic and/or fluorescence sorting), and on-bead cell-binding validation (4). A photo-cleavable linker was incorporated into the library design to enable an on-bead MALDI mass spectrometric
sequencing to be done in one step (15), making this an attractive approach for high throughput or laboratory bench top ligand discovery.

The epidermal growth factor-like domain 7 (EGFL7) is a ~30 kDa protein that is expressed mostly in the endothelium during embryogenesis, physiological and pathological angiogenesis (16-20). Importantly, EGFL7 levels are very low in quiescent endothelium, and its expression is substantially elevated during endothelial regeneration after vascular injury, indicating that EGFL7 carries an important function during angiogenesis (17-19). Increased EGFL7 expression is also observed in response to hypoxia cultured endothelial cells and neonatal rat brain in vivo (21, 22). Besides the endothelium, EGFL7 has also been detected in progenitor cells and neural stem cells (23, 24). Additionally, EGFL7 has also been detected in several tumors and cancer cell lines, including malignant gliomas, hepatocellular, breast, and lung carcinomas (25-27). Overexpression of EGFL7 in mice led to abnormal vasculature remodeling (20), suggesting the possibility that EGFL7 expression contributes to the formation of irregularly shaped, tortuous and leaky blood vessels. This microvessel morphology is characteristic of tumor angiogenesis, and is linked with cancer-specific mortality (28). In patients with malignant glioma, the expression of EGFL7 correlated with high tumor grade and poor prognosis, indicating that EGFL7 can serve as a predictor factor for the clinical progression of the disease (25). Therefore, affinity ligands that specifically target EGFL7 may serve as promising molecular imaging tools to facilitate early diagnosis and monitor disease progression.

Here, we demonstrate the identification of novel EGFL7-targeting peptides through screening an OBOC peptide library. Using our previously established multiplex ‘beads on a bead’ approach (4), we describe a novel peptide, E7-p72 that specifically labeled human tumor and endothelial cells in an EGFL7-dependent manner. Incorporation of E7-p72 onto Cowpea mosaic virus (CPMV) nanoparticles also demonstrates the ability of this peptide to target nanoparticles to sites of high EGFL7 expression.
4.3 Methodology

Cloning recombinant EGFL7

EGFL7 cDNA was amplified by PCR (sense primer: 5’CACCATGAGGGCTCTCAGGAGGTG3’ and anti-sense primer: 5’CTACGAGTCTTTCTTGCAGGAGCAG3’) and cloned into the pENTR/TEV/D-TOPO vector (Invitrogen) according to the manual’s instruction. The TEV-EGFL7 region was cloned into pDEST20 plasmid (Invitrogen) by recombination and the recombinant bacmid DNA was isolated according to the Invitrogen manual’s instruction.

Expression and purification of recombinant GST-EGFL7

Recombinant baculovirus was generated from Spodoptera frugiperda, Sf21 cells that were transfected with 1µg of bacmid DNA using CellFECTIN tranfection reagent (Invitrogen). Plaque titration of the virus was performed according to the standard protocol described in the Invitrogen manual. Sf21 cells were seeded into a T175 cell culture flask with approximately 80% confluency. Upon cell attachment, the medium was removed and the cells were infected with the recombinant baculovirus at a multiplicity of infection (MOI) of 20 for 72 h at 27°C. The cell lysate was harvested in 0.1% NP40, and passed through a 1mL of glutathione-agarose resin. The column was then washed with 25 mL of wash buffer (150 mM, NaCl, 20 Tris–HCl, pH 7.9), and eluted with elution buffer supplemented with 20mM reduced glutathione. Elutions were collected in 1mL fractions and stored at 4°C. All eluted fractions were analyzed using SDS-PAGE and visualized by silver staining.

Generation of EGFL7-magnetic screening beads

All fractions containing EGFL7 recombinant protein were pooled together. 30 ug of anti-GST antibody (Thermo Scientific) was mixed into the protein solution, and incubated with 370
mg of MagnaBind Protein A/G magnetic beads (Thermo Scientific) for 15 minutes at 4°C. After several washes with wash buffer, the conjugation of protein onto magnetic beads was analyzed by treatment of the screening beads with SDS loading buffer and heat, followed by western blot analysis using anti-EGFL7 polyclonal antibody (R&D Systems).

**Synthesis of Fluorescein-Labeled Peptides**

Fluorescein-labeled peptides were prepared following the general protocols outlines previously. Rink amide resin was used with Cys as the C-terminal amino acid, followed by the addition of Fmoc-Ahx-OH (amino-hexanoic acid) as a linker, followed by the amino acids for the sequence of either E7-p72 and E7-p74. Peptides were cleaved using cleavage cocktail comprised of TFA:EDT:TIS (95:2.5:2.5) and precipitated with cold t-butyl methyl ether. Peptides were dye-labeled using maleimide-fluorescein, purified by preparative HPLC (RP-C18) and characterized by ESI+-MS.

**Screening a OBOC library**

OBOC peptide library beads were washed extensively using 70% ethanol, distilled water and binding buffer (50mM Na₂HPO₄, 150mM NaCl, 2mM CaCl₂, 0.2M L-Arginine, pH 7.4) before screening. First, a library enrichment step to remove potential false positive beads was performed by mixing the OBOC library with magnetic beads coated with the anti-GST antibody and GST protein complex for 1 h at 37 °C with constant shaking at 200 rpm. A strong neodymium magnet (K&J Magnetics, Pennsylvania, USA) was placed at the side of the vial for several minutes, and beads that did not respond to the magnet (enriched library) were transferred into another vial using a micropipette. The enriched library was washed five times with binding buffer. The second (EGFL7 specific) screen was performed by adding 50 µg of the EGFL7-coated screening beads into the OBOC library under the conditions described above. A strong magnet was placed at the side of the vial, and the library was screened as described in (4). Positive hits were pooled and washed extensively with ethanol and water. A secondary cell-based screen was performed using EGFL7-overexpressing HT1080-tdT human fibrosarcoma
cells, and EGFL7-low MDA-MB-435-GFP human breast cancer cells as described by Cho and colleagues (4). Beads were visualized under an Olympus IX70 inverted fluorescent microscope, and beads that associated with the red (high EGFL7 expression) HT1080-tdT cells, but not the green (low EGFL7 expression) MDA-MB-435-GFP cells, were manually isolated using a micropipette. Hit beads were collected, washed and peptides were sequenced on-bead using MALDI TOF MS/MS as described in (15) and (4). Isomeric isoleucine and leucine, and isobaric lysine and glutamine are indistinguishable by their mass to charge ratios and must be uniquely identified using alternate methods (29).

**SPR to identify high-affinity peptide for EGFL7**

Peptides were spotted onto SPR chip (GWC Technologies). Different concentrations (1 nM, 20 nM, and 50 nM) of purified EGFL7 were flown over the chip for 10 mins. All procedures were done according to the SPRimager®II array instrument (GWC Technologies) protocol guide. An association curve was generated by plotting the percent change in reflectivity (%ΔR) at 10 mins against the concentration of EGFL7 (nM) using the GraphPad Prism software (version 5).

**Evaluation of cell uptake of FITC-E7-p72 and FITC-E7-p74 peptides by microscopy**

Human HT-1080 fibrosarcoma cells or human umbilical vein endothelial cells (HUVECs) were detached using EDTA and resuspended in DMEM into microcentrifuge tubes. Cells were incubated with 3.3 μM of fluorescein-conjugated E7-p72, E7-p74 and control AGD peptide in suspension for 1 h at 37 °C. Blocking studies were conducted in the presence of 100x excess of non-fluoresceinated peptides. Cells were washed three times with PBS by centrifugation and seeded onto coverslips, washed and mounted. Images were captured using the Zeiss AxioImager Z1 microscope. The boundaries of cells were manually determined and the average fluorescein intensities within the selected region of interest were quantified using Volocity, version 4.0 (PerkinElmer, Massachusetts, USA). All statistics were performed using a one-way ANOVA and Tukey post hoc test.
siRNA knockdown of EGFL7

EGFL7 siRNA (sense: 5'- UGAAGGAAGAAGUGCAGAGUU -3', antisense: 3'-UUACUUCCUUCUUCACGAGU -5') (Sigma, Canada) or scrambled siRNA (negative control) (sense: 5' GAAGTAACACCCGCACCTAUU 3', antisense: 3'-UUCUUCAUUGUGGGCGUGGAU -5') (Sigma, Canada) were used to transfect HT1080 cells. Cells were seeded onto a 6-well plate and transfected with either EGFL7 siRNA at a final concentration of 40 nM using INTEFERin transfection reagent (Polyplus transfection). After 48 hrs, Western blot analysis of transfected cells was performed to confirm knockdown using the anti-EGFL7 polyclonal antibody (R&D systems).

Evaluation of cell uptake of FITC-E7-p72 by flow cytometry

HT1080 fibrosarcoma cells were stably transfected to overexpress EGFL7-v5 protein (HT1080-EGFL7). EGFL7 knockdown in HT1080 cells were achieved using siRNA (HT1080-KD) as previously described. Regular HT1080, HT1080-EGFL7 (overexpressing) and HT1080-KD cells were seeded onto separate wells in a 6-well tissue culture plate to approximately 80% confluency. The cells were incubated overnight in DMEM containing 10% fetal bovine serum and Pen/Strep at 37 °C with 5% CO2. The media in each well were removed and replaced with pre-warmed PBS. FITC-AGD (negative control) or FITC-E7-p72 peptide was added onto cells to a final concentration of 1 µM and incubated for 1 hour at 37 °C with 5% CO2. Cells were washed three times with PBS, fixed with 4% formaldehyde, and the uptake of FITC-labeled peptides were analyzed by flow cytometry using the COPAS flow cytometer (Union Biometrica). Data analysis was performed using FCS express (version 3).

The evaluation of E7-p72 uptake by HUVECs using flow cytometry was performed using the same procedures.
Synthesis of CPMV-PEG-E7-p72 and evaluation of cell uptake of CPMV-PEG-E7-p72 by EA.hy926 endothelial cells using flow cytometry

Propagation and purification of CPMV was performed using standard procedures described in (30). Alkyne was introduced into the peptide using the following procedure: Peptide synthesis was performed on Fmoc-Lys(aloc) functionalized Rink amide resin (0.1 mmol scale) using standard solid phase peptide synthesis (SPPS) chemistry. The Lys(aloc) was deprotected using phenylsilane (296 uL, 24 eq) and Pd(PPh3)4 (10 mg, 0.1 eq) in degassed DCM (2 mL) for 10 min, rinsed with DCM, and repeated once. N3-(PEG)7-COOH (166 mg, 3 eq) was coupled overnight using HCTU (124 mg, 3 eq) and DIPEA (104 uL, 6 eq) in DMF (2 mL). The final Fmoc group was removed with 20% piperidine in DMF. The peptide was cleaved from resin using 95:2.5:2.5 TFA:TIPS:H₂O (2 mL) for 3 h. The peptide was precipitated with cold methyl tert-butyl ether (MTBE), centrifuged, and the ether decanted. The peptide was purified with preparative HPLC, and the fractions lyophilized to yield the peptide as a TFA salt.

Multifunctional CPMV conjugates were synthesized according to the procedures described by Steinmetz at al. (6).

Step 1: AF647 succinimidyl ester was dissolved in DMSO, the labels were used in a molar excess of 1000 to CPMV (2-3 mg mL⁻¹), the reaction was carried out for 4-5 hrs at room temperature in the dark in a in PBS:DMSO mixture of 9:1. Samples were purified by ultrapelleting (Beckman 50.2 Ti rotor, 42000 rpm, 3 hrs, 4 °C). CPMV was re-suspended in PBS.

Step 2: N-(4-Pentynoyloxy) succinimide (provided by Vu Hong, The Scripps Research Institute) was added in a molar excess of 2000:1 per CPMV nanoparticle. Reaction conditions were as described above, after overnight reaction samples were purified by ultrapelleting and re-suspended in PBS and stored at 4 °C.

Step 3: Covalent attachment of PEG and PEG-E7-p72 was achieved using optimized click chemistry protocols. To a buffered solution of CPMV-A647-alkyne (0.4 µM final concentration) azide-PEG-E7-p72 and azide-PEG-E7-p72 (150 µM) in DMSO, respectively, was added in a molar excess of 625:1 per CPMV. For coupling the following reagents were added: amino
guanidine (AMG, 5 mM), CuSO4:TBTA-OH in a molar ratio of 1:5 (500 µM CuSO4, 2.5 mM TBTA-OH), and sodium ascorbate (5 mM). The ligand TBTA-OH (trishydroxypropyl triazole) was provided by Vu Hong (TSRI). The reaction mixture was incubated at room temperature for 60 min on a shaker prior to purification by ultrapelleting (as described above).

CPMV-PEG (control) and CPMV-PEG-E7-p72 were stored in PBS at 4 °C before further use. Flow cytometry to analyze the cell uptake of CPMV-PEG-E7-p72 and control CPMV-PEG (1 µg) was performed. EGFL7 knockdown in EA.hy926 cells was achieved using siRNA (EA.hy-KD) using jetPRIME transfection reagent (Polyplus Transfection). After 48 hrs, regular EA.hy926 and EA.hy-KD cells were seeded onto separate wells in a 6-well tissue culture plate to approximately 80% confluency. EA.hy921 cells were incubated with CPMV-PEG (control) or CPMV-E7p72 at 37°C for 3 hours. Cells were washed three times with PBS, and then detached using 2.5 mM EDTA, and resuspended in flow buffer (PBS with 2mM EDTA, 2% FBS and 0.05% sodium azide). Data analysis was performed using FCS express (version 3).
4.4 Results

A high-throughput approach to screen OBOC library for EGFL7-binding ligands.

We previously described the development of a multiplex ‘beads on a bead’ approach to exploit the tendency of small magnetic screening beads (2 μm diameter) coated with a target protein to reversibly interact with much larger TentaGel beads (90 μm diameter) that comprise random OBOC combinatorial libraries (4). Purified recombinant GST-EGFL7 (target protein) was adsorbed to 2 μm protein A/G-coated magnetic beads via the anti-GST antibody (Figure 4.1a). It was expected that upon mixing of magnetic screening beads containing the target protein with an OBOC library, the screening beads would coat the TentaGel beads in an affinity-dependent manner (Figure 4.1a). Ligands with the highest affinity for the target protein could then be isolated by placement of a magnet at the side of the vial, as they would pool towards the magnet (Figure 4.1b). The affinity of hit peptides for the target protein could then be validated on-bead using live cells prior to sequencing using mass spectrometry technique as we have previously described (4, 15). HT1080-tdT cells (red) that overexpress EGFL7 (EGFL7 positive) and regular MDA-MB-435-GFP cells (green) (EGFL7 negative) were used in this secondary screen to verify the interaction of hit peptides with EGFL7 on living cells (Figure 4.1c). Hit beads that associated strongly with red, but not green cells were isolated either through manual techniques with a micropipette, or by automated sorting using the COPAS large particle flow cytometer.
Figure 4.1. A high-throughput strategy used to screen an OBOC peptide library against recombinant EGFL7 protein. (a) Schematic representation of the beads on a bead approach. Purified recombinant GST-EGFL7 protein was bound onto Protein A/G-coated magnetic beads via an anti-GST antibody, and mixed with a library of TentaGel beads displaying random 8-amino acid peptides. (b) TentaGel beads displaying high affinity peptides that strongly retained magnetic beads on their surfaces were isolated using a magnet. (c) Schematic representation of a secondary cell-based screen using EGFL7-overexpressing HT1080-tdT cells and EGFL7-low HT1080-GFP cells. Positive hit beads were isolated using the COPAS Biosort instrument (Union Biometrica), and peptides were sequenced on-bead using MALDI TOF MS/MS.
The identification of novel EGFL7-targeting ligands.

First, recombinant EGFL7 was purified using the baculovirus expression system as described in the methodology section. Silver staining of SDS-PAGE gels showed the purification of GST-EGFL7 from cell extract to its purified state (Figure 4.2a). The purification of EGFL7 was confirmed by analyzing the eluted fractions by western blot analysis (Figure 4.2b). Next, in order to generate EGFL7 magnetic screening beads, purified EGFL7 were conjugated onto Protein A/G magnetic beads via an anti-GST antibody. Western blot confirmed that purified EGFL7 was successfully conjugated onto magnetic particles (Figure 4.2c).

To isolate novel peptides targeting EGFL7, 1 g of an octapeptide OBOC library was screened using 2 µm EGFL7-coated magnetic beads. The initial magnetic separation step isolated roughly 500 hits, which were subsequently refined by screening them against living cells and sorting. The expression of EGFL7 in EGFL7 over-expressing (EGFL7 positive) HT1080-tdT fibrosarcoma cells and EGFL7-low (EGFL7 negative) MDA-MB-435-GFP breast cancer cells was confirmed by Western blot (Figure 4.2d). In total, 16 library beads that bound to ‘EGFL7 positive’ cells, but not ‘EGFL7 negative‘ cells were isolated and sequenced using a previously described mass spectrometry approach (15). This led to the successful identification of 8 unique peptide sequences. Four of these hit peptides; E7-p72, E7-p73, E7-p74 and E7-p75 were synthesized and purified based on our confidence in the MALDI deconvolution. The sequences of these peptides are displayed in Table 4.1.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequences</th>
</tr>
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<tbody>
<tr>
<td>E7-p72</td>
<td>HYMFLLGH</td>
</tr>
<tr>
<td>E7-p73</td>
<td>SQSSMYPS</td>
</tr>
<tr>
<td>E7-p74</td>
<td>WYKLHPTM</td>
</tr>
<tr>
<td>E7-p75</td>
<td>EWELHAEE</td>
</tr>
</tbody>
</table>
**Figure 4.2. The identification of novel EGFL7-binding ligands through screening an OBOC library.** (a) Silver-stained gel showing the purification of recombinant GST-EGFL7 protein (left). The eluted fractions were subjected to western blot analysis using a polyclonal anti-EGFL7 antibody (right). (b) Western blot analysis confirming the conjugation of purified EGFL7 protein onto the magnetic beads. (c) A secondary cell-based screening approach to identify peptides with high affinity for EGFL7 displayed on cell surface. Western blot analysis using a polyclonal anti-EGFL7 antibody shows negligible EGFL7 level in MDA435-GFP cells and high EGFL7 level in HT1080-tdT cells that overexpress EGFL7 (right). Fluorescence microscopic image of an example hit bead that strongly associated with HT1080-tdT cells that overexpress EGFL7 (red), but have very little interaction with MDA435-GFP cells (green) that do not express EGFL7.
The characterization of high-binding ligands for EGFL7, E7-p72 and E7-p74

The four peptides listed in Table 4.1 were synthesized and further characterized. The interaction of these peptides with purified EGFL7 was simultaneously monitored through surface plasmon resonance (SPR) imaging (GWC Technologies) (Figure 4.3a). A peptide array was prepared by conjugation of each peptide onto individual gold-coated spots on a SPR chip (GWC Technologies), followed by incubation with purified EGFL7. Two (E7-p72 and E7-p74) of the four hit peptides were observed to bind most strongly to EGFL7 in a concentration-dependent manner (Figure 4.3a). E7-p72 and E7-p74 generated the steepest slopes, indicating that EGFL7 bound to these two peptides at the highest rates (Figure 4.3a). The lines produced by E7-p73 and E7-p75 were not significantly different from that of control (Figure 4.3a). Through monitoring the change in reflectivity over time as EGFL7 associates with or dissociates from the spots, the $K_D$ value for E7-p72 and E7-p74 was calculated to be 13.2 nM and 14.3 nM, respectively (Supplementary Figure 4.1). E7-p72 and E7-p74 were conjugated with a fluorescein dye for further uptake analysis. The chemical structures of FITC-E7-p72 and FITC-E7-p74 are as shown in Figure 4.3b.
Figure 4.3. E7-p72 and E7-p74 have highest affinity for EGFL7. (a) Association curves generated from the interaction of hit peptides with purified recombinant EGFL7 using the SPRImager II (GWC Technologies). Association curves were produced by subjecting peptides immobilized onto the SPR chip with different concentrations of EGFL7 (1 nM, 20 nM, and 50 nM). At $t = 600s$ (10 mins), the percent change in reflectivity ($\Delta R$) was plotted against the EGFL7 concentration (nM) using the GraphPad Prism, version 5.0. The slope ($m$) of each line indicates the rate of EGFL7 association with the peptide. (b) Chemical structure of fluorescein-labeled E7-p72 and E7-p74 (right).
FITC-conjugated E7-p72, but not E7-p74 peptide is taken up by HT1080 fibrosarcoma cells in an EGFL7-dependent manner

E7-p72 and E7-p74 were conjugated with a fluorescent dye (fluorescein) and their ability to selectively bind EGFL7 expressing HT1080 fibrosarcoma cells were assessed using fluorescence microscopy and flow cytometry. The uptake of FITC-E7-p72 by regular HT1080 cells was 6 times higher than that of FITC-AGD (control peptide) (Figure 4.4a and b). Blocking with 100 molar excess of non-fluoresceinated E7-p72 peptide significantly reduced the binding and uptake FITC-E7-p72 (Figure 4.4b). No significant uptake of FITC-E7-p74 by HT1080 cells was observed. The uptake of FITC-E7-p72 by HT1080 cells that overexpress EGFL7 (HT-EGFL7) was approximately 2 times higher than that by regular HT1080 cells (Figure 4.4c and d).

Similarly, flow cytometry analysis confirms the affinity of FITC-E7-p72 with HT1080 cells in an EGFL7 dependent manner. The expression of EGFL7 protein in regular HT1080, EGFL7-knockdown HT1080 (HT1080-KD) and EGFL7-overexpressing HT1080 (HT1080-EGFL7) cells was verified by western blot (Figure 4.5a). The fluorescein signal intensity observed in regular HT1080 cells was significantly higher than that in untreated cells (Figure 4.5b). Knockdown of EGFL7 using siRNA significantly reduced the uptake FITC-E7-p72 by cells, while overexpression of EGFL7 increased the uptake of FITC-E7-p72 by more than 4 times (Figure 4.5b).
Figure 4.4. FITC-E7-p72, but not FITC-E7-p74 peptide is taken up by HT1080 fibrosarcoma cells in an EGFL7-dependent manner as evaluated by microscopy. (a)
Fluorescence images showing the uptake of FITC-conjugated E7-p72, E7-p74 and AGD (negative control) (final concentration of 3.3 µM) by HT1080 cells. Left panels: FITC signal from peptide uptake (green). Right panels: merged images of brightfield image, nuclei staining (red) and FITC-conjugated peptide (green). Blocking study was conducted by adding 100x excess of non-fluoresceinated peptides. Scale bar, 10 µm. (b) Bar graph showing the mean FITC intensity of cells from each group (as in (a)). Measurements of peptide uptake were performed by selection of the perimeter of each cell, and then obtaining the mean FITC intensity within selected regions using the Volocity software (Improvision). The uptake of FITC-E7-p72, but not FITC-E7-p74 was significantly higher than that of control AGD (n = 30, p < 0.001). The uptake of FITC-E7-p72 was significantly reduced in the presence of 100x excess non-fluoresceinated E7-p72 peptide (n = 30, p < 0.001). (c) Fluorescence images showing the uptake of FITC-conjugated E7-p72 (final concentration of 3.3 µM) by HT1080 cells and HT1080-EGFL7 overexpressing cells. Left panels: FITC signal (green). Right panels: merged images of brightfield image, nuclei staining (red) and FITC-conjugated peptide (green). Bar graph showing the mean FITC intensity of cells from each group (from (c)). Peptide uptake was quantified as described above. The uptake of FITC-E7-p72 was significantly higher in HT1080-EGFL7 overexpressing cells compared to regular HT1080 cells (n = 30, p < 0.001). Scale bar, 10 µm. (d) All statistics were performed using a one-way ANOVA and Tukey post hoc test.
Figure 4.5. FITC-E7-p72 is taken up by HT1080 fibrosarcoma cells in an EGFL7-dependent manner as evaluated by flow cytometry. (a) Western blot analysis showing the knockdown of EGFL7 by siRNA and overexpression of EGFL7 in HT1080 cells using the EGFL7 polyclonal antibody (R&D Systems). (b) Bar graph showing the uptake of FITC-E7-p72 by regular HT1080, HT1080-KD (EGFL7 knockdown) or HT1080-EGFL7 (overexpressing) cells. Uptake of FITC-E7-p72 was analyzed by flow cytometry using the COPAS flow cytometer (Union Biometrica). All statistics were performed using a one-way ANOVA and tukey post hoc test.
FITC-conjugated E7-p72 is taken up by HUVECs in an EGFL7-dependent manner

As our main objective is to develop E7-p72 as an EGFL7-targeting agent to image angiogenic blood vessels, we sought to study its uptake by human endothelial cells. Flow cytometry analysis shows the specific uptake of FITC-labeled E7-p72 by human umbilical vein endothelial cells (HUVECs) in an EGFL7 dependent manner. First, the expression of EGFL7 protein in regular HUVECs and EGFL7-knockdown HUVECs (HUVECs-KD) was confirmed by western blot (Figure 4.6a). Approximately 41.2% of cells displayed fluorescence intensity above background signal upon treatment with E7-p72 (Figure 4.6b). Knockdown of EGFL7 in HUVECs decreased the number of cells with signal intensity above background level to 20.9% (Figure 4.6b). The average fluorescein signal observed in HUVECs treated with E7-p72 was significantly higher than that in untreated cells (Figure 4.6c). Knockdown of EGFL7 in HUVECs-KD significantly reduced the uptake FITC-E7-p72 by cells by almost 50% (Figure 4.6c).

Taken together, these data suggest that FITC-E7-p72, but not E7-p74 has a high binding affinity for EGFL7 and specifically targets cells expressing EGFL7.
Figure 4.6. FITC-E7-p72 is taken up by HUVECs in an EGFL7-dependent manner as evaluated by flow cytometry. (a) Western blot analysis showing EGFL7 expression and EGFL7 knockdown (by siRNA) in HUVECs using the EGFL7 polyclonal antibody (R&D Systems). (b) Dot plot showing the uptake of FITC-E7-p72 by HUVECs or HUVECs-KD (EGFL7 knockdown). Flow cytometry analysis was performed using the COPAS flow cytometer (Union Biometrica) and the plot was generated using FCS express (version 3). Fluorescence signal higher than the major population of untreated cells were gated for analysis. (c) Bar graph showing the uptake of FITC-E7-p72 by HUVECs or HUVECs-KD. All statistics were performed using a one-way ANOVA and tukey post hoc test.
CPMV-PEG-E7-p72 nanoparticles are taken up by human endothelial cells in an EGFL7-dependent manner

We then, sought to evaluate the ability of E7-p72 to specifically target fluorescent nanoparticle (conjugated with Alexa Fluor 647 near-infrared dyes and PEG to eliminate non-specific binding) to EGFL7-expressing human endothelial cells. Flow cytometry analysis reveals that CPMV-PEG-E7-p72 nanoparticles specifically bind or are internalized by human EA.hy926 endothelial cells in an EGFL7 dependent manner. The expression of EGFL7 protein in regular EA.hy926 and EA.hy-KD (EGFL7 knockdown) was confirmed by western blot (Figure 4.7a). The mean geometric mean fluorescence intensity of cells treated with CPMV-PEG-E7-p72 (43.4) is much higher than that seen in cells treated with control nanoparticles, CPMV-PEG (14.8) (Figure 4.7b). Knockdown of EGFL7 in EA.hy926 (EA.hy-KD) decreased the mean geometric mean fluorescence intensity to 34.6 (Figure 4.7b).

These data suggest that E7-p72 peptide can be used to direct nanoparticles to sites where EGFL7 expression is high. We intend to further evaluate the ability of CPMV-PEG-E7-p72 to target sites of high EGFL7 expression in vivo in a mouse xenograft model of prostate cancer.
Figure 4.7. CPMV-PEG-E7-p72 is taken up by EA.hy926 human endothelial cells in an EGFL7-dependent manner. (a) Western blot analysis showing the EGFL7 expression and EGFL7 knockdown (by siRNA) in EA.hy926 cells using the EGFL7 polyclonal antibody (R&D Systems). (b) Histogram indicating the uptake of CPMV-PEG-E7-p72 and CPMV-PEG (control) by EA.hy926 or EA.hy-KD (EGFL7 knockdown) cells. The mean fluorescence intensity of each group is indicated in this plot.
Characterization of E7-p72 and E7-p72(Daa) in preparation for in vivo studies using human prostate cancer xenograft mouse model

In preparation for in vivo studies, we first examined if E7-p72 can recognize mouse EGFL7 and target mouse cells. We show that the uptake of FITC-E7-p72 by mouse B16F10 mouse melanoma cells was much higher than that of FITC-AGD (control peptide) (Supplementary figure 4.2a). We intend to investigate the ability of E7-p72 to target mouse EGFL7 more rigorously by performing further uptake studies that incorporates cells with differential expression of EGFL7.

The half-life of E7-p72 in serum was determined to be approximately 8 minutes (Supplementary Figure 4.2a). This result prompted us to pursue the development a more stable peptide alternatives that incorporates D-amino acid. Alanine scan studies through enzyme-linked immunosorbent assay (ELISA) show that only the Phe residue in the middle of the peptide was crucial for E7-p72 binding to EGFL7, and that mutation of the Phe to Ala residue decreased the binding of E7-p72 to EGFL7 to a level observed in the negative control peptide (Supplementary Figure 4.2b). Based on our observation that the flanking His residues were not essential for binding to EGFL7, we substituted both L-His (H) in E7-p72 with D-histidines (h) to generate a 2nd generation peptide, E7-p72(Daa). We have demonstrated that E7-p72(Daa) has an improved half life in both human serum (~2.5 hours) and mouse serum (~1 hour) (Supplementary Figure 4.2c). However, further investigation is required to test the efficacy of this peptide in targeting EGFL7 in vitro and in vivo.
4.5 Discussion and conclusion

Here, we report the discovery of novel EGFL7 affinity ligands from screening an OBOC library using our previously developed ‘beads on a bead’ strategy (4). While we have described the utility of the biotin-streptavidin interaction to directly conjugate and display the target protein onto magnetic beads in an unbiased manner, our initial attempts to biotinylate EGFL7 has been unsuccessful. We presume that this issue may be related to the folding pattern of EGFL7, and that it is possible that EGFL7 does not contain any exposed lysine residues on its surface that are available for biotinylation through the N-hydroxysuccinimide ester reaction. As an alternative method, an anti-GST antibody has been used to conjugate recombinant GST-EGFL7 protein onto magnetic beads. Since only the GST epitope was directly linked onto the magnetic bead, we presume that this conjugation did not mask any sites on EGFL7, and that all surfaces of EGFL7 are equally exposed to the ligands on the library beads.

To eliminate false positives, that is promoted by the utility of several extrinsic molecules (ie. magnetic beads, anti-GST antibody and GST protein), we have performed a library enrichment step by first removing all the library beads that interacted with magnetic beads coated with purified GST protein via the anti-GST antibody. Magnetic beads containing EGFL7 are then used to screen the enriched library. To further reduce the number of false positives, an on-bead cell-based assay has been performed to verify affinity of isolated ligands for EGFL7 that are expressed in their native conformation on the cell surface. This step can significantly narrowed down the final number of hits for sequencing analysis, and increased the prospect of attaining high affinity ligands that can target EGFL7 on cell surface.

From the screen, we have isolated four peptide ligands of EGFL7, with no conserved motif observed between peptides. E7-p72 and E7-p74 peptides have exceptional affinity for purified EGFL7 protein, although only E7-p72 binds to and is taken up by cancer cells and endothelial cells in an EGFL7-dependent manner. No conserved motif is observed between peptides, and the E7-p72 sequence is not found in any human proteins. However, a similar motif is detected in sphingolipid delta(4)-desaturase DES1 and DES2 (HYMFLKGH), as well as in G-protein coupled receptor 146 (HYLILLGH). These findings may suggest that these proteins can
be potential ligands of EGFL7, and can therefore, provide important information and give us new insights into the mechanisms by which EGFL7 function.

Here, we describe the synthesis and application of E7-p72 as a first line peptide for molecular imaging of EGFL7 expressing cells. Although this peptide is successful at targeting cancer cells and endothelial cells in an EGFL7 dependent manner, this peptide is composed of L-amino acids, and with a very short half-life of 8 minutes in human serum. While this may not be an issue for applications in vitro, the stability of E7-p72 needs to be optimized prior to utilization in vivo. Nonetheless, E7-p72 can serve as a parent peptide for further development of novel EGFL7 ligands with improved stability and greater targeting efficacy. We are currently in the process of developing and characterizing a 2nd generation peptide, E7-p72(Daa) that incorporates flanking D-histidines. Although we have demonstrated that E7-p72(Daa) has an improved half life in both human and mouse serum, further investigation is required to test the efficacy of this peptide in targeting EGFL7 in vitro and in vivo.

While this novel peptide effectively targets EGFL7-expressing cancer cells and endothelial cells in vitro, we are in the process of evaluating the ability of E7-p72 to target fluorescent nanoparticles to prostate tumors and/or angiogenic sprouts in mice in vivo. As our preliminary results indicate that the uptake of E7-p72 by mouse melanomal cells is significantly higher than that of control peptide, combine with our knowledge that the mouse EGFL7 sequence is highly homologous to human EGFL7 (31), we are optimistic that E7-p72 can be used to target nanoparticles to EGFL7-expressing tumors and angiogenic vessels.
References


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Chapter 5

EGFL7 peptide, E7-C13 for modulating angiogenesis

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Keywords: Peptide, angiogenesis, cancer, receptor tyrosine kinase (RTK), EGFL7, angiogenic inhibitor, therapeutic agent

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5.1 Abstract

Angiogenesis is a promising therapeutic target in cancers, supported by a comprehensive analysis of tumour vasculature in prostate cancers that reported that men with the most irregularly shaped vessels are 17.1 times more likely to develop lethal disease. These vessels specifically express a recently identified 30 kDa secreted protein called epidermal growth factor-like 7 (EGFL7). Here, we describe the identification and characterization of novel EGFL7-derived peptides that can inhibit angiogenesis through interfering with the function of EGFL7. We found two motifs from the EGFL7 sequence that are highly conserved across multiple species, but are not present in its paralog, the EGFL8 protein. We demonstrate that E7-C13 inhibited the morphogenesis of endothelial cells in vitro and also reduced the level of angiogenesis induced by tumor cells in vivo in both the avian embryo and mouse models. Efforts to evaluate the mechanism of action and the efficacy of these peptides in treating tumours in animal models are currently underway. These peptides could provide a basis for a new generation of therapeutic agents for locally advanced prostate cancers.
5.2 Introduction

Angiogenesis is a process where new capillaries are formed from existing blood vessels, and has been implicated in a variety of pathological human diseases, including cancer and diabetic retinopathy. The importance of angiogenesis in the progression of tumorigenesis is well recognized and established (reviewed in (1)). The angiogenic switch is activated during the early stages of tumor development, whereby tumors secrete diffusible angiogenic inducers to signal quiescent vasculature to undergo capillary sprouting. Tumor cells express various angiogenic factors, including vascular endothelial growth factor (VEGF) (2), tumour necrosis factor alpha (TNF-α) (3), and transforming growth factor-alpha (TGF-α) (4), leading to increased angiogenesis. Receptor tyrosine kinases (RTKs) are cell surface membrane proteins that mediate the transmission of extracellular cues from signaling molecules (such as growth factors, cytokines or hormones) into the intracellular environment, thereby modulating processes such as angiogenesis (reviewed in (5)). Activation of RTKs through binding of a ligand on the extracellular domain leads to receptor dimerization and auto-phosphorylation of the receptor, resulting in a series of signaling transduction which ultimately governs several important cellular processes including cell migration, cell proliferation and angiogenesis (reviewed (6)).

A convergent perspective regarding angiogenic activators is that negative regulators, known as angiogenesis inhibitors, play an equally important role in controlling the switch. The significance of angiogenesis inhibitors were first introduced by Bouck and colleagues who demonstrated that tumorigenic cell lines contained much lower levels of inhibitor, which was later identified as a truncated version trombospondin-1 (TSP-1) (7, 8). Several anti-angiogenic agents have been developed to target different stages involved in angiogenesis (reviewed in (9)). Advancement in screening technologies continues to facilitate the discovery of novel anti-angiogenesis agents (10, 11).

Several anti-angiogenesis compounds have been developed, and have either been approved by the FDA (such as Avastin (12), Sutent (13) and Nexavar (14)) or are still currently undergoing clinical trials (such as TNP-470 (15), aflibercept (16), cediranib (17) and pazopanib (18). Nonetheless, despite their ability to effectively treat certain cancers, these drugs each have their own limitations. For example, although Avastin has been demonstrated to significantly
increase the progression-free survival and overall survival of patients with metastatic colorectal cancer (19), it did not provide any benefit to the survival of patients with metastatic breast cancer (20). In addition, it has also been shown that most tumors develop resistance mechanisms to anti-angiogenic drugs (21, 22), most likely as a consequence of long-term administration. As angiogenesis is regulated by several different signaling pathway, blocking a single pathway can lead to resistance when tumors develop an alternative angiogenesis mechanism (reviewed in (23)). Targeting multiple pathways of angiogenesis may overcome or delay resistance, highlighting the importance of combining drug therapies in the clinic. Hence, there remains a great necessity to identify new targets associated with angiogenesis and the development of novel pre-clinical anti-angiogenic agents.

Epidermal growth factor-like domain 7 (EGFL7) is a ~30 kDa protein that consists of an amino-terminal signal peptide domain, an EMI domain and an EGF-domain (containing 2 EGF repeats) (24). EGFL7 is a secreted protein that is predominantly associated with the extracellular matrix (ECM) upon secretion (25). The EGFL7 protein has a parologue, known as EGFL8 (25). EGFL7 is expressed mainly in the endothelium, with high levels observed in endothelial cells during embryogenesis, as well as physiological and pathological angiogenesis. Recent studies show that EGFL7 regulates the development of blood vessels by creating a permissive environment for endothelial cell migration and assembly sprouting of endothelial sprouts (25, 26). EGFL7 is a chemotactant for endothelial cells, and also promotes cell adhesion (25, 27). EGFL7 has been implicated to modulate angiogenesis through inhibition of the Notch signalling pathway (28, 29). *In vivo* study in zebrafish demonstrates that EGFL7 knockdown impairs tubulogenesis through a mechanism that is independent of VEGF signaling (25). Overexpression of EGFL7 in mice results in defective vasculature remodeling, with embryos exhibiting partial lethality and hemorrhaging (29). Altogether, these studies demonstrate that EGFL7 plays a crucial role in vasculature patterning, remodeling and stabilization, highlighting the importance of EGFL7 for angiogenesis.

Recent preclinical studies show that combining anti-VEGF therapy and humanized anti-human EGFL7 monoclonal antibodies led to additional inhibitory effect of tumor growth in human cancer xenograft mouse models when compared to anti-VEGF therapy (Avastin) alone (http://ip.com/patapp/US20110200602). Clinical trials (by Genentech) are currently underway to

Peptide based probes are attractive tools for developing therapeutic agents due to their size, stability, solubility, improved pharmacokinetics and lack of immunogenicity (30). The protein sequence of endogenous angiogenesis inducers or inhibitors contains vital information for designing new anti-angiogenic peptides as therapeutic drugs, as conserved domains or critical amino acid residues are critical for their function (reviewed in (31)). We therefore sought to identify and design novel bioactive anti-angiogenic peptides derived from endogenous human EGFL7 protein. To do this, we performed a Basic Local Alignment Search Tool (BLAST) search to identify regions of local similarity between the EGFL7 sequences from various species, and discovered the E7-C13. Herein, we report a first line peptide against EGFL7, E7-C13, which possesses anti-angiogenesis activity both in vitro and in vivo. This peptide is a promising candidate for developing novel anti-angiogenic agents.
5.3 Methodology

Synthesis of E7-C13 (Ac-GSLLVHSFQQLG-NH₂)

The unsubstituted rink-amide resin was treated with 20% piperdine in DMF to provide a free amine polymeric support ready for coupling. Fmoc-Gly-OH was then coupled to the support using HBTU (3 eq.) as coupling agent in DMF in presence of DIPEA (6 eq.). The removal of Fmoc protective group could be achieved in basic conditions using 20% piperidine solution in DMF. The elongation of the peptide has been carried out in a sequentially manner by alternating deprotection of Fmoc group and coupling of the amino acid. Each individual deprotection/coupling reaction was monitored for completeness prior to the next deprotection/coupling by ninhydrin test. Finally, acetylation of the N-terminal was performed on the solid support using anhydride acetic (Ac₂O), Hunig base with DCM as solvent. The peptide was cleaved and deprotected from the polymeric support using a freshly prepared TFA cocktail containing phenol, EDT and distilled water as scavengers at room temperature. The peptide was precipitated from the acidic cocktail upon addition of tert-butyl methyl ether and was collected by centrifugation. Lyophilisation led to the crude peptide powder which was purified by reverse phase HPLC on a SunFire Prep column (3 x 150 mm, Waters) under linear gradient elution with eluent A consisted of 0.1% TFA in water while eluent B consisted of 0.1% TFA in acetonitrile. The elution was monitored at 220 nm at a flow rate of 20 mL/min. The purity of the final peptide product was checked by analytical HPLC performed on a reverse phase SunFire analytical column (4.6 x 150 mm, Waters) using the mobile phases described above, using a solvent gradient of 10% - 90% B over 15 min with a flow rate of 2 mL/min and detection at 220 nm.

The scrambled peptide (control) (Ac-QVLSGLHQGLFS-NH₂) was synthesized and purified using the same method.

Synthesis of control Flag peptide (H-CSGDYKDDDDD-OH)

The peptide was synthesized using the standard Fmoc chemistry on a multistep automated synthesizer (Appec) at 0.1 mmol scale with Fmoc-Lys(Boc)-Wang resin as polymeric support.
under nitrogen atmosphere. The Fmoc-amino acids were used with the following side-chain protections: Asp-(OtBu), Lys-(Boc), Ser-(tBu), Tyr-(OtBu), Lys(Boc) and Cys-(tBu). Acidic treatment of the peptidyl-resin allowed the cleavage and deprotection of the peptide using TFA with the following scavengers: phenol, EDT, thioanisole and distilled water. The peptide product was purified and the purity checked using same conditions as described above.

**Purification of recombinant EGFL7-myc protein**

Recombinant baculovirus was generated from *Spodoptera frugiperda*, Sf21 cells that were transfected with 1 µg of bacmid DNA using CellFECTIN tranfection reagent (Invitrogen). Plaque titration of the virus was performed according to the standard protocol described in the Invitrogen manual. Sf9 cells were cultured in a shaking incubator in SFM-900 II media (Invitrogen). Cells were infected with the recombinant baculovirus at a multiplicity of infection (MOI) of 20 for 72 hrs at 27°C. After 72 hrs, cells were lysed in lysis buffer (150 mM NaCl, 20 mM sodium phosphate, 0.1% NP40, pH 7.4), and the lysate was collected after centrifugation. Supernatant from myc hybridoma cells were added into the lysate, and the mixture was incubated on ice for 10 minutes. Protein A/G agarose (Thermo Scientific) were washed with lysis buffer, added into the mixture and incubated overnight on a rocker at 4°C. Resin was collected by centrifugation, washed three times with lysis buffer, and eluted in 250 µL of elution buffer (300 mM NaCl, 50 mM Na₂HPO₄, 200 mM L-arginine, 2 mM CaCl₂, pH 4). 250 µL of neutralizing buffer (50 mM sodium phosphate, 200 mM L-arginine, 2 mM CaCl₂, pH 8) was added to each elution, and protein was stored at 4°C before use. All eluted fractions were analyzed by SDS-PAGE and visualized by Coomassie or silver staining.

**Surface plasmon resonance (SPR) study to establish the binding affinity of E7-C13 to purified EGFL7 protein**

E7-C13 and control peptide were spotted onto SPR chip (GWC Technologies). Different concentrations (1 nM, 20 nM, 50 nM and 100 nM) of purified EGFL7 were inserted into the SPR
instrument and allowed to come into contact with the chip for 10 mins. All procedures were done according to the SPRimagır®II array instrument (GWC Technologies) manufacturer’s guide. The percent change in reflectivity (%R) at 10 mins was plotted against the concentration of EGFL7 (nM) to generate an association curve using the GraphPad Prism software (version 5).

**Endothelial morphogenesis/angiogenesis assay**

A 96-well plate was coated with 50 µL per well of Matrigel. After the Matrigel solidified, approximately 2000 human umbilical vein endothelial cells (HUVECs) were seeded onto the surface of the polymerized Matrigel. VEGF (10 ng/ml) and bFGF (10 ng/ml) were added into each well as the angiogenic stimulus. Flag or E7C13 peptide was introduced at a concentration of 200 µM and cells were incubated at 37°C in 5% CO₂ for 6 hours. Cells were then visualized under an inverted microscope and endothelial tube formation was quantified by counting the number of branch points from each well (n = 3). All statistics were performed using a one-way ANOVA and Tukey post hoc test.

**On-plant CAM In vivo angiogenesis assay**

Avian embryos were cultured ex ovo according to the procedures described in Zijlstra and Lewis (32, 33). Collagen containing on-plants were prepared using HT1080 cells as positive angiogenic stimuli and transferred to the CAM of day 9 embryos as per Zijlstra and Lewis (32, 33). The embryos were allowed to incubate undisturbed in a humidified incubator for 3 days, and the on-plants were imaged under the Lumar stereomicroscope (Zeiss Canada, Toronto, Canada). The on-plants were scored as illustrated by Zijlstra and Lewis (32), and the angiogenic index was determined.

**Directed in vivo angiogenesis assay (DIVAA)**

A DIVAA kit was used to assess angiogenesis in 7 week old female Nude mice (Crl:NU-Foxn1nu; Charles River), as per the manufacturer’s recommended protocol (Trevigen, Gaithersburg, MD). Briefly, angioreactor tubes were filled with basement membrane extract
and inserted subcutaneously into mice. After 10 days, mice were sacrificed, angioreactors were removed, and angioreactor contents were transferred into a centrifuge tube. Endothelial cells were labelled overnight in a FITC-Lectin solution, and fluorescence was quantified using a plate reader. All experiments involving animals were approved by the Animal Use Subcommittee at the University of Western Ontario (Protocol No. 2008-101).

**siRNA knockdown of EGFL7**

EGFL7 siRNA (EGFL7-si) (sense: 5`- UGAAGGAAGAAGUGAGAGUU -3`, antisense: 3`- UUACUUCUUCUUCAGUCUC -5`) (Sigma, Canada) or scrambled siRNA (negative control) (sense: 5` GAAGTAACACCCGCACCTAUU 3`, antisense: 3`-UUCUUCAUGUGGGCGAGA -5`) (Sigma, Canada) were used to transfect HUVECs. HUVECs were seeded onto a 6-well plate and transfected with either EGFL7 siRNA at a final concentration of 40 nM using INTEFERin transfection reagent (Polyplus transfection). After 48 hrs, Western blot analysis of transfected cells was performed to confirm knockdown using the anti-EGFL7 polyclonal antibody (R&D systems).

**Cell adhesion assay**

EA.hy926 endothelial-derived cells treated with EGFL7 siRNA or scrambled siRNA (negative control) were detached by treatment with trypsin/EDTA and resuspended in DMEM containing 1% serum. Cells were plated into 96-well plate and treated with 200 µM of Flag, scrambled or E7-C13 peptide for 4 hrs at 37 °C. Cells were then washed three times with PBS and fixed with 4% formaldehyde. Cell nuclei were stained with Hoechst (Pierce) and imaged under the EVOS f1 inverted fluorescent microscope (AMG). The number of cells that remained adhered to the plate was determined using by Volocity, version 4.0. All statistics were performed using a one-way ANOVA and Tukey post hoc test.
Screening for activation of specific tyrosine kinases using a phospho-RTK profiler array system

HUVECs were detached by treatment with trypsin/EDTA and resuspended in EGM. Cells were plated into a T-175 flask and treated with 200 μM of Flag or E7C13 peptide for 4 hrs at 37 °C. Cells were harvested and lysed in Array Buffer 1 according to the Human Phospho-Receptor Tyrosine Kinase (RTK) Array Kit (R&D systems) manufacturer’s guideline. After blocking, each phospho-RTK array membranes were incubated with 100 μg of lysate according to the manufacturer’s guidelines. Membranes were washed, and incubated with anti-phospho-HRP. Activated tyrosine kinases were detected by enhanced chemiluminescence (ECL) as per manufacturer’s guidelines.
5.4 Results

Sequence analysis of EGFL7 protein and identification of E7-C13 peptide

EGFL7 has a molecular weight of ~30 kDa and is important for a role in vascular development and angiogenesis (reviewed in (34)). In search for conserved sequences in EGFL7 that could be responsible for its function in angiogenesis, we compared the sequences of EGFL7 protein from six different species: human, cow, mouse, rat, chicken, and dog using the ClustalW multiple sequence alignment (Figure 5.1a). We observed that several domains remain highly conserved across these species (Figure 5.1a). We then compared the protein sequences of human EGFL7 and its paralog, EGFL8 using the ClustalW multiple alignment sequence (Figure 5.1b). We distinguished the E7-C14 and the E7-C13 sequences, which are unique to EGFL7 but not present in EGFL8, and is conserved across the six species that we analyzed (Figure 5.1a and b). In human EGFL7, the E7-C14 peptide is located between amino acids 213–226, while the E7-C13 peptide is located between amino acids 237–248 (Figure 5.1c).
Figure 5.1. Sequence analysis of EGFL7 protein and identification of E7-C13 peptide. (a) EGFL7 protein sequence homology between six different species (human, cow, mouse, rat, chicken and dog) using the ClustalW multiple sequence alignment tool. The E7-C14 and E7-C13 sequences are indicated in a black box. (b) Protein sequence alignment between human EGFL7 and its paralog, EGFL8 using the ClustalW multiple sequence alignment tool. The E7-C14 and E7-C13 sequences are indicated in a black box. (c) Protein structure of EGFL7 (signal peptide: orange box, EMI domain: blue box, EGF domain: red box, E7-C14 peptide sequence: purple and E7-C13 peptide sequence: green).
The inhibition of HUVECs morphogenesis by E7-C13 peptides *in vitro*

E7-C14 and E7-C13 were synthesized as described in the methodology section. The effects of E7-C14 and E7-C13 peptides on angiogenesis were investigated first using *in vitro* matrigel angiogenesis assay. Human umbilical vein endothelial cells (HUVECs) were allowed to spontaneously sprout and form tubes on solid matrigel in the presence of VEGF and bFGF. Treatment with E7-C13 resulted in significant decrease in tube formation compared to controls (H2O and Flag peptide), while E7-C14 only exhibited a modest inhibition (Figure 5.2a). HUVECs treated with either H2O or Flag interacted with each other and formed sprouts, while cells treated with E7-C13 remained relatively unchanged (Figure 5.2a). E7-C14 also displayed slight inhibition in the level of HUVECs sprouting, although this reduction was not significant compared to control. The level of angiogenesis was quantified by counting the number of branch points formed in each groups. E7-C13 significantly decreased the formation of branch points by approximately 4 times (Figure 5.2b). We showed that E7-C13 significantly inhibited endothelial cells morphogenesis *in vitro*. 
Figure 5.2. E7-C13 inhibits sprouting and tube formation of HUVECs in vitro. (a) *In vitro* angiogenesis assay demonstrating a significant decrease in the level of sprouting and tube formation in HUVECs on Matrigel in the presence of VEGF and FGF after treatment with E7-C13 (but not E7-C14) compared to controls (Flag peptide or H20) (at a concentration of 200 µM). (b) Bar graph showing the number of branch points that was formed by HUVECs from each group. The level of angiogenesis was quantified by counting the number of branch points (indicated by arrows). All statistics were performed using a one-way ANOVA and tukey post hoc test.
Characterization of E7-C13 peptide

Recombinant EGFL7 protein was purified as described in the methodology section. The chemical structure of E7-C13 is illustrated in Figure 5.3a. The association of E7-C13 with purified EGFL7 protein was determined using surface plasmon resonance (SPR). SPR analysis shows that the association of E7-C13 with EGFL7 is significantly stronger than that of control peptide, with a 50% maximal binding at saturation ($K_{50}$) of approximately 30 nM. (Supplementary Figure 5.1). To advance to in vivo studies, we examined the plasma stability of E7-C13 peptide. E7-C13 was incorporated into 100% human serum, and the amount of peptide remaining at each time point was quantified over a period of 7 hrs using high-performance liquid chromatography (HPLC). The plasma half-life of E7-C13 was calculated to be approximately 5.5 hours in 100% serum (Figure 5.3b).
Figure 5.3. Synthesis and characterization of E7-C13. (a) Chemical structure and sequence of E7-C13. Structure was generated using the ChemDraw software. (b) Serum stability plot demonstrating the peptide stability of E7-C13 in 100% human serum. Peptide was treated with serum at a final concentration of 50 µg/mL at 37°C. At each time point, 200 µL of serum was retrieved, and serum proteins were precipitated in cold ethanol. The supernatant containing the peptide was retrieved, frozen in dry ice and lyophilized. Samples were resuspended in 200 µL of H₂O and 40 µL of MeCN, and analyzed by HPLC. Graph was generated using the linear function in GraphPad Prism. The half-life of E7-C13 is 327 minutes (5.5 hrs).
E7-C13 inhibits angiogenesis *in vivo* using the avian CAM model

Next, the anti-angiogenic efficacy of E7-C13 was evaluated *in vivo* in the avian chorioallantoic membrane (CAM) model. HT1080 cells were cultured onto fiber-mesh to stimulate the recruitment of blood vessels from the CAM onto the mesh. Visible blood vessels growing into the collagen on-plants containing HT1080 fibrosarcoma cells (Figure 5.4a). E7-C13 significantly reduced the number of new blood vessels that entered the on-plant at a concentration of 200 µM compared to a vehicle control (Figure 5.4b). The level of angiogenesis observed upon treatment with E7-C13 is comparable to that of background angiogenesis, which occurs in control on-plants.
Figure 5.4. E7-C13 inhibits angiogenesis in the avian CAM model. (a) *In vivo* angiogenesis assay demonstrating that E7-C13 inhibits angiogenesis using the chicken chorioallantoic membrane (CAM) model. HT1080 cells were cultured onto collagen-containing fiber-mesh to stimulate the recruitment of blood vessels from the CAM onto the mesh. A negative control group with no HT1080 cells was also used. Visible blood vessels growing into the collagen on-plants are indicated by red arrows. (b) Bar graph showing the level of angiogenesis observed in each group (n > 32). The angiogenic index was quantified by counting the percentage of grids within the mesh that contains visible blood vessels (# of squares containing blood vessels/total # squares). All statistics were performed using a one-way ANOVA and tukey post hoc test.
E7-C13 inhibits angiogenesis in mice using DIVAA

The anti-angiogenic potential of E7-13 was investigated in mice using the directed *in vivo* angiogenesis assay (DIVAA). Angioreactor tubes (semiclosed silicone cylinders that are approximately 1 cm in length) were filled with basement membrane extract (BME). HT1080 cells were added to stimulate angiogenesis into the angioreactors. E7-C13 significantly inhibited the growth of new blood vessels into the angioreactor at a concentration of 200 µM compared to a vehicle control (positive control) (Figure 5.5). The level of angiogenesis observed after treatment with E7-C13 is comparable to that of background angiogenesis that is observed in negative control angioreactors that contained no HT1080 cells.

![Graph showing percent angiogenesis](image)

**Figure 5.5.** E7-C13 inhibits angiogenesis in mice using the directed *in vivo* angiogenesis assay (DIVAA). Bar graph showing the percent angiogenesis observed in angioreactor tubes (n = 8) from each group. Angioreactors were filled with basement membrane extract (BME) and HT1080 cells to stimulate angiogenesis. BME (-ve) is a negative control that contained only
BME without cells. All statistics were performed using a one-way ANOVA and tukey post hoc test.

**E7-C13 does not alter the adherent property of human endothelial cells.**

A study in zebrafish revealed knockdown of EGFL7 resulted in impaired vascular tube formation due to the failure of angioblasts to separate and retain extensive tight junctions (25). It was shown that EGFL7 promotes endothelial cell adhesion, although the strength of adhesion was significantly weaker compared to other cell adhesion proteins, such as fibronectin and collagen (25). This suggests that EGFL7 provides a permissive substrate that predominantly favors motility over stable adhesion to allow the local migration of angioblasts during the process of tube formation. Because E7-C13 is a peptide that is derived from the sequence of EGFL7, we examined whether E7-C13 inhibits angiogenesis through direct impediment of EGFL7 activity. Cell adhesion assay using EA.hy926 human endothelial cells treated with Flag (control), scramble (control) or E7-C13 peptide (200 µM) shows that E7-C13 did not affect the attachment of cells on culture plates (Figure 5.6a). siRNA knockdown of EGFL7 in cells significantly decreased cell adhesion (Figure 5.6a). Vinculin staining of EA.hy926 cells reveals no difference between cells treated with scramble (control) or E7-C13 peptide in the formation of focal adhesions on glass coverslip (Figure 5.6b).
**Figure 5.6. E7-C13 does not affect cell adhesion of EA.hy926 human endothelial cells.** (a) Cell adhesion assay showing that E7-C13 (200 µM) does not alter the adherent properties of EA.hy926 endothelial cells compared to Flag or scramble peptide. Knockdown of EGFL7 in EA.hy926 cells using siRNA significantly decreased cell adhesion (p < 0.01). Cell nuclei were stained with Hoechst dye, imaged and counted using the Volocity software (version 6.1.2, Improvision). All statistics were performed using a one-way ANOVA and tukey post hoc test. (b) Vinculin staining of EA.hy926 cells showing focal adhesion formation on glass coverslip. Left panel: Vinculin (orange) and DAPI (blue). Right panel: merged images of vinculin (orange), WGA plasma membrane stain (green) and DAPI (blue). Scale bar, 10 µm.

**RTK activation profile of HUVECs treated with E7-C13**

To elucidate the molecular mechanism in which E7-C13 is modulating angiogenesis, we screened an array of 49 different anti-human receptor tyrosine kinase (RTK) antibodies to determine the profile of tyrosine kinase activation upon treatment of HUVECs with E7-C13. Lysate from HUVECs treated with E7-C13 at a concentration of 200 µM showed differential activation of several RTKs compared to that from Flag (control) (Figure 5.7a). The intensity of
each spot was quantified using the Volocity software (version 6.1.2, Improvision), and the level of phosphorylation of each RTK was compared according to the coordinates displayed in Figure 5.7b. We observed that E7-C13 treatment results in a decrease in phosphorylation of anaplastic lymphoma kinase (ALK), fibroblast growth factor receptor 3 (FGFR3), Axl and vascular endothelial growth factor receptor 1 (VEGFR-1).

Figure 5.7. Profiling RTK activation in HUVECs upon treatment with E7-C13. (a) Array membranes displaying 49 different anti-human RTK antibodies incubated with lysates from HUVECs treated with either control Flag or E7-C13 peptide (200 µM) for 4 hrs (R&D systems). Phosphorylated RTKs were detected by incubation with pan anti-phospho-HRP followed by treatment with enhanced chemiluminescence. (b) Phospho-RTK array coordinates (left) and their respective RTKs (right table).
5.5 Discussion and conclusion

We report here the identification of a novel E7-C13 peptide that is derived from the EGFL7 protein sequence that exhibits anti-angiogenesis potential. Initially, we identified two different sequences, E7-C14 and E7-C13, which are both highly conserved across several different species and unique to EGFL7, but not present in its paralog, EGFL8. Protein sequence alignment using the NCBI basic local alignment search tool (BLAST) blastp algorithm reveals that these sequences are not found within other human protein sequences. Since EGFL7 is a very important regulator of vasculogenesis (reviewed in (34)), we postulated that these unique peptide sequences that is highly conserved across multiple species would have biological importance that assumes a crucial function in modulating angiogenesis. Out of the two peptides, we found that E7-C13 exhibited a much greater anti-angiogenesis potential compared to E7-C14 in in vitro angiogenesis assays.

Here, we describe the synthesis and application of E7-C13 as a first line peptide that is directly derived from the sequence of EGFL7 as an angiogenesis inhibitor. This peptide is composed of L-amino acids, and with a half-life of ~5.5 hours in human serum, which may attribute to the high working peptide concentration that is required for anti-angiogenic effect in vivo. Nonetheless, E7-C13 can serve as a parent peptide for further development of novel anti-angiogenic agents with greater efficacy and potency. As reviewed by Soto and colleague, the conversion of peptide to clinically useful drugs often involves chemical modifications, such as introduction of D-amino acids, non-natural amino acids and peptide cyclization in order to improve peptide stability (35). The efficacy of E7-C13 in inhibiting angiogenesis through intravenous administration route is yet to be determined.

We have established that E7-C13 has a high binding affinity for purified recombinant EGFL7 protein through surface plasmon resonance analysis. Since EGFL7 is a secreted protein that is mostly deposited onto the ECM, we therefore, postulate that E7-C13 interacts directly with EGFL7 on the surface of endothelial cells, leading to the inhibition of its function in modulating angiogenesis. Nonetheless, further investigations are required to specifically address and validate this hypothesis. EGFL7 has been shown to promote endothelial cell adhesion and
the formation of focal adhesions (25). Since we observed that E7-C13 neither affects endothelial cell adhesion nor the formation of focal adhesions \textit{in vitro}, it is possible that E7-C13 represses angiogenesis through other EGFL7-signaling pathways without influencing the adherent property of cells.

We sought to uncover the mechanism of action of E7-C13 by screening an array containing 49 different receptor tyrosine kinase (RTK) antibodies to simultaneously monitor the increase or decrease in phosphorylation of each RTK upon treatment of human endothelial cells with E7-C13. We observed that E7-C13 decreases the activity of ALK, FGF3, Axl and VEGFR-1. ALK has been identified to be a receptor of for the growth factor, Pleiotrophin (PTN) (36) that has been implicated to serve as a rate-limiting factor for angiogenesis (37, 38). Overexpression of FGFR3 in myeloma and bladder cancer patients is correlated with increased disease pathogenesis, poor clinical response and decreased survival (39, 40). Axl has been shown to play an important role in angiogenesis and tumorigenesis through the angiopoietin/Tie2 and the Dickkopf signaling pathways (41). Similar to the phenotype we observed with E7-C13, Axl knockdown results in the impairment of tube formation in human endothelial cells (41). VEGFR-1 is essential for the organization of embryonic vasculature, and that disruption of of the VEGFR-1 gene results in severe abnormalities in blood vessel formation (42). Although there are currently no clinically approved drugs that specifically target and hamper the activity of these RTKs, inhibitors of these RTKs have exhibited great promises for cancer therapy. For example, inhibition of ALK with a potent drug induced regression of established lymphomas (43), while monoclonal antibodies against Axl attenuated the growth of non-small cell lung carcinoma xenografts (41). These reports strengthen our belief that E7-C13 can serve as a promising anti-angiogenic agent for treating cancers.

The process that E7-C13 undertakes to deactivate these RTKs is currently unclear, and more investigations are required to unveil the exact mechanism that leads E7-C13 to inhibit angiogenesis. Uncovering the mechanism of action of E7-C13 could potentially allow us to link the function of EGFL7 to specific RTK pathways, therefore giving us insights into novel mechanisms by which EGFL7 promotes angiogenesis and tumorigenesis. Identifying the specific targets of E7-C13 could also help us establish the potential for using E7-C13 to treat patients that are resistant to therapeutic drugs that specifically inhibits other RTK activities. Ultimately, the
use of E7-C13 can be expanded to incorporation into nanoparticles to target EGFL7 for potential applications in imaging and drug delivery.

References


Chapter 6

Discussion and Conclusion

6.1 Summary and significance

Ligand-mediated targeting has revolutionized the field of nanotechnology to facilitate early diagnosis, monitoring of disease progression, and drug delivery. My research goals focus on the targeting aspect in the development of nanotechnology. This dissertation described the strategies that we have designed and utilized to rapidly and accurately discover novel peptides for targeting tumors. As metastasis contributes to greater than 90% of cancer mortalities, non-invasive detection of angiogenic factors expressed during the earliest stages of tumor development can aid in the diagnosis of neoplasms before they metastasize. Therefore, my research goals involve designing a high-throughput and unbiased ‘beads on a bead’ approach to facilitate the isolation of novel ligands against $\alpha_v\beta_3$ integrin or EGFL7 via screening an OBOC combinatorial peptide library.

Conventional high-throughput methods often involve the immobilization of the target protein in a specific orientation, thus biasing its display and surface exposure to ligands during the screening process. My first research objective was to develop an unbiased multiplex ‘beads on a bead’ strategy to isolate, characterize and validate high affinity ligands from one-bead one-compound (OBOC) libraries (1). This scalable approach enriches for non-canonical ligands and incorporates multiple selection and validation parameters, including on-bead binding validation. To establish this strategy, we performed proof of principle experiments by exploiting the well-established affinity of $\alpha_v\beta_3$ integrin, a protein that is overexpressed on tumors and angiogenic blood vessels, for the RGD peptide, a motif found in several ECM proteins. Peptides derived from the RGD sequence have been demonstrated to target $\alpha_v\beta_3$-expressing tumors for non-invasive imaging. However, RGD peptides have been shown to affect the biology of cancer cells, which can lead to more severe tumor progression. We sought to generate peptides targeted to $\alpha_v\beta_3$ integrin that do not contain the RGD motif to allow the detection of nascent tumors without affecting the tumor biology. To screen for peptides against $\alpha_v\beta_3$ integrin, we employed the
‘beads on a bead’ method to screen an OBOC library using $\alpha_i\beta_3$-coated magnetic beads. Positive hits consisted of library beads coated with magnetic beads, and these were isolated from the negative library beads using either a strong magnet (bulk isolation) or a large particle flow cytometry instrument (high-throughput isolation). An on-bead cell assay was used to validate the affinities of peptides with native $\alpha_i\beta_3$ integrin on the cell surface of $\alpha_i\beta_3$-expressing MDA-435 breast cancer cells. The hit peptides were cleaved and sequenced "on bead" using a MALDI-TOF/MS technique that we have previously described (2), and the binding affinities of each peptide were determined using surface plasmon resonance (SPR). We reported the discovery of novel peptide ligands that target $\alpha_i\beta_3$ but do not contain the RGD motif, and demonstrated that, in contrast to RGD, they did not affect cancer or endothelial cell biology (1). This strategy is useful for both laboratory-based benchtop or large scale high throughput approaches. The peptides identified here represent novel targeting agents for integrins that can be applied to cancer imaging or treatment without the risk of increased tumor invasion and metastasis.

Several studies have demonstrated much success in using whole cell binding assays to screen OBOC libraries to isolate ligands specific to cell surface receptors of numerous human cancer cell lines (3), ie. Jurkat T-leukemia cells (4), T-lymphoma cell (5), and breast cancer cells (6). Nonetheless, conventional methods for isolating few positive hits from billions of OBOC library beads through manual techniques remain impractical and challenging. The COPAS large particle biosorter carries great potential for high-throughput and rapid isolation of surface binding ligands from a random OBOC library. However, the biggest challenge associated with this strategy is the dissociation of cells from the beads upon flowing them through the tubes of the instrument. Overcoming this issue will enable an easy and high-throughput automated sorting of high-affinity ligands from OBOC libraries. We showed that bound cells can be reversibly cross-linked onto beads through chemical fixation, and then easily sorted using the COPAS biosorter without affecting the sequence deconvolution of on-bead peptides using MALDI-TOF/TOF mass spectrometry. This finding enables one to discover novel affinity ligands through screening combinatorial libraries against living cells, and obtain peptide sequences from positive hits very quickly.

EGFL7 is a protein that is dramatically upregulated in the endothelium of tumors. The expression of EGFL7 correlates with poor prognosis in several different cancers. Studies have
shown that the vasculature in prostate cancers have shown that men with the most irregularly shaped vessels are 17.1 times more likely to develop lethal disease, and these vessels specifically express EGFL7. Taken together, these findings show that EGFL7 is an important biomarker for clinically relevant neoplasms and metastases. We hypothesize that molecular imaging using peptides targeted to EGFL7 will allow for non-invasive detection of early cancers and distant metastases. To test this, we screened an OBOC library to identify novel high-affinity peptide ligands of EGFL7 through screening a combinatorial peptide library using the ‘beads on a bead’ approach (1). We coated magnetic nanobeads with full-length recombinant EGFL7, mixed these with the peptide library beads that each displayed a unique octapeptide, and isolated the most highly interacting library bead "hits" with a magnet. An on-bead cell-binding assay was used to validate the peptide hits using an EGFL7-expressing HT1080 fibrosarcoma cell line. Hit peptides were then sequenced using a novel in-house MALDI-TOF/MS technique (2), and their binding affinity to EGFL7 was quantified using surface plasmon resonance (SPR). A high-affinity peptide ligand, E7-p72 was shown to bind and be taken up by cancer cells and endothelial cells in an EGFL7-dependent manner, and the knockdown of EGFL7 or the presence of an excess of unlabeled peptide significantly decreased this uptake. This lead candidate could provide a basis for a new generation of sensitive molecular imaging agents for the early and non-invasive diagnosis of cancers.

Our growing understanding of EGFL7 function has also led us to design and generate a peptide that inhibits angiogenesis. To do this, we searched for bioactive peptides derived from EGFL7 with the hypothesis that they would interfere with EGFL7 function, leading to the inhibition of angiogenesis. We identified bioactive EGFL7 sequences by searching for highly conserved motifs across several species, but are not present in the paralog of EGFL7, the EGFL8 protein. Particularly, one high-affinity ligand, E7-C13 inhibited angiogenesis in vitro (using human primary endothelial cells) and in vivo (using both the avian embryo chorioallantoic membrane (CAM) and mouse models). This peptide could provide a basis for a new generation of therapeutic agents for locally advanced cancers.
6.2 Future work

We have reported here a versatile ‘beads on a bead’ screening strategy for the discovery of novel affinity ligands from OBOC libraries, and demonstrate its utility by identifying several high affinity peptide ligands for $\alpha_v\beta_3$ integrin. As described in ‘chapter four’ of my thesis, we have also used this approach to screen for peptides that target the EGFL7 protein, confirming its usefulness in the identification of novel ligands against any target protein of interest. We intend to continue using this approach to screen against several different cell surface targets.

The $\alpha_v\beta_3$-targeting peptides, LCE62 and LCE64 described in this dissertation could provide a basis for a new generation of tumor targeting agents to image newly developed tumors, or treat locally advanced cancers. Although these peptides target breast cancer cells in vitro in a $\alpha_v\beta_3$ dependent manner without affecting the biology of the cells, the efficacy of these peptides to target $\alpha_v\beta_3$-expressing tumors in vivo still needs to be further evaluated. In order to do this, serum stability assays need to be performed to establish the half-lives of these peptides. As these peptides consist only L-amino acids, modifications on the sequences with non-natural amino acids may be required to enhance the stability of peptides in serum. Ultimately, these peptides can be conjugated with a fluorophore, or attached onto the surface of nanoparticles loaded with contrast agents and/or therapeutics for applications in molecular imaging and/or drug delivery of $\alpha_v\beta_3$-expressing cancers.

We have also demonstrated that screening an OBOC combinatorial library directly using fluorescently labeled cells, followed by a rapid automated isolation of high affinity hits using the COPAS large-scale flow cytometer can be achieved through chemical cross-linking of the cells onto hit beads prior to sorting. In collaboration with Dr. Leonard Luyt and Dr. Eva Turley from the London Regional Cancer Centre, we have used this approach to screen for novel peptides against the receptor for hyaluronic acid mediated motility (RHAMM) using fluorescently labeled RHAMM-positive cells (green) and RHAMM-knockout cells (red). RHAMM is a cell surface receptor for hyaluronan, and regulates growth factor signaling (7, 8). RHAMM is also required for cellular locomotion of ras-transformed fibrosarcoma cells, T-lymphocytes, malignant B cells and breast carcinoma cells (9). Therefore, identify novel peptides with high affinity for RHAMM can be useful for the development of molecular imaging probes to facilitate the diagnosis and
monitoring of certain cancers. Hit beads that interacted most strongly with green cells, but not red cells were isolated, and the peptides were sequenced (2). These peptides are currently being further characterized and evaluated for their ability to target RHAMM-expressing cells for the development of new molecular imaging agents in Dr. Turley’s laboratory.

We have reported the discovery of a novel EGFL7 targeting ligand, E7-p72, as a first line peptide for molecular imaging of EGFL7 expressing cells. While E7-p72 effectively targets cancer cells and endothelial cells in an EGFL7 dependent manner in vitro, we are in the process of evaluating the ability of E7-p72 to bind EGFL7-expressing solid tumors as well as angiogenic sprouts in mice. Currently, we are developing EGFL7-targeting CPMV nanoparticles (CPMV-PEG-E7-p72) for molecular imaging through the incorporation of E7-p72 peptide and near infrared fluorophore onto the surface of the viral capsid. We intend to establish a xenograft model of human prostate cancer in nude mice, and then evaluate the ability of CPMV-PEG-E7-p72 to specifically target and illuminate the site where the tumor is developing. At the endpoint, tumors will be resected, frozen and cryo-sectioned. Sections will be stained with anti-mouse PECAM antibody to visualize the endothelium, and the localization of CPMV-PEG-E7-p72 will be determined through confocal microscopy. In addition, in collaboration with Dr. Leonard Luyt, we are also in the process of labeling E7-p72 with radionuclides (ie. gallium-68) to develop new imaging probes for positron emission tomography (PET) imaging of tumor development and/or angiogenesis.

On going work is also currently underway to develop and characterize a 2nd generation peptide, E7-p72(Daa) that incorporates flanking D-histidines residues that has a significantly improved serum stability property. The binding affinity of E7-p72(Daa) to EGFL7, as well as its uptake by EGFL7-expressing cells in vitro and by tumor cells and/or angiogenic vessels in vivo still needs to be further evaluated.

We also described identification of E7-C13, a first line peptide directly derived from the sequence of EGFL7 that inhibits angiogenesis. Although we have demonstrated that E7-C13 inhibits angiogenesis in vitro (using matrigel morphogenesis assay) and in vivo (using the collagen on-plant avian embryo CAM model (10) and the directed in vivo angiogenesis assay in mice), the efficacy of E7-C13 in inhibiting angiogenesis through intravenous administration
route is yet to be determined. We are currently in the process of identifying the signaling pathways in which E7-C13 uses to inhibit angiogenesis by monitoring the changes in phosphorylation level in 49 different receptor tyrosine kinases (RTK) upon treatment of human endothelial cells with E7-C13. Our preliminary findings show that E7-C13 decreases the activity of anaplastic lymphoma kinase (ALK), fibroblast growth factor receptor 3 (FGF3), Axl and vascular endothelial growth factor receptor 1 (VEGFR-1). Efforts to validate these results through western blot analyses are currently in progress.
References

Supplementary Figure 2.1. LCE60, LCE62 and LCE64 have high affinity for $\alpha_v\beta_3$ integrin.
Association and dissociation curves generated from the interaction and dissociation of RGD, LCE60, LCE61, LCE62, and LCE64 with $\alpha_v\beta_3$ integrin using the SPRimmer II (GWC Technologies). Association curves were obtained by exposing peptides immobilized onto the
SPR chip with $\alpha_v\beta_3$ integrin. At $t = 1900s$, the dissociation curves were obtained by washing the chip with only buffer. The fitted curves were generated using the ‘association-then-dissociation’ equation in GraphPad Prism, version 5.0.

From Chapter 3

Supplementary figure 3.1. Cells remained bound onto the RGD-containing library bead after fixation and sorting. TentaGel beads containing a series of RGD-containing peptides incubated with MDA-MB-435 GFP cells (left), fixed with 3% formaldehyde (middle) and sorted using the COPAS biosorter (right).
Supplementary Figure 3.2. Establishing the sorting parameters for the remaining test library beads coated with intact cells. Dot plot showing the the cell-bead population and sorting profiles. The upper panel shows the two distinct bead and cell population (indicated by arrows) and the beads were gated for sorting. The bottom panel shows the green fluorescence intensity of each bead. The sort gate was established using negative control TentaGel beads that has undergone cell treatment, fixation and washes so that only beads with cells attached (higher fluorescent intensities) were sorted. RGD-containing TentaGel beads with MDA-MB-435 GFP
were then inserted into the COPAS instrument, and beads with the highest association with cells were sorted into a 96-well plate.

Supplementary Figure 3.3. On-bead MALDI-TOF/TOF MS sequencing of the remaining test peptides before and after fixation. MS/MS spectra of RGD-containing peptides before fixing with formaldehyde (left), and after fixing plus sorting (right). Peptide sequences from both samples were successfully attained. Fragments labeled b_j and y_j were calculated by complementarity.
Supplementary Figure 4.1. E7-p72 and E7-p74 have nanomolar affinity for EGFL7 protein. Association and dissociation curves generated from the interaction and dissociation E7-p72 and E7-p74 with purified EGFL7 (15.6 nM) using the SPRimager II (GWC Technologies). Association curves were obtained by exposing peptides immobilized onto the SPR chip with $\alpha_v\beta_3$ integrin. At $t = 1700s$, the dissociation curves were obtained by washing the chip with binding buffer. The fitted curves were generated using the ‘association-then-dissociation’ equation in GraphPad Prism, version 5.0. The $K_D$ values for each peptide was also calculated and indicated in this plot.
Supplementary Figure 4.2. Characterization of E7-p72 and E7-p72(Daa) in preparation for \textit{in vivo} studies using human prostate cancer xenograft mouse model. (a) Fluorescence images showing the uptake of FITC-E7-p72 and FITC-AGD (negative control) (final concentration of 3.3 \( \mu \)M) by HT1080 cells. Left panels: FITC signal from peptide uptake (green). Right panels: merged images of brightfield image, nuclei staining (red) and FITC-conjugated peptide (green). (b) Bar graph generated from ELISA-based E7-p72 alanine scan study. E7-p72 mutants were produced by substituting each amino acid in the E7-p72 sequence with an alanine residue. Peptides were immobilized onto a maleimide-activated 96-well plate and incubated with purified EGFL7. Level of EGFL7 in each well was detected using anti-EGFL7 antibody, followed by TMB substrate. The plate was inserted into a microplate reader, and values were obtained at 450 nm. (c) Serum stability curves demonstrating the peptide stability of E7-p72 and E7-p72(Daa) in 100\% human and mouse serum. E7-p72(Daa) contains the same sequence as E7-p72, with the exception that both flanking L-histidine residues were substituted with D-histidines. Peptides were treated with serum at a final concentration of 50 \( \mu \)g/mL at 37\( ^\circ \)C. At each time point, 200 \( \mu \)L
of serum was retrieved, and serum proteins were precipitated in cold ethanol. The supernatant containing the peptide was retrieved, frozen in dry ice and lyophilized. Samples were resuspended in 200 µL of H2O and 40 µL of MeCN, and analyzed by HPLC. Graph was generated using the exponential function in Microsoft Excel. The half-lives of each peptide are indicated.

From Chapter 5

Supplementary Figure 5.1. E7-C13 has high binding affinity for recombinant EGFL7 protein. Association curves generated from the interaction of E7-C13 or control peptide with EGFL7 using the SPRimager II (GWC Technologies). Association curves were obtained by exposing peptides immobilized onto the SPR chip with EGFL7 at different concentrations (1 nM, 20 nM, 50 nM and 100 nM). At t = 600s (10 mins), the percent change in reflectivity (%R) was plotted against the concentration of EGFL7 (nM) to generate an association curve using the GraphPad Prism, version 5.0.
Curriculum Vitae

Choi-Fong Cho

EDUCATION

• PhD Medical Biophysics (UWO) (Honours - Program in Molecular Imaging) (Sept 2007 - transferred in June 2012)
• PhD Oncology (UOA) (June 2012 - Dec 2012)
  • Degree anticipated: December 2012
  • University of Western Ontario, London ON, Canada; University of Alberta, Canada
  • Schulich School of Medicine and Dentistry
  • Supervisor: Dr. John Lewis
• BSc Biochemistry Honours (Sept 2003 - Apr 2007)
  • Degree awarded: June, 2007 (with distinction)
  • University of Guelph, Guelph ON, Canada
  • College of Physical and Engineering Science
• High school diploma (Jan 2001 - Jun 2003)
  • Centennial Collegiate Vocational Institute, Guelph ON, Canada
  • GPA: 92%

RESEARCH EXPERIENCE

PhD Research Project (Sept, 2007 - current)
• Established a novel strategy to screen an OBOC library for targeting peptide ligands for molecular imaging using magnetic beads
• Established a novel strategy to screen an OBOC library directly against cells for targeting peptide ligands using a large particle flow cytometer
• Discovered and validated novel peptide ligands that target various cancer targets, ie. \( \alpha \nu \beta 3 \) integrin, Rhamm, and EGFL7
• Discovered and validated novel peptide ligands that inhibit angiogenesis in a chicken embryo model of cancer
• Discovered and validated a novel peptide that inhibits cancer cell migration

Honour’s Research Project (Jan - Apr, 2007)
Department of Molecular and Cellular Biology, University of Guelph
• Investigated bacterial exotoxins, a class of virulence factors produced by pathogenic bacteria that are toxic to eukaryotic cells for the potential development of therapeutics against bacterial infections and diseases
• Solubilized and purified a novel exotoxin called Thermotoxin for the ultimate goal of obtaining its crystal structure
Research Assistant - Funded by NSERC USRA (May - Aug, 2005)
Department of Chemistry, University of Guelph
- Investigated Ruthenium-based catalysts for the conversion of waste products to more useful sources of energy in the oil industry
- Worked independently on an assigned research project by performing various experiments in effort to synthesize catalysts to be tested
- Successful in obtaining crystals for structural analysis of two different catalysts

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**Contributions to Research and Development**

**Publication**


**Patent**


**Book chapter (Submitted)**


**Manuscripts in preparation**


2. Iyengar, B., Fung, L., **Cho, C.F.**, Ablack, A., Ryu, W., and Lewis, J.D. The Drosophila ortholog of EGFL7, blade runner, is required for wing structure integrity


**Published abstracts**


Oral presentations


2. The Discovery of EGFL7-Binding Peptide Ligands for Molecular Imaging Using a Novel "Beads on a Bead" Approach. Oncology Research & Education Day. 2009. (Award winning presentation)


Poster presentations


