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Tumor-targeting peptides from combinatorial libraries*

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Abstract

Cancer is one of the major and leading causes of death worldwide. Two of the greatest challenges infighting cancer are early detection and effective treatments with no or minimum side effects. Widespread use of targeted therapies and molecular imaging in clinics requires high affinity, tumor-specific agents as effective targeting vehicles to deliver therapeutics and imaging probes to the primary or metastatic tumor sites. Combinatorial libraries such as phage-display and one-bead one-compound (OBOC) peptide libraries are powerful approaches in discovering tumor-targeting peptides. This review gives an overview of different combinatorial library technologies that have been used for the discovery of tumor-targeting peptides. Examples of tumor-targeting peptides identified from each combinatorial library method will be discussed. Published tumor-targeting peptide ligands and their applications will also be summarized by the combinatorial library methods and their corresponding binding receptors.

Keywords

Tumor-targeting peptide; Biological library; Phage-display peptide library; One-bead one-compound peptide library; High throughput screening; Cell surface receptor

1. Introduction

Breakthrough advances have been achieved in cancer diagnosis and treatment in the last decade including the recently FDA-approved immunotherapeutic agents, which have provided patients with new hope. However, cancer continues to be the second cause of death in the US (584,881 *vs* 611,105 in heart disease from the 2015 Fast Stats provided by CDC), and it is expected to surpass heart disease to become the No. 1 killer by 2030. Conventional chemotherapies have low specificity towards cancer cells and therefore exhibit serious toxic side effects. Target-specific delivery of chemotherapeutic drugs to the tumor cells can help improve the outcome of existing anti-cancer drugs. Widespread use of targeted therapies and molecular imaging in the clinic requires high affinity, tumor-specific agents as effective

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targeting vehicles to deliver therapeutics and imaging probes to the tumor sites. Tumor-targeting agents can be antibodies, proteins, peptides, peptidomimetics, glycopeptides, peptoids, aptamers or small molecules. Several cell surface-targeting antibodies have been approved by the FDA as vehicles to deliver radionuclides (e.g. Zevalin or Bexxar, anti-CD20 antibodies loaded with ^{90}Y or ^{131}I, respectively), toxins (e.g. Adcetris, an anti-CD30 antibody-MMAE conjugate directed against systemic anaplastic large cell lymphoma and Hodgkin's lymphoma), or cytotoxic chemotherapeutic agents (e.g., Trastuzumab emtansine) to the cancer cells. Cancer-targeting antibodies have proven success in the clinic, but they also suffer some limitations because (i) the Fc region of the antibodies binds to the reticuloendothelial system resulting in significant toxicities to liver, bone marrow, and spleen; (ii) antibodies against the cancer cells have difficulty in infiltrating the entire tumor mass due to their large size (M.W. ~160,000 Da); (iii) they are difficult to manufacture in large-scale; therefore, they are expensive. Tumor-targeting peptides are efficient alternative vehicles for selective delivery of high dose of chemotherapeutic drugs or diagnostic agents to tumor sites while sparing normal tissues. Several peptide hormones have already been used for tumor targeting. For example, octreotide, a cyclic octapeptide analogue of somatostatin, has been used for radiotargeting of neuroendocrine tumor [1]. AN-152, a linear peptide analogue of LHRH, has also been used to target LHRH receptor of ovarian cancer, breast cancer and prostate cancer [2]. Peptides consisting of only eukaryotic amino acids in general are not stable *in vivo*, but their stability against proteolysis can be significantly improved if they contain D-amino acids and unnatural amino acids, are cyclized, and/or are *N*-and *C*-terminally blocked. Advantages of peptides over currently-used biomolecules such as antibodies are their rapid blood clearance, increased diffusion and tissue penetration, chemical stability, and ease of synthesis in large scale. In addition, they can be readily conjugated to cytotoxic drugs, radionuclides, or toxins in a chemically defined manner.

The subject of using combinatorial libraries to discover tumor-targeting ligands has been reviewed by us and other investigators in the past [3–9].Inthis review, we attempt to give an update on combinatorial library technologies and newly identified tumor-targeting peptides. Additionally, we categorize the peptide ligands according to their interacting receptor(s) and library screening.

2. Overview of approaches to discover tumor-targeting peptides

Tumor-targeting ligands generally target one of the following three sites: (i) cancer cell surface receptors, (ii) the tumor's extracellular matrix, and (iii) tumor vessel endothelial cell surface receptors. Many of these targeting molecules can be developed through (i) the use of native ligands or their analogues such as octreotide against somatostatin receptors, bradykinin analogues against bradykinin receptors, AN-152 against LHRH receptors, and folic acid against the folate receptor; (ii) computer-aided design if the X-ray crystallographic structure of the cancer-associated receptor or a related receptor is known, in combination with medicinal chemistry, and/or (iii) screening combinatorial libraries. The combinatorial library method allows rapid identification of tumor-targeting ligands from a large number of diverse compounds. In this review, we shall focus on different library technologies and their use for discovery of tumor-targeting peptide ligands. The phage-display peptide library [10]

and the OBOC combinatorial peptide library [11] are the two most popular approaches that have been successfully applied to discover tumor-targeting peptides. Other biological-display peptide libraries such as yeast-display, bacteria-display, ribosome-display, and mRNA-display peptide libraries have also been used but are less common. In addition to OBOC library, peptide nucleic acid (PNA)-encoded solution phase peptide library and peptide microarrays are two other synthetic chemical libraries that have been used for the discovery of tumor-targeting ligands. The former takes advantage of its ease in decoding (see below). Peptide microarrays have been mainly used for ligand optimization and structure–activity relationship studies. Other synthetic combinatorial library methods such as positional-scanning peptide library have also been used to discover cancer targeting peptides but are much less often applied. A summary of the advantages and disadvantages of different library approaches is shown in Table 1. Live cancer cells or cancer-related proteins are commonly used as probes to screen combinatorial libraries. The advantages of using live cells for library screening include (i) the target receptors on cell surface are in the native state; (ii) cloning, expression, and purification of cell surface targets are not needed; (iii) the cell surface target can be an unknown receptor; (iv) targeting ligands with cell-penetrating ligands can be identified; and (v) functional ligands that trigger downstream signaling can be readily identified, particularly with the OBOC method. Therefore, most cancer-targeting peptides target cancer cell surface and have been discovered from cell-binding assays. Soluble cancer-associated proteins, if available, can also be used as probes for identification of ligands, although their specificity against other proteins needs to be verified. The technology of biological-display, OBOC, PNA-encoded solution-phase peptide libraries and peptide microarray, as well as their applications in the discovery of cancer-targeting peptides will be discussed below.

3. Biological library methods

The surface profile difference between cancer cells and their nonma-lignant counterparts can serve as an excellent molecular address for targeted delivery of therapeutic agents, diagnostic agents or both to cancer cells. The biological-display system is an efficient tool in discovering novel tumor-targeting peptides via high-throughput screening. The peptide-displaying microbes can be considered as peptide-covered micro-particles. The peptides displayed on the surface of the microbes can be directly used to screen live cancer cells and/or purified receptor proteins. The sequence of the peptide hits can be easily determined by DNA sequencing of the microbial plasmid. The biological-display system includes phage, yeast, bacteria, ribosome, mRNA, and mammalian cell-display [12]. Phage-display peptide library remains the most commonly used combinatorial library technique to discover tumor-targeting peptides. To the best of our knowledge, mammalian cell-display has not been applied to the discovery of tumor-targeting peptides so far; therefore, it will not be discussed in this review.

3.1. Phage-display peptide library method

3.1.1. Methodology—A phage-display peptide library is comprised of a heterogeneous mixture of billions of bacteriophage clones, each carrying a different foreign DNA insert and therefore displaying a different peptide on its surface [10]. Peptides are typically displayed

at the *N*-terminus of the pIII or pVIII proteins [4,13]. Inverted pVIII proteins have been used to display peptide with free carboxy terminus [14]. The pVIII "landscape" (all pVIII proteins express peptides) has also been used as peptide-displaying coat proteins [4]. Techniques for generating chimera phage with both peptide-displaying coat proteins and normal wild-type coat protein have been developed. The M13 filamentous phage is the most widely used phage-display system due to its high capacity for replication and ability to accommodate large foreign DNA. The fd filamentous phage, which is closely related M13 phage, is also commonly used for pIII and pVIII display [10,15]. The less commonly used T7 lytic phages that lyse their bacterial hosts have also been used for peptide library display. In T7 phage, peptide sequences are typically displayed as C-terminal fusions of the 10B capsid protein. T7 libraries exhibit less bias than filamentous phage libraries and longer peptide libraries (12-20mer) are most often used for the discovery of cell binding peptides. In contrast, short peptide libraries (7-12mer) are more popular in M13 phage display.

The phage-display library method has several advantages: (i) it can display combinatorial peptide libraries with huge permutations (10^9); (ii) the size of the grafted peptide is not limited by the constraints as in the case of a synthetic peptide library; (iii) it can take advantage of known protein folds *(e.g.* zinc-finger fold, conotoxin fold, immunoglobulin fold, or cystine-knot) by grafting random oligopeptides on such tertiary folds; (iv) the method is highly efficient, inexpensive, and amenable to both short and long peptides, linear and simple cyclic peptides (disulfide formation with two L-cysteines), and can be carried out in most molecular biology laboratories; (v) phage-display peptide libraries are commercially available, *e.g.,* Ph.D.-7, Ph.D.-12 and Ph.D.-C7C (cyclic with a disulfide bond) libraries can be purchased from New England Biolabs (Ipswich, MA, USA); (vi) phage-displayed libraries can be screened with *in vivo* selection techniques in xenograft models; (vii) the standard library can be easily generated by simply growing the microorganisms; (viii) usage of this method may identify novel ligands even without prior knowledge about the identity of the target receptor on cell surface; and (ix) recent advances in chemical modification of phage-display libraries with thiol-reactive compounds or crosslinkers prior to screening enable introduction of limited cyclization and derivatization to the displayed peptides [16-18].

However, despite the many advantages, this biological library approach suffers some important limitations: (i) only the natural L-amino acid peptide libraries (comprised of 20 eukaryotic amino acids) can be incorporated into the phages. Such peptides are generally susceptible to proteolysis particularly if the *N*- and *C*-termini are not blocked; (ii) because of bias in genetic codons, the peptide library is not totally random; (iii) screening assays of the phage-display libraries are generally limited to binding and a few functional assays such as protease substrate determination; (iv) complicated bicyclic, compact scaffolding, branched structures, or molecules with special chemistry of cyclization are more difficult with this method; and (v) optimizing a phage-display peptide into a proteolytic stable molecule while still retaining a high binding activity and specificity to the cell surface receptor is not a trivial matter.

Fig. 1 illustrated the process of selecting phage clones that bind a specific target, which is called "biopanning" including *in vitro, in vivo,* and *ex vivo* biopannings. Kay et al. have

published a step-by-step screening protocol which facilitates new investigators to learn this technology [19]. The following factors need to be taken into account because they may affect the quality of hits identified from screening phage-display libraries: the number of phages used, stringency of selection process, the number of selection rounds, competitive selection and subtractive panning. Panning of a phage-display library with living cells provides the option to select phages that bind and internalize inside the cells, which can be achieved by optimizing the washing techniques [20]. Decoding of peptide ligands identified from phage-display libraries can be achieved with DNA sequencing, which is simple and inexpensive.

3.1.2. Discovery of tumor-targeting peptides from phage-display peptide library—Tumor-targeting peptides can be discovered from phage-display libraries via one of the three screening approaches: *in vitro*, *in vivo* or *ex vivo* selections (Fig. 1). Cancer-associated proteins, specific cancer cell lines, patient tissues, and tumor xenograft mouse models have been successfully used as screening probes. *In vivo* selection of phages has recently shifted from using xenograft mouse models to transgenic and clinically relevant patient-derived xenograft (PDX) models.

Unbiased bio-panning of phage-displayed peptide libraries has yielded a number of peptides that bind cancer cells and cancer-associated antigens presented on cancer cell surface, tumor vasculature or tumor lymphatic vessels. Different peptide ligands have been identified for a variety of tumor types including breast, lung, thyroid, head and neck, liver, prostate, bladder, colon and gastric cancers, osteo-sarcomas, as well as pancreatic ductal adenocarcinomas and squamous cell carcinoma. Table 2 lists some tumor-targeting peptides that were identified from *in vitro* selection of phage-display peptide library using purified receptor as a screening probe. Examples of tumor-targeting peptides that target specific receptor(s) using live cells, tissues, or tumors as screening probes are listed in Table 3. Phage-display library screening also yields many tumor-targeting peptides of which the binding partners have not yet been identified. This latter group of pep-tides will not be covered here but can be found in other reviews [3–7].

3.1.2.1. Epidermal growth factor receptor (EGFR)-binding peptides: EGFR is a cell surface protein that binds to epidermal growth factors and is over expressed in a variety of human cancer cells, thus making it an excellent target for drug delivery. Li et al. screened a phage-display peptide library and identified a peptide named GE11 (YHWYGYTPQNVI) that binds to EGFR specifically and efficiently with a dissociation constant (K_D) of approximately 22 nM [21]. GE11 has a much lower mitogenic activity towards EGF. GE11 is internalized preferentially into cancer cells with high expression level of EGFR and is furthermore accumulated in EGFR over expressing tumor xenografts *in vivo* after i.v. administration. In gene delivery studies, GE11-conjugated polyethylenimine (PEI) was found to deliver genes efficiently into EGFR over expressing cells and tumor xenografts. Taken together, GE11 could be potentially used as a safe and efficient tumor-targeting vehicle for selective drug delivery mediated through EGFR.

Hamzeh-Mivehroud et al. recently identified two short peptide ligands (SYPIPDT and HTSDQTN) against EGFR from screening of a phage-display peptide library Ph.D.-7 [22].

EGFR expressing A-431 cells were used as the matrix in a cell-based subtractive biopanning approach. The identified peptides were able to inhibit the EGF-induced phosphorylation of EGFR in a concentration-dependent manner. The results of affinity binding experiments showed that EGF was able to inhibit competitively the binding of peptide-bearing phage to A-431 cells.

3.1.2.2. αvβ6 integrin-binding peptides: Integrin αvβ6 is highly expressed in many malignancies but is usually expressed at low or undetectable levels in normal adult tissues. It supports epithelial cell proliferation during inflammation, fibrosis, wound healing and carcinoma progression [23]. Overexpression of integrin αvβ6 usually correlates with malignant potential and poor prognosis [24]. Integrin αvβ6 can also increase cancer metastasis by promoting cancer cell invasion and migration. Because the expression pattern of integrin αvβ6 is restricted to tumors and other pathological tissues, it has become a promising diagnostic and therapeutic target.

A peptide specifically targeting lung cancer cells, named H2009.1 (RGDLATLRQLAQEDGVVGVR), was isolated by Oyama et al [25] through panning of a Ph.D.-20 peptide library. However, its cellular receptor target was not identified at that time. Later, Elayadi et al. found out that integrin αvβ6 is the binding receptor of this peptide [24]. H2009.1 is able to mediate cell-specific uptake of a fluorescent nanoparticle via this receptor. Nothelfer et al. identified a shorter αvβ6-targeting peptide, namely, HBP (SPRGDLAVLGHK), through screening a Ph.D.-12 phage-display peptide library against HNO223 head and neck squamous cell carcinoma cell (HNSCC) line [26]. HBP-1 (SPRGDLAVLGHKY) with a tyrosine added to the C-terminal of HBP for easy radiolabeling with ^{131}I showing preferential binding to αvβ6 over αvβ3 integrin. ^{125}I-labeled HBP-1 showed binding to 5 different HNSCC cell lines. [^{131}I]-Labeled HBP-1 accumulated rapidly in 2 different HNSCC tumor xenografts, with stable uptake until 45 min after intravenous administration. Peptide histochemistry by HBP-1 was positive for HNSCC tissue sections but negative for normal tissues. Goodman and co-workers reported the discovery of an unexpected non-RGD recognition motif for integrin αvβ6 [27]. They compared the recognition profiles of recombinant αvβ6 and αvβ3 integrins by screening Ph.D.-7 and Ph.D.-12 peptide libraries. As predicted, phages binding strongly to αvβ3 were found to contain ubiquitous RGD sequences. However, in addition to RGD-containing phages, one-quarter of the phages isolated from the Ph.D.-12 library contained the distinctive consensus motif DLXXL for αvβ6. A synthetic DLXXL peptide, RTDLDSLRTYTL, was determined to be a selective inhibitor of RGD-dependent ligand binding to αvβ6 in an isolated receptor assay (IC_{50} = 20 nM), and in a cell adhesion assay (IC_{50} = 50 μM). DLXXL peptides were specific inhibitors of αvβ6-fibronectin interaction since synthetic scrambled or reversed DLXXL peptides were inactive. Further studies revealed that a 7-mer peptide sequence RG/TDLXXL is the minimum motif required for high affinity and selectivity towards αvβ6 and additional flanking amino acids resulted in further improvement in binding affinity and specificity.

Because linear peptides may suffer from *in vivo* instability and rapid *in vivo* clearance, several peptides cyclized by disulfide bond have been recently developed that exhibit high affinity for αvβ6 [28]. Several αvβ6-targeting ligands have been labeled with radionuclides

for *in vivo* PET or SPECT imaging in animal models of cancer and other diseases. For a review, please refer to ref. [29].

3.1.2.3. Neuropilin-1 (NRP-1)-bindingpeptides: NRP-1 is a membrane-bound receptor and plays an essential role in normal neuronal and vascular development [30,31]. NRP-1 has also been implicated as a novel mediator of the primary immune response [32]. The expression of NRP-1 can be stimulated in response to tissue injury or hypoxic conditions [33]. NRP-1 was found to be highly expressed in a variety of solid tumors, such as prostate, breast, pancreatic, lung, ovarian, and gastrointestinal carcinomas [34]. In addition, the expression of NRP-1 is elevated in acute lymphoblastic leukemia (ALL) and acute myelogenous leukemia (AML) [35], and is correlated with poor survival of AML patients [36]. NRP-1 expression was found increased in the bone marrow of ALL and AML patients compared with normal bone marrow. Ligands that bind to NRP-1 should potentially exhibit an increased homing specificity towards leukemic bone marrow. Increased expression of NRP-1 was found correlated with tumor growth and vascularization *in vivo* and with invasiveness in human cancer. Therefore, NRP-1 could serve as an excellent target for effective receptor-mediated drug delivery. Teesalu et al. reported discovery peptide RPARPAR from *ex vivo* screening with T7 phage. NRP-1 was found to be the cellular receptor of this peptide [37]. Wang and colleagues isolated two peptide sequences GGKRPAR (P4) and RIGRPLR (P7) with superior binding affinity and specificity relative to the RPARPAR peptide by using a microfluidic phage selection (MiPS) system [38]. Compared to conventional biopanning methods, the MiPS is a more efficient system to identify peptides with higher affinity and specificity by applying stringent selection conditions against minimal amount of target cells [38]. By screening of human T-cell lymphoma Molt-4 cells with a phage-display peptide library CX_7C, Karjalainen et al. discovered a motif F(F/Y)XLRS (X = any amino acids) targeting NRP-1 [35]. A cyclic peptide CGFYWLRSC, when linked to a pro-apoptotic peptide $(klaklak)_2$ (wherein small letters stand for D-amino acids) decreased cell viability at a relatively low μM level in human leukemia and lymphoma cell lines, while the control, a mixture of peptides CGFYWLRSC and $(klaklak)_2$, did not show cell killing effect at the same concentration.

3.1.2.4. αvβ3 integrin-binding peptides: The αvβ3 integrin is a receptor expressed on the surface of various normal and cancer cells, and binds to extracellular matrix proteins displaying the RGD sequence [39]. It plays a key role in angiogenesis and metastasis of human tumors. Peptide ligands targeting αvβ3 integrin have great potential for developing cancer targeting therapy and imaging. Phage-display peptide library screening has successfully been used to identify peptide ligands targeting αvβ3 integrin. By screening a phage-display library, a nonapeptide named RGD-4C (CDCRGDCFC) was identified to be highly selective against αv integrins [40]. Witt et al. screened phage-display peptide libraries with a human rhabdomyosarcoma (RMS) cell line RD, and discovered RMS-I (CQQSNRGDRKRC) targeting αvβ3 integrin expressed on the RD cells [41]. This peptide binds to RMS and other tumor cell lines, but not to normal skeletal muscle cells and fibroblasts. Sugahara et al. screened a cyclic CX7C peptide library displayed on T7 phage against tumor blood vessels in metastasis mouse models of human prostate cancer [42]. After three rounds of ex *vivo* selection with cell suspensions from bone tumors, followed by

one *in vivo* selection for homing to the bone tumors, a tumor-homing peptide named iRGD (internalizing RGD) with sequence CRGDKRGPDC was discovered. It has been proposed that iRGD homes to tumors involving three steps: first iRGD bound to αν integrins on tumor endothelium via RGD motif, and was then cleaved by proteolysis to expose a binding motif for neuropilin-1 (NRP-1), which mediates penetration into tissue and cells [42]. MRI imaging with iRGD peptide-decorated superparamagnetic iron oxide nanoworms in mice bearing 22Rv1 orthotopic xenograft has shown significantly improved sensitivity in tumor imaging. *In vivo* anti-tumor efficacy study with iRGD-coated Abraxane demonstrated that 8-fold more Abraxane was found accumulatedinthe tumors than that of non-targeted-Abraxane. iRGD was able to bind to tumor vessels and then spread into the extravascular tumor parenchyma, whereas conventional RGD peptides only delivered the cargo to the blood vessels.

Optimization of RGD cyclopeptides through changing the ring size and amino acid chirality, and introducing constrained building blocks such as *N*-methylated amino acids led to the discovery of Cilengitide, a head-tail cyclic peptide with sequence [RGDf-N(Me)V-] [43–45]. Cilengitide binds strongly and relatively selectively to αvβ3 integrin. Preclinical studies of Cilengitide in mice demonstrated efficacious tumor regression [46]. Phase II studies also demonstrated that Cilengitide is a potential monotherapy in patients with recurrent glioblastoma [47]. However, in a phase III clinical trial investigating Cilengitideas anti-tumor therapyin combination with standard chemo-radiotherapy, it did not improve overall survival in patients with newly diagnosed glioblastoma [48]. Nevertheless, αvβ3 integrin remains a potential therapeutic and imaging target for cancer. RGD peptides could still be developed as vehicles to deliver drug payload or imaging probes to tumor sites. Cilengitide, however, is a head-to-tail cyclic peptide without any handle for attachment. One residue will need to be modified to introduce a handle.

3.1.2.5. Interleukin 13 receptor α2 (IL-13Rα2)-binding peptides: IL-13Rα2 is a plasma membrane receptor that is over-expressed in a majority of patients with glioblastoma multiforme (GBM) but not found in normal brain [49]. Debinski's group screened a disulfide-constrained heptapeptide phages display library, and identified several peptide ligands; one of these peptides, CGEMGWVRC, was found to bind to IL-13Rα2 with the highest specificity [50]. Surprisingly, the linear form of this peptide bound to IL-13Rα2 even more avidly than the disulfide-cyclized form. Furthermore, they found this linear peptide is capable of homing to both subcutaneous and orthotopic human GBM xenografts expressing IL-13Rα2 when administered intravenously [50].

3.1.2.6. Vascular endothelial growth factor receptor 3 (VEGFR-3)-binding peptides: VEGFR-3 belongs to class III receptor tyrosine kinase family and is initially expressed in all embryonic endothelia, but its expression in the blood vessel endothelium decreases during development. Human VEGFR-3 is up-regulated in a variety of human cancers. Inhibiting the signal pathway of VEGFR-3 could prevent the growth and metastasis of tumor [51,52]. To identify novel ligands with specific binding capabilities to VEGFR-3, Qin and coworkers screened a phage-display peptide library against VEGFR-3 and discovered a consensus peptide motif CSDXXHXWC (X is any amino acid). Peptide P1,

CSDSWHYWC, exhibited the highest affinity to VEGFR-3 in phage ELISA. Chemically synthesized P1 can bind to VEGFR-3 positive carcinoma cells with specificity [53].

3.1.2.7. Platelet-derived growth factor receptor β (PDGFRβ)-binding peptides: The PDGFRβ is a transmembrane glycoprotein in the receptor tyrosine kinase family. It is an important factor for regulating cell proliferation, cellular differentiation, cell growth and development. PDGFRβ is implicated in tumor growth through angiogenesis activation and in early stages of fibrosis. PDGFRβ is upregulated in various solid tumors, and its signaling plays a key role in the regulation of tumor interstitial fluid pressure [54]. This receptor represents an attractive and potentially valuable target for treatment and molecular imaging in oncologic and fibrotic diseases.

Peptide IPLPPPSRPFFK targeting PDGFRβ has been identified through biopanning with a Ph.D.-12 library against the recombinant extracellular domain of PDGFRβ [55]. PDGFR-P1 (IPLPPPSRPFFKY-NH_2) is a synthetic peptide with a tyrosine was added to the C-terminal of the identified peptide for radiolabeling = with ^{125}I or ^{131}I. *In vitro* studies demonstrated a higher binding to PDGFRβ-expressing BxPC3 and MCF7 cells as well as PDGFRβ-transfected-HEK cells in comparison to negative control wtHEK293 and CaIX-transfected HEK cells. Binding was inhibited up to 90% by the unlabeled PDGFR-P1 peptide. *In vivo* biodistribution experiments were performed in subcutaneous BxPC3 tumor mouse model in Balb/c nude mice. *Ex vivo* distribution studies revealed a higher accumulation in BxPC3 tumors than in most normal organs. Therefore, PDGFR-P1 is a promising candidate for targeting human PDGFRβ.

3.1.2.8. Protein tyrosine phosphatase receptor type J (PTPRJ)-binding peptides: Mutations or overexpression of protein tyrosine kinases (PTKs) often result in cell malignant transformation [56]. Protein tyro-sine phosphatases (PTPs) that antagonize the oncogenic PTK signaling are considered potential tumor suppressors and, consequently, potential targets for novel anticancer therapies [57]. PTPRJ is a receptor protein tyrosine phosphatase whose expression is strongly reduced in many cancer cell lines and tumor specimens [58]. Using PTPRJ-His-6 recombinant protein as a probe to screen a phage display library Ph.D.-C7C, Paduano et al. isolated two peptide ligands PTPRJ-pep19 (CHHNLTHAC) and PTPRJ-pep24 (CLHHYHGSC), which could induce mitogen-activated protein kinase (MAPK) dephosphorylation and inhibit cell growth of HeLa and human umbilical vein endothelial cell (HUVEC) cells [59]. In a subsequent work, they developed a panel of nonapeptide analogues based on the sequence of PTPRJ-pep19; one of this PTPRJ-19.4 (CHHALTHAC) was able to dramatically reduce cell proliferation and effectively trigger apoptosis of both HeLa and HUVEC cells, as well as inhibiting *in vitro* tube formation on Matrigel [60].

3.1.2.9. VAV3-binding peptides: VAV3 is a guanine nucleotide exchange factor and activates the Rho GTPase pathway to promote invasion, proliferation, and survival. Expression level of VAV3 in recurrent glioblastoma is higher than that of primary glioblastoma [61]. Elevated VAV3 expression correlates with higher grade tumor and poor prognosis. Self-renewing glioma-initiating cells (GICs) in glioblastoma were found to express high level of VAV3, at the apex. Targeting VAV3 by ribonucleic acid interference

decreased GIC growth, migration, invasion and *in vivo* tumorigenesis. A VAV3-targeting peptide SSQPFWS was identified by the Rich group through *in vivo* biopanning of the Ph.D.-7 library in mice implanted subcutaneously with patient-derived glioblastoma xenografts [61]. This peptide specifically internalized into GICs. When labeled with a fluorescent dye, this peptide was found to be able to identify and sort functional GIC cells from bulk and unsorted glioma culture using FACS.

3.2. Yeast-display peptide library

3.2.1. Methodology—Yeast-display technique was first published by the Wittrup lab [62]. Like other directed evolution display technologies, yeast-display relies on an intimate linkage between genotype (plasmid encoding the gene) and phenotype (protein scaffold expressed on the cell surface) [63]. Among different yeast strains and many cell wall anchors that have been reported inyeast-display, Aga2 subunit of the mating protein a-agglutinin in *Saccharomyces cerevisiae* is most commonly used. In this system, mutant proteins or library peptides are displayed as fusion protein with Aga2p, which in turn are covalently linked via two disulfide bonds to Aga1p subunit that anchors on the yeast cell wall. Incorporation of a hemagglutinin (HA) and/or c-myc tag adjacent to the displayed protein/peptide enables easy detection with immunoflu-orescence (see Fig. 2). In a yeast-display library, each yeast cell displays ~50,000 copies of the mutant proteins or peptides; in some cases, the expression level may be lower.

Library screening in general involves incubation of the library with target-coated magnetic beads or fluorescently labeled soluble target, followed by isolation of the positive yeast through magnetic separation or fluorescence activated cell sorting (FACS) [64] (Fig. 3).

3.2.2. Discovery of tumor-targeting peptides from yeast-display peptide library

3.2.2.1. αvβ3 integrin-binding peptides: Silverman et al. replaced a six amino acid loop in agouti-related protein (AgRP) with a nine amino acid loop containing an RGD motif, created a yeast surface display library by randomizing the residues flanking the RGD sequence, and identified six clones with high affinity for αvβ3 integrins [65]. Binding data showed that the engineered AgRP peptides bound to cells expressing αvβ3 integrins with affinities ranging from 15 nM to 780 pM and were highly specific for αvβ3 over other integrins [65].

3.2.2.2. Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4)-binding peptides: CTLA-4, an inhibitory receptor expressed by T lymphocytes, has emerged as a target for the treatment of metastatic melanoma. In a recent research, Maaβ et al. isolated ten different cystine-knot peptides that bind to the extracellular domain of CTLA-4 from knowledge-based combinatorial library based on the cystine-knot peptide McoTI-II. Cystine-knots (also known as knottins) are small, compact peptides (typically 20-60 amino acids) that consist of a core of at least three disulfide bonds that are interwoven into a “knot” conformation [66]. The most potent peptide MC-CT-010 (SPRCKYSHVPCRRDSDCPGKCICRGNGYCG) containing four cyclic loops was conjugated with neutravidin, and fused to antibody Fc domain or the oligomerization

domain of C4b binding protein, resulting in enhanced dissociation constants in the nM range [67].

The tumor-targeting peptides identified by the yeast-display library method are summarized in Table 4.

3.3. Bacteria-display peptide library

3.3.1. Methodology—Display of heterologous peptides on the surface of bacterial cells was reported as early as 1986 [68]. Bacteria-display library (up to 10^{11} different peptides) utilizing membrane flagella to display random pep-tides enables bound clones to be readily eluted from the immobilized target by mechanical shearing of the flagella even if the peptide-target interaction is very strong. In contrast, elution of phages can only be achieved by breaking the peptide-target interaction, which could be problematic if the affinity is extremely high. Furthermore, bacteria are easier to cultivate with a selection marker that helps to prevent library contamination [69]. In bacteria-display peptide libraries, peptides are typically fused to the C- or *N*-terminus, or inserted into the middle of one of the four bacterial proteins (FliTrx, CPX, OmpA, or invasion). Bacterial surface display is a well-established methodology for the discovery and optimization of peptides with desired activities such as binding to cancer cells [70]. The subject of bacteria-display library has previously been reviewed [71–73]. A step-by-step protocol of the design and screening of bacteria-display peptide library has been published by the Daugherty group [74]. Bacteria-display combines the advantages of phage- and yeast-display since large libraries can be constructed efficiently in *Escherichia coli* [75]. *E. coli* is ideal for display libraries because it grows quickly and is easy to manipulate. Similar to yeast-display, bacteria that display target-binding peptides can be enriched from large libraries using sequential magnetic-activated cell sorting (MACS) and FACS [76]. A screening process of bacteria-display peptide is shown in Fig. 4.

3.3.2. Discovery of cancer targeting peptides from bacteria-display peptide library

3.3.2.1. Vascular endothelial growth factor (VEGF)-binding peptides: VEGF is a potent angiogenic factor and an essential growth factor for vascular endothelial cells [77]. VEGF is up-regulated in many tumors and plays important role in tumor angiogenesis and vascular permeability. VEGF-mediated signaling occurs in tumor cells, and this signaling contributes to key aspects of tumorigenesis, including the function of cancer stem cells and tumor initiation. Combination therapies using anti-VEGF therapies with chemotherapy and/or radiotherapy are effective against many types of tumor. To identify peptide ligands specific for VEGF, Kenrick and Daugherty constructed and screened bacteria-display peptide libraries, which were displayed on *E. coli* strain MC1061 on the *N*-terminus of circularly permuted OmpX (CPX) [76], by one round of Magnetic selection (MACS) followed by four rounds of FACS. They discovered a core motif of WE/DWE/D that conferred binding to VEGF from a random 15mer peptide library. A focused library $X_6W(E/D)W(E/D)X_9$ was constructed and screened. The resulting pep-tides from screening exhibited a consensus of CSR(F/L)(V/L)MWEWECF (account for 12 of 19 amino acid positions). A third library was constructed based on the consensus from the second generation of the form X_4CX_4(M/

I)W(E/D)W(E/D)C(I/L/M/F)X_3. A potent peptide named 3.30 (WPVRCSRFVMWEWECFLRA, K_D = 470 nM) was identified. This peptide has a comparable binding affinity to peptide v114 (VEPNCDIHVMWEWECFERL, K_D = 230 nM), which was affinity-matured using phage-display library approach. These two peptides share a high consensus motif CXXXVMWEWECFXR(A/L).

The tumor-targeting peptides identified by the bacteria-display library method are summarized in Table 5.

3.4. Ribosome-display and mRNA-display peptide libraries

3.4.1. Methodology—Ribosome-display is an *in vitro* selection and evolution technology for large libraries of proteins and peptides [78]. An important feature of this technology is that the RNA encoding the peptide library is translated *in vitro* while peptides and their corresponding RNA remain associated with the ribosome. The sequence information for the peptide of interest can be selected by affinity purification of the resulting peptide–ribosome–mRNA (PRM) complex [79]. Kawasaki first reported this technology in a patent application in 1991 [80]. In 1994, Mattheakis et al. used it to select peptide ligands against an antibody [81]. Heyduk et al. recently reported the improvement of ribosome-display method by using next generation sequencing (NGS) for decoding of the isolated PRMs [82]. A single NGS analysis can reveal the identity of millions of sequences in complex nucleic acid mixtures. Read count of a given pep-tide sequence is proportional to the relative abundance of a sequence in thesample. This method greatly reduces the number of selection rounds that were required to identify specific ligands. Since ribosome-display procedures are performed entirely *in vitro*, there are two main advantages over other biological-display technologies. First, the diversity of the peptide library is not limited by the transformation efficiency of bacterial cells but only by the number of ribosomes and different mRNA molecules present in the test tube. Second, random mutations can be introduced easily after each selection round. Fig. 5 demonstrates generation and screening of ribosome-display peptide library.

In mRNA-display library method, the mRNA is first ligated to a PEG linker connected to puromycin and translated *in vitro* [83]. The ribosome stalls at the junction between RNA and DNA. Puromycin then binds to the A-site of ribosome and attacks the peptidyl-tRNA at the P-site. The nascent peptide is thereby transferred to puromycin. The resulting covalently linked mRNA–peptide complex has the puromycin-linker-mRNA on one side of the tunnel, and the peptide on the other side of the tunnel. It is then reverse transcribed and used for selection. The DNA strandisrecovered from target-bound complexes by hydrolyzing the complementary mRNA at high pH, and then amplified by PCR [83]. Since all the steps of mRNA-display are *in vitro* and cell-free, library size is not limited by the need to transform bacteria and in principle can reach as large as ~10^{13}. Furthermore, using synthetic tRNAs pre-charged with desired unnatural amino acids, the PURE (protein synthesis using recombinant elements) system [84] enables the incorporation of D-, α-hydroxyl and *N*-methyl amino acids, *N*-substituted glycines (peptoid building blocks), and amino acids with reactive side chains for library cyclization into the macrocyclic or linear peptide libraries. Fig. 6 demonstrates generation and screening of ribosome-display peptide library. Since the

peptide and mRNA are not topographically segregated, the mRNA can potentially interfere with the peptide.

3.4.2. Discovery of tumor-targeting peptides from ribosome-display peptide library

—Rong and Wen reported a ribosome-display system to isolate specific anti-tumor peptides from a designed random DNA library with affinity to membrane model [85]. Pore-forming peptides Mast21 and MastoparanX were chosen as positive control peptides to optimize the artificially synthesized tumor liposome cell membrane model. After 6 rounds of successive selection with improved membrane model (PE/PS, 3:7) and ribosome-display system, several peptides were obtained. Anti-tumor effects of the isolated peptides on non-small cell lung cancer cell line NCI-H460 were analyzed with MTT assay. *In vitro* experiment confirmed that two peptides SeqA3 (MKYDWEGVRDMFRRCLWISLRSWCVH) and SeqB3 (MKYDWRCLAGHAIKGWALRSHLAVYD) showed anti-tumor effects against NCI-H460 cells, with IC_{50} of 22.5 μM and 11.3 μM, respectively, compared to that of normal human fibroblast cell line CCD-27SK at a concentration greater than 100 μM.

3.4.3. Discovery of tumor-targeting peptides from mRNA-display peptide library

3.4.3.1. Interleukin-6 (IL-6)-binding peptides: IL-6 is a multifunctional cytokine and plays an important role in the host immune defense and the modulation of growth/differentiation in various malignancies [86–88]. Clinical studies have revealed that increased serum IL-6 concentrations in patients are associated with advanced tumor stages and short survival in patients [86]. Therefore, blocking IL-6 signaling is a potential targeted therapeutic strategy for cancer (i.e., anti-IL-6 therapy). Kobayashi et al. isolated a novel peptide inhibitor against IL-6 by *in vitro* selection using mRNA-display technology. Of a total of 39 clones analyzed, 12 had an identical sequence of NQQLIEEIIQILHKIFEIL, which was named CA11. After the amino acid sequence of CA11 was partially randomized and submitted for further selection, a new peptide RA07 (INTLLSEINSILLDIISLL) was obtained. RA07 specifically interacted with IL-6 and prevented the IL-6/IL-6R complex from binding to gp130 [89].

3.4.3.2. Vascular endothelial growth factor receptor 2 (VEGFR2)-binding peptides: Suga's group recently selected inhibitors of VEGFR2 from libraries produced in a PURE translation system with 16 natural amino acids and four backbone-modified unnatural amino acids (cycloleucine, D-phenylalanine, D-tyrosine, *N*-methyl-histidine, and *N*-methyl-phenyl-alanine) [90]. The most potent compound L1 is a cyclic head-to-tail thioether macrocyclic peptide, which showed relatively high serum stability and was much more potent than previously reported small molecule (“monomer”) antagonists obtained from combinatorial libraries [91-93]. It blocked VEGF-induced HUVEC proliferation with an IC_{50} of 60 nM and inhibited angiogenesis as measured by the HUVEC tube formation assay.

The tumor-targeting peptides identified by the ribosome- or mRNA-display library method are summarized in Table 6.

4. OBOC peptide library

4.1. Design, synthesis and decoding of OBOC peptide library

Unlike phage-display technology, which limits the peptide library to L-amino acids and simple configuration structure, the OBOC technology offers a lot more structural possibilities, *e.g.* linear, cyclic, branch and macrocyclic peptide libraries, as well as peptide libraries comprised of both natural and unnatural amino acids (L-/D-, α-/β-/-γ-amino acids and amino acids with posttranslational modifications such as phosphorylation and glycosylation). Fig. 7 shows examples of different peptide libraries that can be constructed and encoded using OBOC library approach. OBOC library is synthesized on solid phase such as TentaGel resin beads from Rapp Polymere (Tübingen, Germany). Standard solid phase peptide synthesis employing Fmoc-chemistry and split-mix strategy are commonly used for the synthesis of OBOC peptide libraries (Fig. 8) [11,94]. Each 80-100 μm bead displays only one chemical entity and contains approximately 100 pmol of the same compound.

To successfully apply OBOC peptide library approach to the discovery of cancer-targeting peptides, good library design is important. Since the peptide concentration on bead surface is very high that may lead to identification of low-affinity ligands, a down-substitution method [95,96] employing topologically segregated bi-layer beads [97] has been developed to reduce loading only on bead surface while keeping full loading in the bead interior for decoding. Cell surface is generally negatively charged due to presence of the sialic acids and phospholipid head groups. To avoid non-specific anionic–cationic interactions between the cells and the peptide–bead surface, the amount of basic amino acids (i.e. lysine and arginine) in the library construction can be lowered, but not eliminated because basic residues may be required for binding or internalization. This is certainly true for the RGD motif of αvβ3, αvβ5 or α5β1 integrin and many cell-penetrating peptides. To reduce the rate of false positive beads, Kodadek's group introduced the concept of redundant OBOC libraries, in which more than one bead displays an identical compound [98]. If compounds isolated from the screening of a redundant library are present more than once, they are likely to be high quality ligands. While useful, redundant libraries limit the number of unique compounds that can be synthesized and screened at one time.

Tumor-targeting ligands can be discovered through screening OBOC libraries with live cancer cells (see below) or with an extracellular domain of cell surface receptors. In general, fully random peptide libraries (i.e., each amino acid has an equal chance to present in the library) are screened for the discovery of initial hits. Subsequent lead optimization can be achieved through screening-focused libraries, e.g. using motifs of the initial hits. Positive beads are picked up manually under a stereo-microscope or by automated sorting with a COPAS instrument [99]. If the target is a protein, it can be used to decorate magnetic nanoparticles, which in turn can be used as probes to detect and isolate positive beads with magnetic bead sorting [100,101]. After positive beads are isolated, chemical decoding can be performed directly with Edman microsequencing if coding tag consists of α-amino acids [102]. Alternatively, coding tag can be released from a bead for decoding via mass spectrometry (MS) [103,104]. A third method is partial Edman degradation-mass

spectrometry (PED-MS) method developed by Pei et al. [104], in which partial Edman chemistry is performed on beads prior to release of "ladder sequences" for MS analysis. For OBOC peptide library comprising long sequence *(e.g.,* >15mer), huge permutation or complex structure, decoding by MS remains a significant challenge. In that case, traditional automatic Edman microsequencing may be preferred, but it is slower and more expensive.

The main advantages of the OBOC method are (i) a large number (10^6-10^8) of compounds which can be rapidly synthesized and screened concurrently without the need for any special equipment, and therefore can be employed in any chemical or biochemical laboratory; (ii) OBOC library can be screened using either one or a combination of both on-bead binding and solution phase functional assays (via a cleavable linker) [105]; (iii) both binding and functional ligands *(e.g.,* pro-apoptotic agents) [106,107] can be discovered. OBOC library can also be used for discovery of cell penetrating peptide ligands, but a cleavable linker and a reporting probe are needed in between the library compound and beads; (iv) OBOC combinatorial libraries are based on synthetic chemistry; therefore, it enables incorporation of D-amino acids, unnatural amino acids, many other organic building blocks, and investigation of cyclic, turned or branched peptides and secondary structures which can confer enhanced resistance to proteolytic degradation, a key requirement for clinical applications; (v) tumor-targeting ligands identified by OBOC library approach are not limited to peptides, but also *N*-methylated peptides [108], glycopeptides [109-111], peptide tertiary amides [112,113], peptidomimetics [114,115], and peptoids [116]; and (vi) the OBOC method can be used for rapid optimization of the initial lead compounds, whether they are native or identified from biological or synthetic peptide libraries. In OBOC method, each library compound is tethered to the solid support via a linker such as polyethylene glycol. In tumor imaging and drug delivery applications, the linker could be used as a convenient handle to connect the cancer targeting ligand with the therapeutic or diagnostic payload. However, unlike phage-displayed libraries, it is technically difficult to screen tumor-targeting ligand in live animals with OBOC technology.

4.2. Screening of OBOC library

4.2.1. Screening of OBOC library with live cancer cells—To identify ligands that bind to the surface of cancer cells, the direct way is to incubate OBOC library beads with live cancer cells and inspect under a microscope for cell-bound beads (Fig. 8). The screening process includes 5 steps: extensive washing of the library beads, incubating beads with cells, isolating positive beads, stripping of the cells from beads and decoding the peptide sequence of each positive bead. Beads with the appropriate ligands that bind to the cell surface receptors will be coated by a monolayer of cells and considered as "hits". In order to eliminate the false "hits" due to non-specific binding, Lam and co-workers have developed two "subtraction screening" methods [117]. In the first method, the OBOC library beads are first screened with cancer cells, the positive beads coated with cancer cells are picked and recycled by stripping the cells off with 8 M guanidine hydrochloride followed by thorough washing. The recycled beads are then incubated with "normal" cells. Those beads that bind to both cancer and normal cell types are considered false positive. The second method is termed "dual-color screening method" which involves labeling the cancer cells with a fluorochrome *(e.g.* calcein AM) and mixing them with unlabeled "control" cells prior to

screening the bead-library. Those beads that only bind to the fluorescent cells are considered "true positive" beads. In addition to screening for cell binding ligands, OBOC libraries can screen for both cell attachment and cell functions *(e.g.,* cell signaling or apoptosis). One may use a green fluorescent protein (GFP) transfected cell line in which GFP will be expressed upon activation of a specific cell-signaling pathway. For the discovery of pro-apoptotic peptides, caspase-3 fluorescent substrate may be used to identify beads that are coated with cells undergoing apoptosis. This OBOC library method can be applied to both suspension and adherent cells, as well as fresh cancer cells isolated from patient blood, pleural fluid, ascites, or biopsy specimens.

Traditionally, positive beads identified from screening of OBOC library are picked up manually with a micropipette. However, it could be tedious and time-consuming if there were too many beads to pick. To speed up the screening process, fluorescence-based sorting platform such as the Complex Object Parametric Analyzer and Sorter (COPAS, from Union Biometrica) has been developed. Lewis group reported high-throughput screening of OBOC peptide libraries using intact cells. They evaluated a COPAS large particle biosorter for high-throughput sorting of beads that were bound by MDA-MB-435 GFP breast cancer cells [118]. When an OBOC library of GRGDXX was screened against human cancer cells that express αvβ3 integrin, cells bound to beads were rapidly dissociated when sorted through the COPAS instrument. However, after the bound cells were reversibly cross-linked onto the beads with 3% formaldehyde, they have successfully sorted positive beads together with cells. This approach should facilitate screening of OBOC library with live cells to identify novel peptide ligands against cell surface targets in their native state. Major drawbacks of COPAS screening are that the equipment is expensive and generally not available in most laboratories, and formaldehyde fixation may preclude sequential screening with other cells. Furthermore, overfixation can potentially destroy the coding tags.

4.2.2. Screening of OBOC library with cancer-associated protein—Cancer-associated soluble proteins can be screened for binding to OBOC peptide libraries. For target protein that cannot be visualized directly through a microscope, a reporter system such as an enzyme, fluorophore, colorimetric dye or radionuclide conjugated to the target protein is needed to identify the interacting beads. A general approach is to use biotinylated protein and probe with streptavidin-alkaline phosphatase (AP) employing an enzyme-linked colorimetric assay. The selection process is relatively simple and involves the catalysis of the colorimetric substrate bromochloro-indolyl phosphate (BCIP) by the bead-bound AP, turning the positive beads turquoise (Fig. 8). A two-step subtraction approach is used to eliminate false positive beads that bind to streptavidin [117]. If an antibody to the target protein is readily available, a secondary antibody conjugated to AP can be used as the reporter system. The success of OBOC library screening is dependent on the stringency of the screening conditions and effective negative control. COPAS bead sorting can be used for screening of OBOC library with soluble proteins [119]. Alternatively, bead sorting can be achieved with the aid of streptavidin-coated magnetic nano-particles. Bulk magnetic separation can be performed on the bench of any lab at low cost without the need for expensive specialized equipment.

4.3. Discovery of tumor-targeting peptides from OBOC peptide library

We and other researchers have reported the use of the whole-cell bead binding assay to identify cell surface ligands against a number of different cancer cell lines, including both adherent and non-adherent cells.We have also performed experiments on fresh cancer cells isolated from patients. A variety of peptide libraries have been designed, synthesized and screened including libraries consisting of all L-amino acids or D-, both L- and D-amino acids. Some of these libraries are linear; others are cyclic (Fig. 7). Upon further optimization with focused libraries, we have found that unnatural amino acid substitution and/or addition of organic moieties often lead to the development of ligands with higher affinity and specificity. Tumor-targeting ligands identified from OBOC peptide library are summarized in Table 7.

4.3.1. α3β1 integrin-binding peptides—Overexpressionofα3β1 integrin has been reportedinseveral cancer types, such as glioblastoma [120], ovarian cancer [121], breast cancer [122–124], lung cancer [125], and melanoma [126], and has been associated with poor prognosis, tumorigenesis, tumor metastasis, invasion, and resistance to cancer treatment [127–130]. Thus, α3β1 integrin has been investigated as a promising cancer-specific biomarker and therapeutic target.

Through screening of random OBOC libraries, we previously discovered a cyclic motif cDGXGXXc (c stands for D-cysteine) that binds to the α3 sub-unit of α3β1 integrin on ovarian adenocarcinoma cell lines ES-2, SKOV-3, and CaOV-3. We subsequently synthesized and screened two secondary libraries based on this motif and identified several new peptides with higher affinity towards these cell lines including a cyclic peptide named OA02 with the sequence cdG-HoCit-GPQc (wherein HoCit is L-homocitrulline) [131]. OA02-decorated paclitaxel-loaded nanomicelles showed improved tumor-targeting in the SKOV-3 mouse model compared with drug-loaded nanomicelles without OA02 [121]. We subsequently synthesized four OBOC peptide libraries based on cdGXGXXc and screened against MDA-MB-231 breast cancer cells. LXY1 with sequence cdGLG-Hyp-Nc (wherein Hyp is L-hydroxyproline) was identified with high binding affinity (K_D = 0.4 μM) and specificity to α3 integrin [132]. Based on the established SAR information, two highly focused OBOC cyclic peptide libraries were further designed, synthesized, and screened against MDA-MB-231 breast cancer cells under stringent conditions. A novel cyclic peptide LXY3 (cyclic cdG-Tyr(3-NO_2)-G-Hyp-Nc) with a high binding affinity (IC_{50} = 57 nM) was identified [133]. Moreover, the targeting efficiency and specificity of LXY3 to the breast adenocarcinoma tumors in mouse xenografts were further confirmed by *in vivo* andexvivo near-infraredfluorescence optical imaging. Using OBOC library approach followed by optimization with medicinal chemistry, we have very recently developed a cyclic nonapeptide ligand named LXY30 (cyclic cdG-Phe(3,5-diF)-G-Hyp-GcR), which showed further improved *in vivo* tumor targeting property [134]. Chemical structures of OA02 and LXY30 are shown in Fig. 9. Tumor-targeting of LXY30 has been verified by *in vitro* binding to SKOV3 cells (Fig. 10A) and clinical ovarian tumor tissue (Fig. 10B), as well as *in vivo* tumor uptake in SKOV3 xenograft mouse model (*in vivo* and *ex vivo* optical images are shown in Fig. 10C and D, respectively).

4.3.2. α4β1 integrin-binding ligands—α4β1 integrin is a non-covalent heterodimeric transmembrane receptor, recognizing the QIDS and ILDV sequences in the vascular cell adhesion molecule-1 (VCAM-1) and fibronectin, respectively [135, 136]. Activated α4β1 integrin is expressed in lymphomas, leukemias, sarcomas, and melanomas [137]. It strengthens tumor cell adhesion to vasculature endothelium and facilitates tumor cell extravasation, thus promoting dissemination of tumor cells to distal organs [137–139]. α4β1 integrin prevents the apoptosis of malignant chronic lymphocytic leukemia (CLL) cells [140] and plays important roles in the drug resistance of both multiple myeloma [141] and acute myelogenous leukemia [142]. Anti-α4 integrin antibody has been found to inhibit multiple myeloma growth in a murine model [143]. Furthermore, α4β1 is expressed on proliferating but not on quiescent endothelial cells in angiogenesis during tumor development [144]. Therefore, α4β1 integrin is an attractive drug target against cancers, especially lymphoid malignancies. High-affinity and high-specificity ligands of α4β1 integrin can be used to noninvasively image α4β1 integrin-expressing tumors and to develop α4β1-targeting anti-cancer therapy.

Through screening random OBOC peptide libraries against live Jurkat T-lymphoid leukemia cells, we have identified peptide ligands which contain LD(V/I/F) motif against α4β1 integrin [145]. However, these ligands from random peptide libraries have relatively low affinity, and the binding is barely detectable on α4β1-expressing cells using radiolabeled or fluorescence-labeled ligands. Using a highly focused OBOC combinatorial peptidomimetic library in conjunction with a high stringency screening method, we have successfully identified a high-affinity (IC_{50} = 2 pM) and high-specificity tri-peptide derived molecule LLP2A (Fig. 9) that bind to activated α4β1 integrin of lym-phoid cancers [115]. LLP2A provides an important tool to noninvasively monitor α4β1 expression and activity during tumor progression and has been used successfully to detect α4β1-expressing tumors *in vivo* [115,146–154].

4.3.3. αvβ3 integrin-binding peptides—Lam and colleagues have designed several cyclic RGD-based OBOC peptide libraries and screened against αvβ3 integrin receptor transfected K562 myeloid leukemia cells using whole cell on-bead screening. A new RGD peptide, cGRGDdvc (LXW7) [155], cyclized by a disulfide bond and with a built-in handle at the carboxyl terminus, was identified. LXW7 shows high specificity against αvβ3 integrin and comparable binding affinity (IC_{50} = 0.68 μM) to some of the well-known RGD "head-to-tail" cyclic pentapeptide ligands reported in the literatures. It binds to both U-87MG glioblastoma and A375M melanoma cell lines, both of which express high levels of αvβ3 integrin. Optical *in vivo* imaging study with LXW7-biotin/streptavidin-Cy5.5 complex showed higher tumor uptake in U-87MG glioblastoma and A375M melanoma xenografts, and lower uptake by liver when compared to biotinylated RGD cyclo-pentapeptide ligands. Further optimization of LXW7 led to development of LXW64 with sequence cGRGDd-nal1-c (where nal1 stands for D-1-naphthylalanine). LXW64 exhibits 6.6-fold more potent binding affinity against αvβ3-expressing U-87MG cell *in vitro* and better *in vivo* tumor targeting compared to LXW7 [108].

RGD-based peptides may affect the biology of tumors resulting in increased tumor aggressiveness, invasiveness [156] and micrometastases [157]. Cancer cells treated with

RGD-labeled iron oxide nanoparticles lose intercellular contacts and have decreased cell adhesion [158]. Low concentrations of RGD peptide can enhance tumor growth *in vivo* by promoting VEGF mediated angiogenesis [159]. Therefore, there is a great need to identify new non-RGD peptides that do not negatively affect tumor biology yet potently bind to αvβ3 integrin. The Lewis group recently identified several non-RGD peptides against αvβ3 integrin containing a (K/H)_(K/H) motif, through screening an OBOC octapeptide library via a multiplex "beads on a bead" library screening approach [160]. In this method, αvβ3 integrin was first biotinylated incompletely to average one biotin per molecule. The biotinylated integrin was then immobilized to 2 μm streptavidin-coated beads that are both magnetic (from iron oxide core) and fluorescent (from rhoda- mine), followed by incubating with OBOC peptide library. The positive beads, with the highest affinity peptides for αvβ3, were isolated by magnetic sorting and flow-based fluorescence separation. The positive beads were further validated with binding to αvβ3 integrin-expressing live cells, prior to MS decoding. The two peptides with the highest affinity, LCE62 (MAFKHKAH) and LCE64 (KTKKVHSQ), have K_D value of 10.8 ± 1.2 and 4.7 ± 0.3 nM, respectively, against MDA-MB-435 breast cancer cells that express αvβ3 integrin. No significant uptake of FITC-LCE62 and FITC-LCE64 was observed in αvβ3-knockdown MDA-MB- 435 cells as well as αvβ3-low expression HT-1080 fibrosarcoma cells. Interestingly, peptides LCE62 and LCE64 do not appreciably alter the morphology, cell adhesion, and cell spreading of MDA-MB-435 cells, nor do they affect the tube formation of HUVEC cells *in vitro.* Taken together, non-RGD peptides LCE62 and LCE64 could be utilized as vehicles that effectively deliver imaging and therapeutic agents to αvβ3-expressing tumor cells without negatively affecting the biology of tumor cells.

4.3.4. Aminopeptidase N (APN)-binding peptides—APN is a cell membrane protein that plays a key role in tumor angio- genesis [161]. Wang et al. reported a continuous-flow microfluidic method for OBOC combinatorial peptide library screening [162]. They used an integrated screening strategy based on a lab-on-chip system that included high-throughput positive peptide isolation, magnetic bead sorting, single bead trapping, and *in situ* MS decoding. A 7mer OBOC peptide library containing ~2×10^5 peptide beads was screened within 4 h, and 140 peptides targeting APN with a conserved YXXY sequence at the N-terminal were discovered. Peptide AP-1 (YVEYHLC) has high affinity (K_D = 37.5 nM) and high specificity for APN. *Ex vivo* and *in vivo* optical imaging demonstrated high tumor uptake in a HepG2 liver cancer xenograft mouse model.

4.3.5. CD21-binding peptides—CD21 receptor is a cell surface marker of malignant B cell lymphoma. The Kopecek group has identified four heptapeptide ligands of CD21 through screening a 7mer peptide OBOC library with a two-step fluorescence screening method [163]. The binding affinities of selected peptides, YILIHRN (B1), PTLDPLP (B2), and LVLLTRE (B3), were in the μM range as determined by a fluorescence quenching assay. Peptide B1 was conjugated to N-(2-hydroxypropyl)-methacrylamide (HPMA) copolymer via linkers with different lengths containing one to four repeats of the 8-amino-3,6-dioxaoctanoic acid. The HPMA copolymer-B1 conjugates with three repeats of linker in the spacer showed optimal bio-recognition by the CD21 receptor.

5. PNA-encoded solution phase peptide library

5.1. Methodology

In 2004, Winssinger et al. reported a self-assembled PNA-encoded peptide library method [164]. In this method, peptides were first prepared by the “split-mix” synthesis method and then cleaved from the resin to form a PNA-encoded solution phase peptide library such that each peptide was linked to a PNA coding tag via a hydrophilic linker. The peptide library was then mixed with the protein of interest followed by exposure to a planar oligonucleotide microarray of predetermined sequences [165]. Alternatively, the PNA-encoded soluble peptide library can be first hybridized to the oligonucleotide microarrays and then mixed with the target protein. Decoding can be achieved via direct read-out from the oligonucleotide microarray. Svensen et al. recently reported a related PNA-encoded peptide library, which allows the identification of versatile cell-penetrating homing peptides and/or cell surface receptor ligands [166]. The PNA-tagged peptide library was first incubated with cells, which were then lysed, PNA extracted and hybridized with random DNAs, followed by amplification of the hybridized DNA for decoding *via* microarray hybridization readout. The advantages of this method include easy decoding and only a tiny amount of tags are needed for decoding. The disadvantage for this method is that since the peptide and PNA code are not topographically segregated, interference by the PNA coding tag could be problematic.

5.2. Discovery of tumor-targeting peptides from PNA-encoded solution phase peptide library

5.2.1. αvβ5/αvβ3 integrins- and CCR6-binding peptides—Svensen and Bradley reported discovery of peptide ligands targeting αvβ5/αvβ3 integrins and receptor CCR6 by screening a PNA-encoded peptide library (Library-1) with human D54 cells (overexpressing αvβ5 and αvβ3 integrins) and HEK293T-CCR6 (over-expressing CCR6) [166,167], respectively. Library-1, Ac-FQX4X3YX2X1IK-PNA17-fluorescein, consisted of 10,000 nonapeptide-PNA conjugates with each variable amino acid (X = 10 l- or D-amino acids) encoded by a PNA-triplet. Library screening identified two peptides with sequences of FQSIYPpIK [166] and FQpIYIIIK [167] bound to D54, and one peptide with sequence of FQIPYIIIK [166] bound to HEK293T-CCR6 cells, respectively. Their binding to specific cells was confirmed with flow cytometry analysis when cells were incubated with the FAM-labeled peptides or with CCR6 antibodies. These three peptides did not show cytotoxicity at 100 μM as assessed with standard MTT assays.

6. Peptide microarray

6.1. Methodology

Peptide microarrays are usually prepared by printing peptides on a solid surface *(e.g.* glass slides, cellulose sheets, polymer-based membranes, microchip, etc) through *in situ* synthesis *(e.g.,* SPOT synthesis), *in situ* immobilization, or chemical ligation. Details on this technology can be found in the previous reviews [168,169]. Peptide microarrays enable investigators to rapidly analyze, in parallel, molecular interactions between immobilized molecules and complex biological mixtures. Screening is accomplished by a binding assay

with cells or fluorescent-labeled protein. The peptide sequences of immobilized peptides on microarrays are known; therefore, decoding is not required. Another advantage of peptide microarray is peptides with different lengths or structures can be included in the same library to achieve maximal structure-activity relationship (SAR) information. Peptide microarrays enable rapid identification of critical amino acid residues for target binding and determination of the minimal binding sequence. Although there is no report so far, microfluidic technology holds the promise to prepare peptide microarray more efficiently. Work is currently underway in our laboratory to develop microfluidic print-head [170,171] that allows solid phase peptide synthesis on solid planar substrates using standard Fmoc-chemistry.

One main drawback of peptide microarray is that only a relatively small number of peptides can be generated and screened, therefore this technology is a medium throughput approach and mainly used for ligand optimization and SAR studies. Immobilization of peptides on a planar surface might influence their conformation, resulting in a decrease of their target affinities. The results provided by an array might be biased by unequal peptide quantity (concentration) or surface immobilization.

6.2. Discovery of tumor-targeting peptides from peptide microarray

6.2.1. PDGFRfi-binding peptides—Peptide microarrays have been successfully applied for optimization of the PDGFRp binding peptide PDGFR-P1 (IPLPPPSRPFFKY), which was originally identified from a phage-display peptide library [172]. A peptide array randomizing PDGFR-P1 by replacement of each amino acid with 20 natural amino acids was constructed via spotting. Fc-tagged PDGFRp and fibroblast growth factor receptor (FGFR, as a negative control target) were incubated with the peptide arrays. After washing, spot intensity was determined using an Fc-specific antibody conjugated to horseradish peroxidase. Selected derivatives and fragments of PDGFR-P1 were chemically synthesized, radiolabeled, and evaluated in cell-based assays, using PDGFRβ-overexpressing BxPC3 (human pancreatic carcinoma cells) and control MCF7 cells (human breast cancer expressing FGFR). Through addition of a D-Tyr to the N-terminus and replacement of Ser at residue 7 with Arg, the binding affinity of the resulting peptide yG2 (yIPLPPPRRPFFK, wherein y = ^{125}I-tyrosine) against BxPC3 cells was increased (K_D = 0.57 μM for yG2 *vs* 3.43 μM for PDGFR-P1). Serum stability of yG2 was found to improve 20-fold ($t_{1/2}$ = 80 min for yG2 and 4 min for PDGFR-P1).

7. Perspectives

Cancer cell-targeting peptides identified from combinatorial libraries are excellent vehicles for delivery of therapeutic agents or imaging probes to the primary or metastatic tumor sites. Therapeutic payload includes radionuclides, potent cytotoxic peptides such as MMAE, therapeutic oligonucleotides such as siRNA and microRNA, biologics such as IL-2, nanocarriers encapsulating chemotherapeutic agents, and therapeutic check-point blockade antibodies. To be effective clinically, the cancer cell-targeting peptides need to have a high affinity and a high specificity to their target tissue(s). Non-specific targeting of these peptides to circulating blood cells, plasma proteins, or normal organs should be low. They

should be stable in circulation and tissue proteases. For tumor imaging, uptake of the imaging probes into the cancer cells may not be needed. However, for many of the aforementioned therapeutic payloads, cancer cell uptake is essential. Although nanotherapeutics without targeting ligands can be passively taken up by the tumors via the "enhanced permeability retention (EPR)" effect, *in vivo* xenograft studies demonstrated that the addition of targeting ligands can enhance intra-tumoral distribution and cellular uptake of the nanotherapeutics into the tumor cells, resulting in better tumor response [121].

Drug resistance often develops with cancer treatment. This could be due to the lack of targeting to the "tumor stem cells", emergence of mutant tumor cells that evade the targeting ligands or therapeutic payloads, or regional inaccessibility of some parts of the tumor to the therapeutic agent. Some of the solutions to this problem include (i) development of ligands that target all tumor cells including "tumor stem cells", (ii) the use of therapeutic radionuclides that can exert "bystander effects", (iii) the use of multiple different therapeutic pay-loads, and (iv) the addition of immunotherapy, such as checkpoint blockade, resulting in active targeting by the host's immune cells.

It is now clear that the tumor microenvironment plays a very important role in oncogenesis and tumor progression. Thus, there is a need to develop peptides that target specific cellular or acellular components within the tumor microenvironment. Therapeutic payloads against these targets are probably very different from those used in cancer cell targeting.

There is an increasing interest in developing therapeutic peptides or proteins that block intracellular protein-protein interactions. Peptide libraries have been used to discover such blocking agents. However, *in vivo* tumor-specific delivery of these peptides inside the target tumor cells could prove to be challenging. Cell-penetration peptides (CPP) have been used as the delivery agent, but CPPs are often nonspecific and they, together with the therapeutic payloads, will likely be taken up by a large number of circulating blood cells before they ever reach the tumor sites. One approach to overcome this hurdle is to use nanocarriers to protect the CPP-drug conjugates during the circulation and release them at the tumor sites. Another approach is to create "pro-CPP" that does not bind and enter circulating blood cells, but can be enzymatically activated at the tumor sites to become CPP. To further enhance tumor cell delivery of such drugs, one may incorporate both pro-CPP and tumor-targeting peptides to the final drug conjugate. Development of trifunctional peptides equipped with (i) tumor-targeting, (ii) CPP or pro-CPP, and (iii) protein-protein blocking functions could be very challenging. One approach is to incorporate one or more of these functional motifs *(e.g.* tumor-targeting and CPP functions) in the OBOC library design and screen for ligands that bind strongly to the target protein. Ligands identified from such libraries are expected to be able to target and penetrate target tumor cells *in vivo* as well as block specific protein-protein interaction inside the tumor cells.

Although many short peptides *(e.g.* <20mer) identified from combinatorial library screening are foreign to the host, they probably are not immunogenic unless they are conjugated to a carrier protein and administered together with immune adjuvants.

There is no set rule for optimal length and structure of peptides for receptor binding; it all depends on the nature of the receptors and the corresponding ligands. For example, integrins often bind to relatively short peptide motifs, such as -RGD-, which is the minimal binding motif for αvβ3, αvβ5, αvβ6, αvβ3, and αvβ1 integrin. Binding specificity and affinity of this tripeptide motif to specific integrin(s) depend greatly on the nature and chirality of the residues flanking the RGD sequence as well as the overall size of cyclization. Many peptides with RGD motifs have been discovered by both OBOC and phage-display library methods (Tables 2, 3, 4, 7).

Although structural information of the receptor is not needed in combinatorial library approaches, it could be very helpful. Many related peptides are often discovered from combinatorial library screening. If structural information of the receptor and binding affinity of the peptides are available, computational chemistry can guide rational design of peptides and focused peptide libraries; this can greatly facilitate the discovery process.

There have been great advances in the drug delivery field in recent years. Peptidic and non-peptidic ligands that bind to target cells continue to play a very important role in this field. In addition to cancer targeting, there are needsin other disease areas. For example, there is an urgent need for targeted-delivery of antibiotics to bacterial biofilms or pathogens hiding inside host cells. As stem cell biology advances, there is a great need for efficient delivery of therapeutic stem cells to target organ sites [173]. Undoubtedly, combinatorial technologies discussed in this review will play a significant role in the development of efficacious vehicles for drug, nucleic acid, and cell deliveries in these therapeutic areas.

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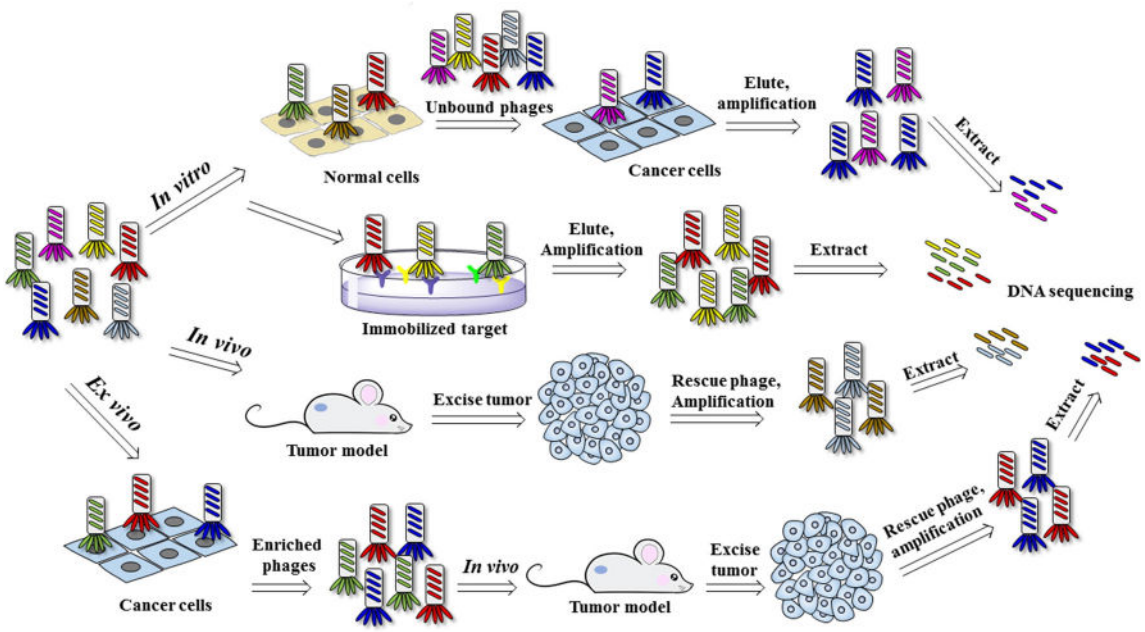

Fig. 1.
Biopanning of phage-display peptide library.

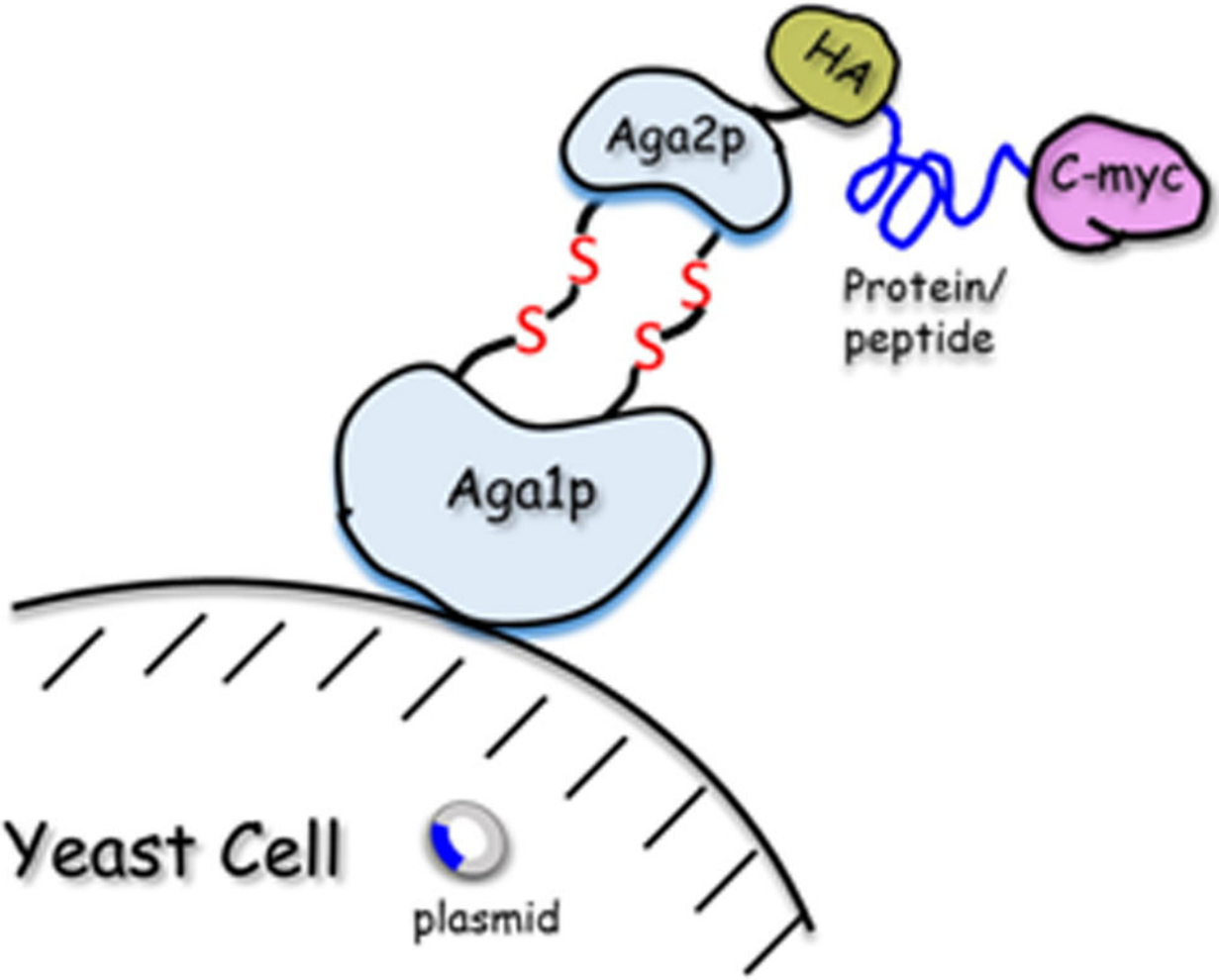

Fig. 2.
Display surface of the yeast cell.

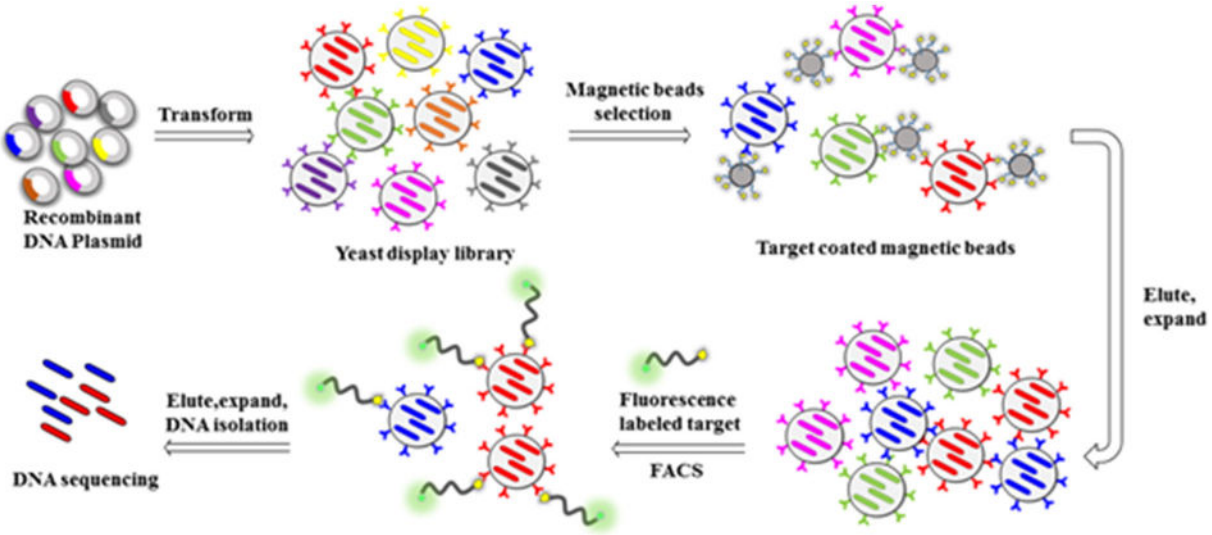

Fig. 3.
Construction and screening of yeast-display peptide library.

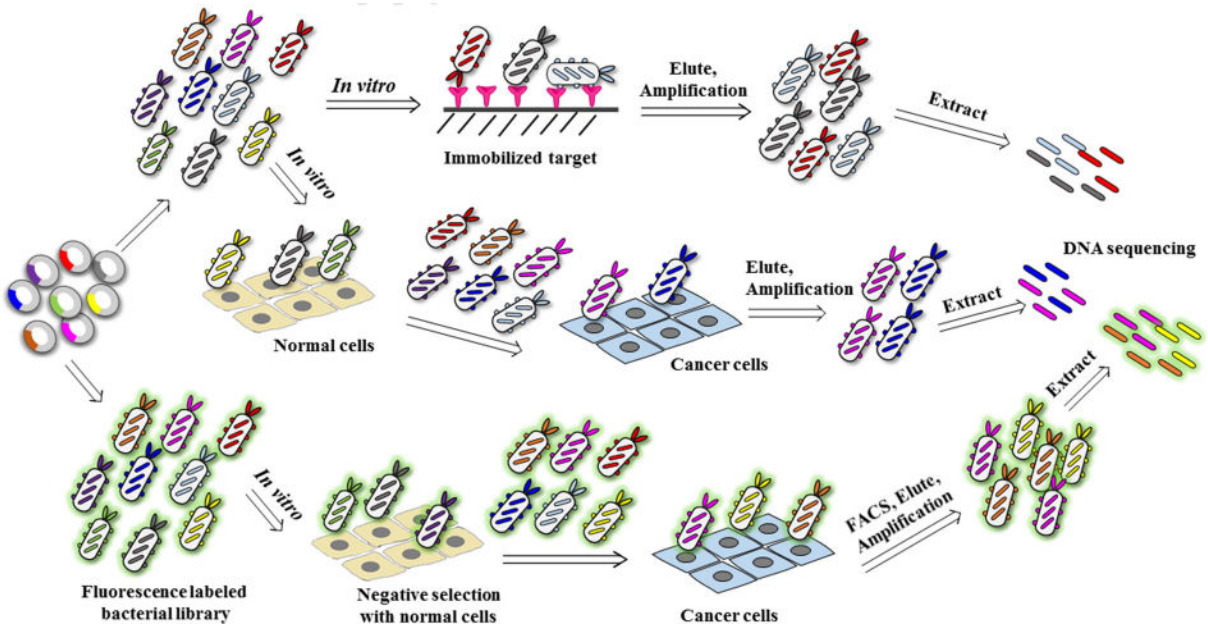

Fig. 4.

Construction and screening of bacteria-display peptide library.

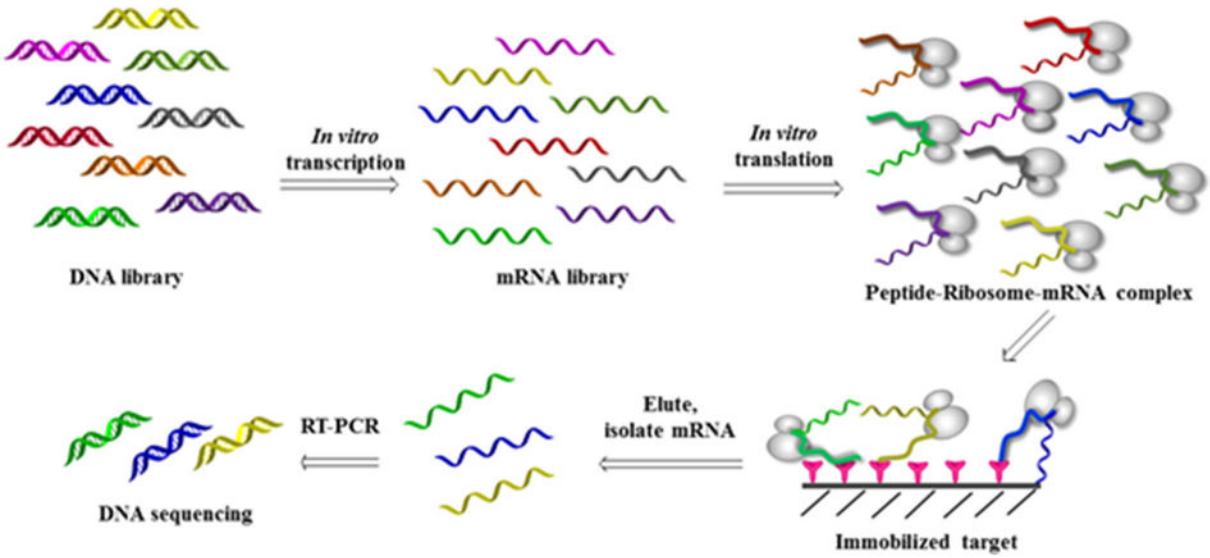

Fig. 5.
Construction and screening of ribosome-display library.

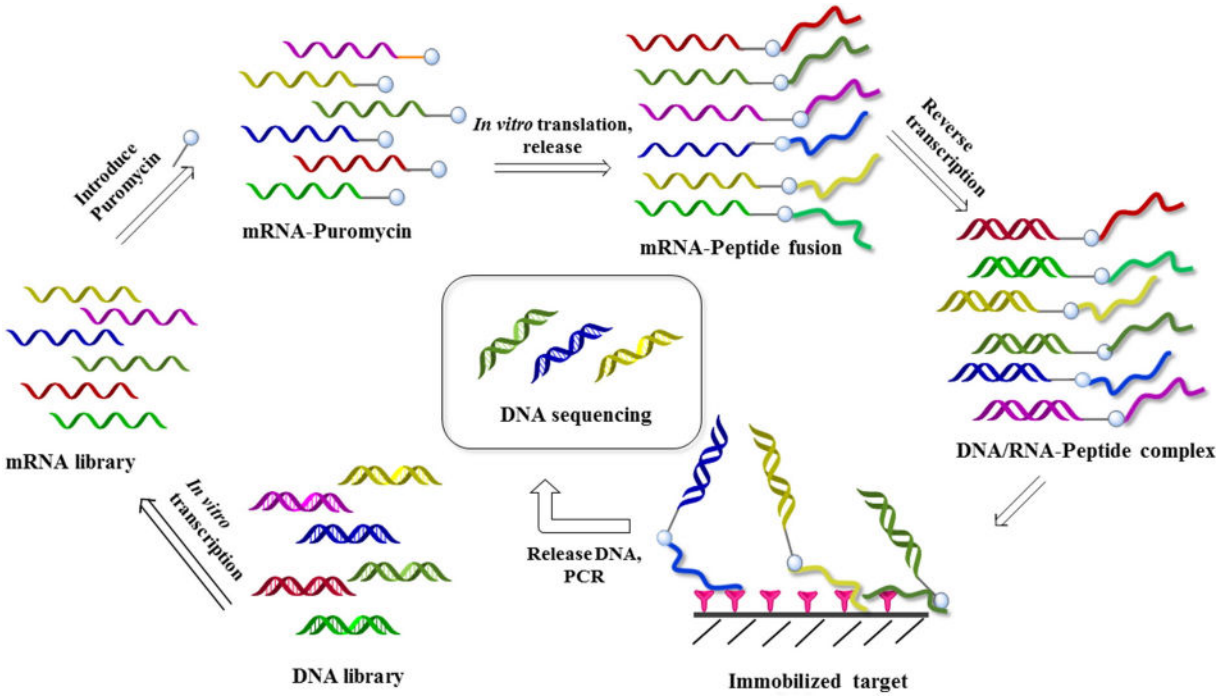

Fig. 6.
Construction and screening of mRNA-display library.

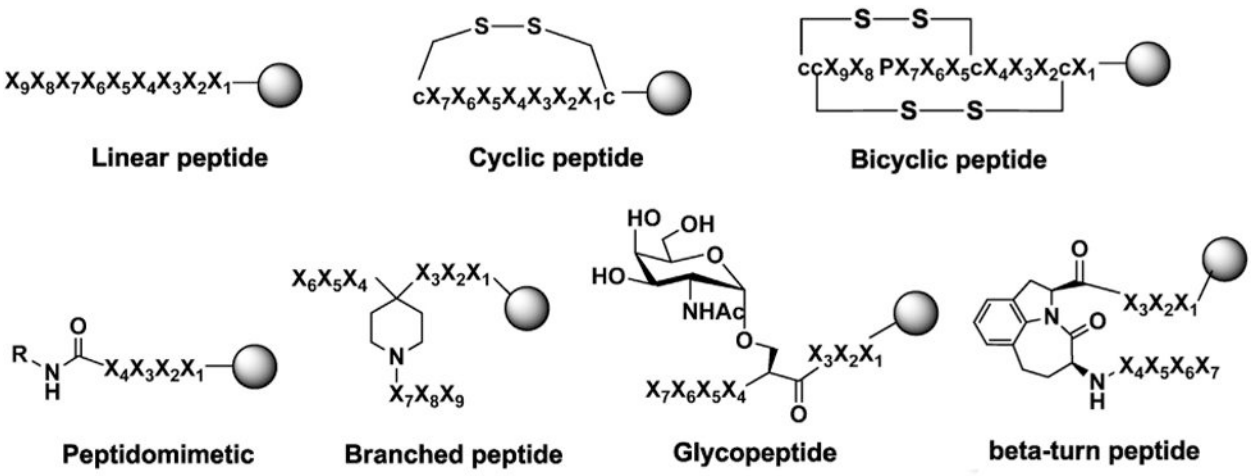

Fig. 7.

Examples of synthetic OBOC peptide and peptidomimetic libraries for discovery of tumor-targeting ligands. X_1–X_9 stand for natural and unnatural amino acids, L- or D-amino acids. c represents D-cysteine, K stands for L-lysine. R: alkyl or aryl.

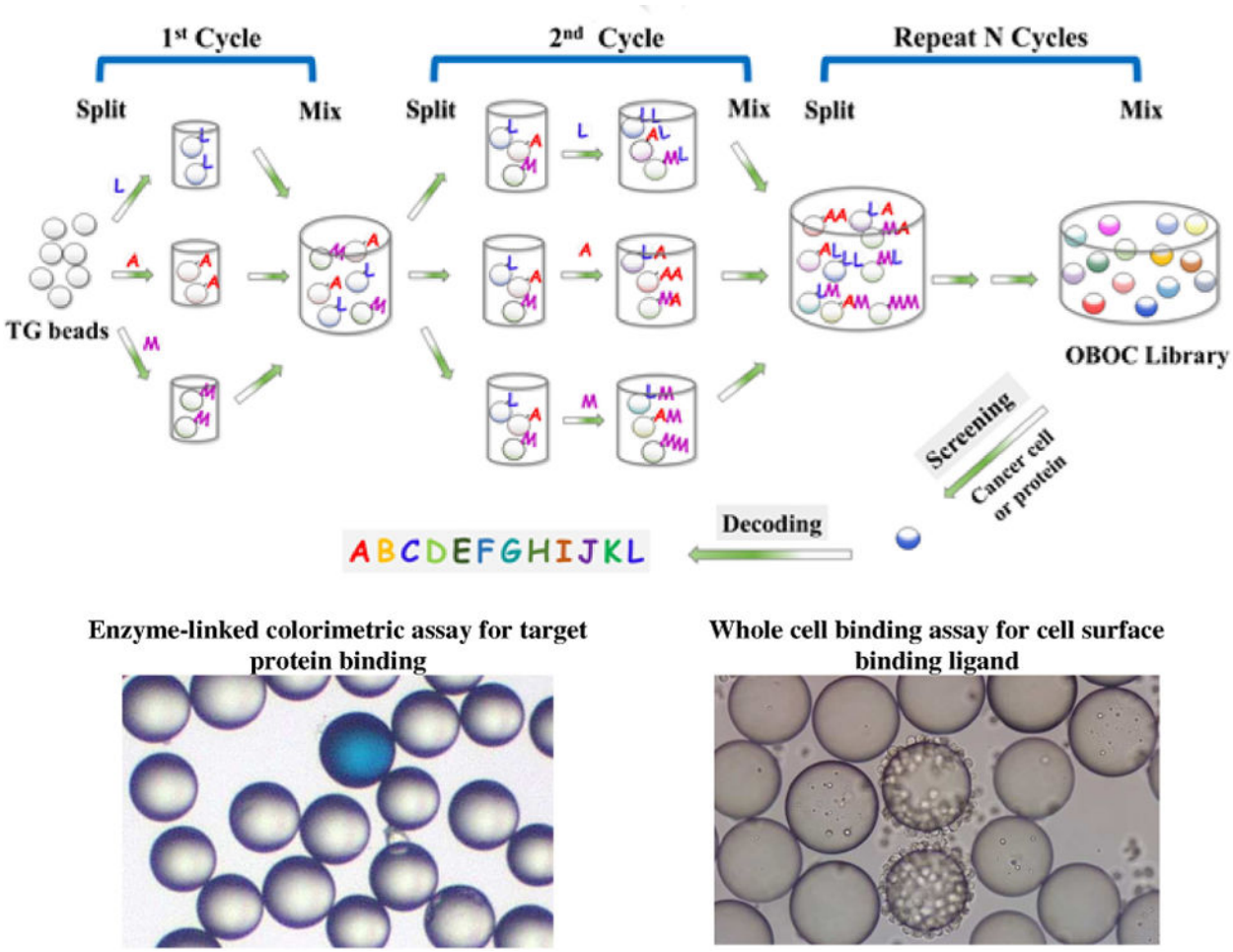

Fig. 8.

Synthesis and screening of an OBOC combinatorial peptide library. Eukaryotic amino acids use standard single-letter codes. B and J represent unnatural amino acids.

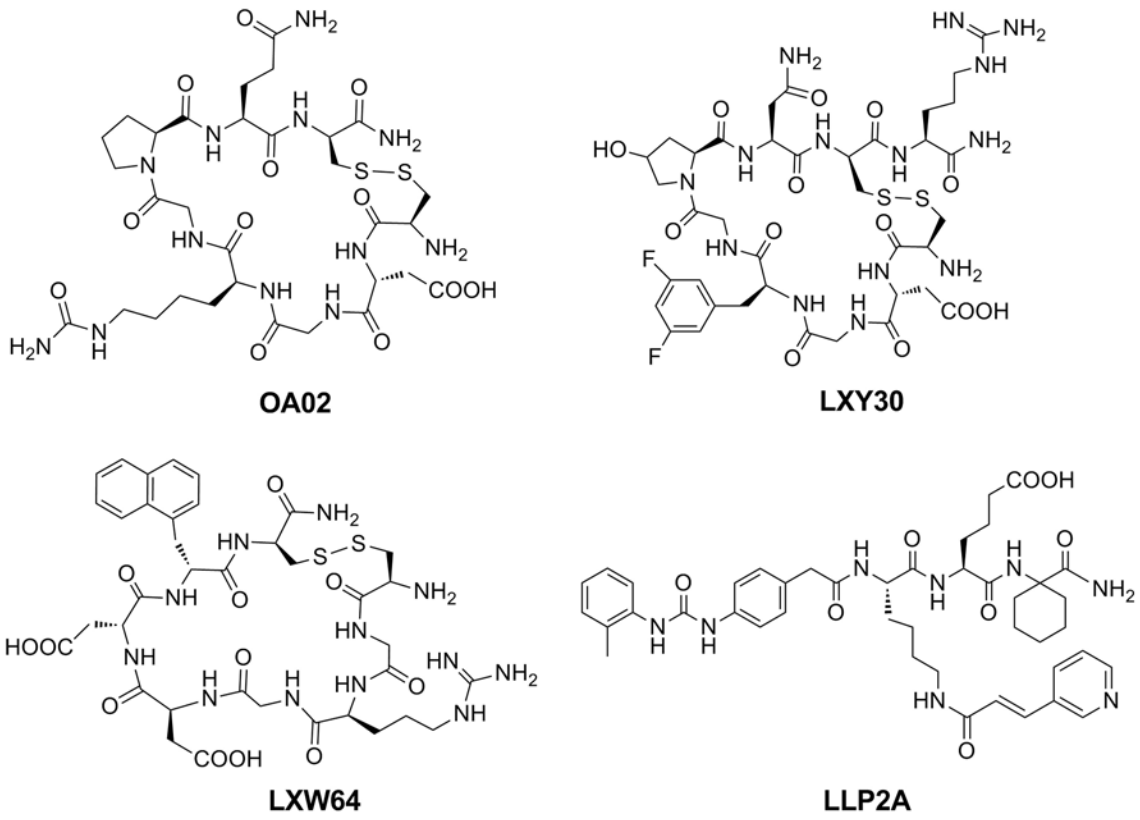

Fig. 9.

Chemical structuresof selected tumor-targeting ligands identified by the OBOC library approach. OA02 and LXY30 are peptide ligands of α3β1 integrin. LXW64 and LLP2A are ligand of αvβ3 and α4β1 integrin, respectively.

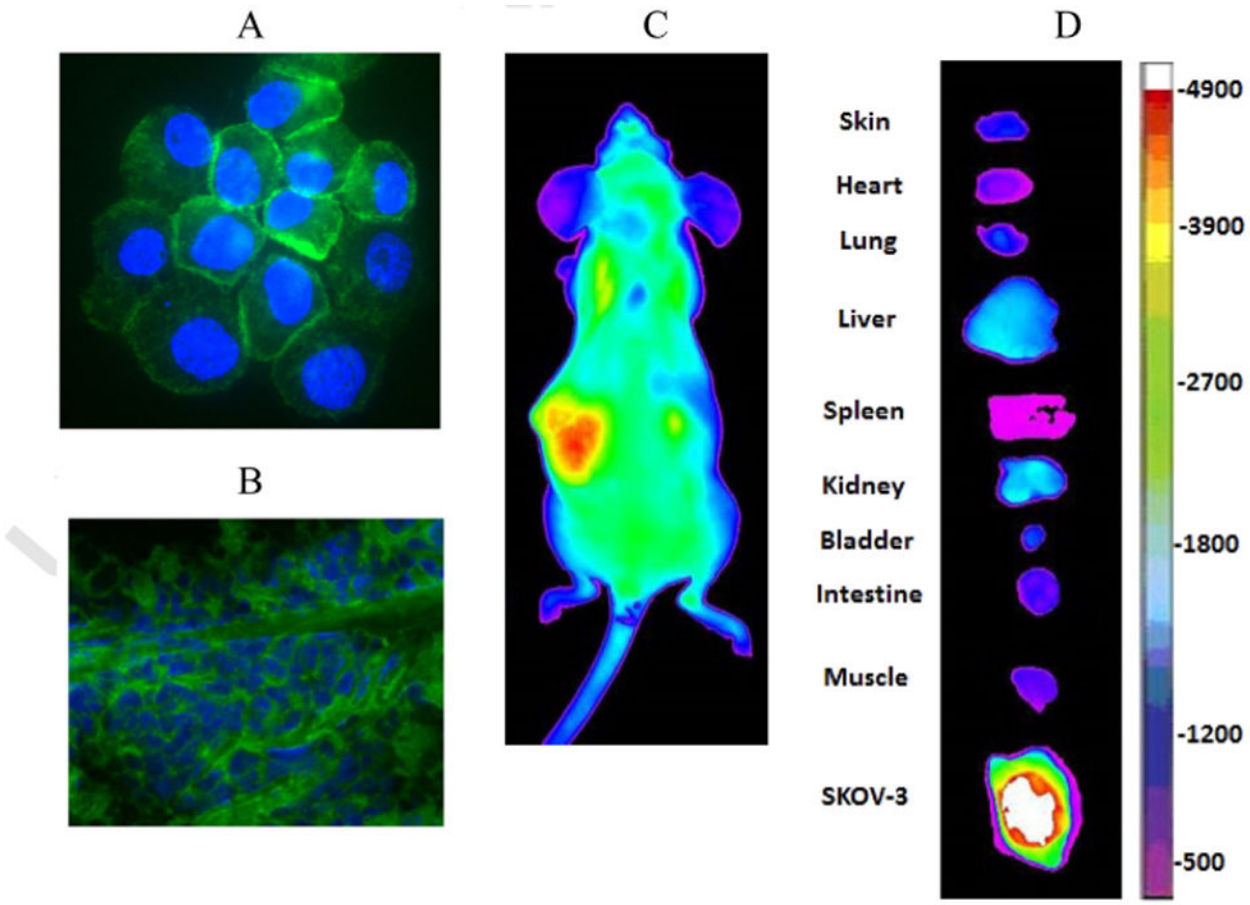

Fig. 10.

Applications of tumor-targeting peptides identified by OBOC library approach (examples). A: *in vitro* staining of SKOV3 cells with LXY30-Biotin-SA-PE; B: Staining of human ovarian tumor tissue with LXY30-Biotin-SA-Alexa488 (1 μM of LXY30-Biotin). Green fluorescence: Positive staining with Alexa488. Blue fluorescence: Nuclei counter-stained with DAPI; C: *In vivo* optical imaging of SKOV3 subcutaneous tumor with LXY30-SA-Cy5.5; D: *Ex vivo* optical imaging of SKOV3 subcutaneous tumor and normal organ.

Table 1

Comparison of different combinatorial peptide library methods.

Combinatorial library methods		Advantages	Disadvantages
Biological library	Phage-display	• High capacity (up to 109) • Able to display long peptides (e.g. 30 amino acids) with tertiary folds possible • Multiple selection options (in vitro, in vivo and ex vivo) • Libraries are commercially available, or can be generated in many laboratories • Easy to handle • Inexpensive	• Not totally random because of bias in genetic codons • Typically limited to peptide libraries with eukaryotic amino acids • Unable to generate complicated structures • Limited to binding and simple functional assay Two hosts (phage and bacterium) are needed • Identification of consensus sequences among phage clones is unlikely
	Yeast-display	• Offers easy secretion and purification of displayed peptides • Yeast grows rapidly and has the ability to perform posttranslational modification • Use of FACS for screening because most libraries include sequences for fluorescent protein tag	• Limited library size • Unable to display highly stable structures of long peptides
	Bacteria-display	• Easy to manipulate • *E. coli* grows rapidly and provides up to 10^{11} peptide entities • Only one host is needed • Quantitative screening can be achieved with FACS when bacteria is fluorescent-labeled • Library amplification does not require reinfection • Commercially available	• Limited to *in vitro* biopanning screening • Library size is limited (10^5) if other bacteria are used rather than *E. coli* • Complex bacterial cell surface can interfere with binding of displayed peptide • Limited by access to a flow cytometer with cell sorting capabilities
	Ribosome-or mRNA-display	• No need for cellular transformation • Easy mutagenesis for PCR • Newer system can incorporate unnatural amino acids • High library diversity	• Screening limited to selection conditions that keep the display complex intact • Low display efficiency
Chemical library	OBOC	• Not limited to natural amino acids; highly efficient synthesis and screening • Each peptide is spatially separable, therefore multiple different motifs can be identified	• Linker effect unpredictable until tested • Chemical structure of positive beads has to be analyzed • Cannot be used for *in vivo* selection in animals

Combinatorial library methods	Advantages	Disadvantages
	• Applicable to both binding and functional assays • Inexpensive • Lead ligand can be rapidly optimized • Can be easily synthesized by experienced peptide chemist • Built-in PEG linker can be used to link diagnostic and therapeutic agents • Multiple use possible	• Library not commercially available
PNA- encoded solution phase peptide library	• Library decoding on DNA chip is highly efficient • Able to split-mix synthesis to generate the library	• Synthesis of PNA coding tag is cumbersome • PNA cannot be amplified by standard PCR • Library size is limited • Require special DNA chip for decoding • Limited to binding and simple functional assay • Not commercially available
Peptide microarray	• Replicates of peptide chips can be made • Microassay possible to save expensive and precious assay reagents • Peptide chips are commercially available or can be custom-- made • Moderately expensive	• Library size is limited • Spotting technique is rapid but requires synthesis of individual compound separately • *In situ* synthesis not widely available • Limited to on chip binding and some functional assays • Peptide chip generally not recyclable for subsequent use • Possible linker effect

Table 2

Tumor-targeting peptides identified from phage-display peptide libraries by *in vitro* biopanning against purified receptors.

Receptor	Sequence*	Ref	Library	Application	
				Imaging	Therapy
GPC3	DHLASLWWGTEL (TJ12P1)	[174]	Ph.D.-12	[174]	–
PD-L1	NYSKPTDRQYHF** (^{D} APP1)	[175]	Ph.D.-12	[175]	[175]
β-Catenin	AC#AQKLDGC#SYISWSC#G (BC1) AC#SGWWPKC#QGYIPGC#G (BC2) AC‡APGVYRC‡NQNFIWC‡G (BC3)	[176]	ACX_6CX_6CG	–	–
PDGFRβ	IPLPPPSRPFFK	[55]	Ph.D.-12	[55]	–
PKCδ	LMNPNNHPRTPR (PKC-bp)	[177]	Ph.D.-12	–	–
PTPRJ	***C***HHNLTHA***C*** (PTPRJ-pep19) ***C***LHHYHGS***C*** (PTPRJ-pep24)	[59]	Ph.D.-C7C		
	CHHALTHA***C*** (PTPRJ-pep19.4)	[60]	Ph.D.-C7C	–	–
TfR 1	SPRPRHTLRLSL (B18)	[178]	Ph.D.-12	–	–
Tie 2	TMGFTAPRFPHY	[179]	Ph.D.-12	–	[179]
CD-21	RMWPSSTVNLSAGRR (P1)	[180]	15mer	–	–
VEGFRI (Flt-1)	NGYEIEWYSWVTHGMY (SP5.2)	[181]	16mer	–	–
IL-10 RA	FRSFESCLAKSH	[182]	PH.D.-12	–	–
EGFR	YHWYGYTPQNVI (GE11)	[21]	Ph.D.-12	[183,184]	[21,183,185–189]
	QHYNIVNTQSRV[a]	[190]	Ph.D.-12	–	–
	QRHKPRE	[191]	Ph.D.-7	[191]	–
FGF8b	HSQAAVP (P12)	[192]	Ph.D.-7	[192]	–
aFGF	AGNWTPI (AP8)	[193]	Ph.D.-7	–	–
bFGF	PLLQATL (P7)	[194]	Ph.D.-7	[194]	–
IL-6Rα	LSLITRL (S7)	[195]	Ph.D.-7	–	[195]
α5β1	***C***RGD***C***L	[196]	6mer	–	–
	GA***C***RGD***C***LGA (synthetic peptide)				
	CRRETAWA***C***	[197]	CX_7C	–	–
	GA***C***RRETAWA***C***GA (synthetic peptide)				
α6β1	VSWFSRHRYSPFAVS (P3)	[198]	15mer	–	–
αvβ3/αvβ5	CDCRGDCFC (RGD-4C)	[40,199]	CX_9	[200–202]	[199,201–204]

Receptor	Sequence*	Ref	Library	Application	
				Imaging	Therapy
	HGRFILPWWYAFSPS (P-30)	[253]	15mer	–	–
Fibrin-fibronectin complexes	***C***GLIIQKNE***C*** (CTL1)[f]	[254]	CX_8C	[254]	–
	CNAGESSKN***C*** (CTL2)[f]				
FGFR	AESGDDY***C***VLVFTDSAWTKI***C***DWSHFRN (C19)	[255]	26mer	–	–
	MQLPLAT	[256]	Ph.D.-7	–	–
	CRALLRGAPFHLAE***C***	[257]	15mer	–	–
E-selectin	IELLQAR	[258]	Ph.D.-7	–	[258]
MMP2-processed collagen IV	TLTYTWS	[259]	Ph.D.-7	–	–
PSA	CVAYCIEHHCWTC (C-4)	[260]	$CX_3CX_4CX_2C$	–	–
Notch1 NRR	AC[#]ERYQGC[#]FSVGGYC[#]G (NRR17)	[261]	ACX6CX6CG	–	–
CD44	THENWPA (CV-1)	[262]	Ph.D.-7	–	–
	WHPWSYLWTQQA (RP-1)	[263]	Ph.D.-12	–	–
FGF3	VLWLKNR (FP16)	[264]	Ph.D.-7	–	–
Extradomain-B fibronectin	***C***TVRTSAD***C*** (ZD2)	[265]	Ph.D. C7C	[265]	–
APRIL	AAAPLAQPHMWA (sAPRIL-BP1)	[266]	Ph.D.-12	–	[266]
p16	SHSLLSS	[267]	Ph.D.-7	–	–
pre-miR-21	ALWPPNLHAWVP	[268]	Ph.D.-12	–	–

*Cysteine residues that form disulfide bonds are indicated in bold and italic

**Mirror-image phage display strategy was used. Synthetic D-version of the IgV domain of PD-L1 was used as screening target D-version of this peptide targets PD-L1.

#Bicyclic peptide cyclized by1,3,5-tris(bromomethyl)benzene.

‡Bicyclic peptide cyclized by1,3,5-triacryloyl-1,3,5-triazinane.

[a]ICR-62 mAb was used as a panning probe;

[b]12H23 mAb was used as a panning probe.

[c]Bicyclic peptides binding to the ECD of Her2.

[d]L-26 mAb was used as a panning probe;

[e]N-12 mAb was used as a panning probe;

[f] human plasma clots were used for panning probes; GPC3: Glypican-3;T antigen: Thomsen-Friedenreich glycoantigen; PS: Phosphatidyl serine; IL-10 RA: human interleukin-10 receptor alpha; APN: Aminopeptidase N; PPP2R1A: phosphatase 2 regulatory subunit A, α-isoform; CXCR2: CXC chemokine receptor-2; TfR1: human transferrin receptor 1; IL-6Rα: Interleukin 6 receptor chain *α*; Notch1 NRR, negative regulatory region in Notch 1. p16: Protein p (16INK4a).

Table 3

Tumor-targeting peptides identified from phage-display peptide libraries by screening cancer cells or tumors.

Receptor	Sequence[a]	Ref	Library	Selection	Cells or tumors	Cancer type	Imaging	Therapy
HER2	LTVSPWY	[269,270]	Ph.D.-7	*In vitro*	SKBR3	Breast cancer	[271,272]	[269,273]
α-Enolase	SSMDIVLRAPLM (pHCT74)	[274]	Ph.D.-12	*In vitro*	HCT116	Colorectal cancer	–	[274]
EGFR	FPMFNHWEQWPP	[275]	Ph.D.-12	*In vitro*	U-87 MG	Glioblastoma	[275]	–
	SYPIPDT (P1)	[22]	Ph.D.-7	*In vitro*	A431	Epidermoid	–	–
	HTSDQTN (P2)					carcinoma		
MUC1	***C***LFMRLAW***C***	[276]	CX_7C	*In vitro*	B16 cells cocultured with B-1 lymphocytes	Melanoma	–	–
Nucleolin	DMPGTVLP	[277,278]	Landscape phage-display libraries f8/8 and f8/9	*In vitro*	MCF-7	Breast cancer	–	[277,279]
	DWRGDSMDS							
	VPTDTDYS							
	VEEGGYIAA							
GP130	VTWTPQAWFQWV (VTW)	[280]	PH.D.-12	*In vitro*	U-87 MG	Glioblastoma	–	[281,282]
Nestin	AQYLNPS	[283]	Ph.D.-7	*In vitro*	Glioma stem cells	Glioblastoma	–	[283]
Cadherins	***C***SSRTMHH***C***	[284]	CX_7C	*In vitro*	B16-F10-Nex2	Melanoma	–	[284]
α4β1	***C***PLDIDFY***C***		CX_7C	*In vitro*	Kasumi-1	Leukemia	–	–
α5β1	***C***PIEDRPM***C*** (RPMrel)	[285,286]	CX_7C	*In vitro*	HT29	Colon cancer	[285]	[286]
αvβ6	RGDLATLRQLAQEDGVVG-VR	[24,25]	Ph.D.-20	*In vitro*	H2009	NSCLC	[287]	[288–293]
	(H2009.1) SPRGDLAVLGHK (HBP)	[26]	PH.D.-12	*In vitro*	HNO223	Head and neck cancer	[26]	–
	SPRGDLAVLGHKY (HBP-1)							
αvβ3 (RMS-I)	***C***QQSNRGDRKR***C*** (RMS-I)	[41]	CX7–10C	*In vitro*	RD	Rhabdo-myosarcoma	[41]	–
IL-13Rα2	***C***MGNK***C***RSAKRP (RMS-II) ***C***GEMGWVR***C***	[50]	Ph.D-C7C	*In vitro*	G26-H2 and SnB19-pcDNA cells	GBM	[50]	–
VPAC1	GFRFGALHEYNS (VP2)	[294]	Ph.D.-12	*In vitro*	CHO-K1 cell transfected wi VPAC1	th Colorectal cancer	–	–
IGHC	***C***TLPHLKM***C***	[295]	CX C	*In vitro*	Raji	Lymphoma	–	–
HSPG[b]	ASGALSPSRLDT (OSP-1)	[296]	Ph.D.-12	*In vitro*	143B	Osteosarcoma	[296]	–
Adenoviral receptor	SWDIAWPPLKVP	[297]	PhD-12	*In vitro*	A172	Glioblastoma	–	–
GRP78	***C***TVALPGGYVRV***C*** (Pep42)	[298]	CX3-12C	*In vitro*	Me6652/4	Melanoma	–	[298,299]
GRP78	ETAPLSTMLSPY (GMBP1)	[300]	Ph.D.-12	*In vitro*	SGC7901	Gastric cancer	–	[300]

Receptor	Sequence[a]	Ref	Library	Selection	Cells or tumors	Cancer type	Imaging	Therapy
GRP78	GIRLRG	[301]	Ph.D.-7	*In vivo*	Irradiated GL261 gliomas i mice	n Murine glioma	[301]	[301]
APP	***C***PGPEGAG***C***	[302]	CX C	*In vivo*	Mammary tissue	Breast cancer	–	–
IL-11Rα	***C***GRRAGGS***C***	[303]	CX_7C	*In vivo*	Prostate from a human patient	Waldenström macro-globulinemia	[304]	[305]
PDGFRβ[b]	CRGRRST (RGR)	[306]	CX7C	*In vivo*	angiogenic islets in RIP1-Tag2 mice	Pancreatic istets	–	[307]
APN (CD13)	***C***NGRCVSGCAGRC (NGR)	[199,308]	CX_9	*In vivo*	MDA-MB-435 xenografts	Breast cancer	–	[199,309–317]
p32/gC1qR	***C***GNKRTRG***C*** (LyP-1)	[318,319]	CX7C	*Ex vivo*	MDA-MB-435 xenograft	Breast cancer	[211]	[210,320–323]
TIP-1	HVGGSSV	[324,325]	Ph.D.-7	*In vivo* *In vivo*	Irradiated and SU11248-	LLC, murine	[325–328]	[325,327]
α2bβ3	RGDGSSV	[329]	Ph.D.-7	*In vivo*	treated LLC and GL261 tumo irradiated LLC and GL261 tumors	rs glioblastoma LLC, murine glioblastoma	[329]	[329]
α3β1[b]	SWKLPPS	[330]	Ph.D.-7	*In vivo*	AZ-P7a tumor	Gastric cancer	–	[330]
αvβ3 αvβ5	***C***RGDKRGPD***C*** (iRGD)	[42]	CX_7C	*In vivo*	PC-3 xenograft	Prostate cancer	[331]	[331,332]
NRP-1	GGKRPAR (P4)	[38]	Ph.D.-7	*In vitro*	PC-1	Prostate cancer	–	–
	RIGRPLR (P7)							
	CGFYWLRS***C***	[35]	CX_7C	*In vitro*	Molt-4	Lymphoma	–	[35]
	RPARPAR	[37]	Ph.D.-7	*Ex vivo*	PPC-1 human prostate	Prostate cancer	[333]	[333]
				In vivo	carcinoma xenograft			
MMP2-processe collagen IV	d TLTYTWS	[259]	Ph.D.-7	*In vivo* *In vitro*	*In vivo* LLC tumor-bearing mouse, then *in vitro*	Lewis lung carcinoma	–	[259]
VAV3	SSQPFWS	[61]	Ph.D.-7	*In vivo*	MMP2-processed collagen glioblastoma xenograft	IV xxx	[61]	–
CRKL	YR***C***TLNSPFFWEDMTHE***C***-HA	[334]	20mer	*In vivo*	DU145 xenograft	Prostate cancer	–	[334]
Plectin-1	KTLLPTP (PTP)	[335]	Ph.D.-7	*Ex vivo*	PDAC cells from the Kras/p53 mouse	PDAC	[335,336]	–

IGHC: immuno-globulin heavy chain; HSPG: heparin sulfate proteoglycans; PDAC: Pancreatic ductal adenocarcinoma; IL-13Rα2: Interleukin 13 receptor α2; GBM: glioblastoma multiforme; LLC: Lewis lung carcinoma; GRP78: glucose-regulated protein 78; APP: aminopeptidase P; NRP-1: neuropilin-1; APN: aminopeptidase N.

[a]Cysteine residues that form disulfide bonds are indicated in bold and italic.

[b]Candidate cellular receptor.

Table 4

Tumor-targeting peptides identified from yeast-display peptide library.

Receptor	Sequence	Ref	Library type
Mcl-1	RPEIWMTQGLRRLGDEINAYYAR (MS1) RPEIWLTQSLQRLGDEINAYYAR (MS2)	[337]	Bim-BH3 variants
	RPEIWLTQHLQRLGDEINAYYAR (MS3)		
	RPEIWIAQEIDRIGDEVNAYYAR (MB1)	[338]	Bim-BH3
CTLA-4	SPR^{3}***C***KYSHVP^{2}***C***RRDSD^{1}***C***PGK^{3}***C***I^{2}***C***RGNGY^{1}***C***G (MC-CT010)[a]	[67]	oMCoTI-II mutant *Saccharomyces cerevisiae*
$\alpha_v\beta_3$ Integrin	G^{4}***C***VRLHES^{3}***C***LGQQVP^{1}***C***^{4}CDPAAT^{3}***C***Y^{2}***C***TGRGDEKLR^{2}CY^{1}***C***R (6C)[α]	[65]	AgRP mutants *Pichia pastoris*
$\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_5\beta_1$ Integrin	G^{3}***C***PRPRGDNPPLT^{2}***C***KQDSD^{1}***C***LAG^{3}***C***V^{2}***C***GPNGF^{1}***C***G (EETI2.5F)[α]	[339]	EETI-II mutant *S. cerevisiae*
Bcl-X_L	RPEIWVAQELKRNGDEFNAYYAR (BCL-X_L)	[338]	Bim-BH3

[a]Disulfide bonds formed between ^{1}***C*** and ^{1}***C***, ^{2}***C*** and ^{2}***C***, ^{3}***C*** and ^{3}***C***, ^{4}***C*** and ^{4}***C***, respectively. Mcl-1: myeloid cell leukemia 1. CTLA-4: cytotoxic T lymphocyte-associated antigen 4.

Table 5

Tumor-targeting peptides identified from bacteria-display peptide library.

Receptor	Peptide sequence[a]	Ref	Cancer type	Cell line used for selection	Library type
VEGF	GPGPCSRLVMWEWECFAAL WPVRCSRFVMWEWECFLRA	[76]	N/A	N/A	CPX
NS	IAVAPGWLWEEE (Hep1)	[340]	Liver cancer	HepG2	FliTrx
	KELCELDSLLRI (Hep2)				
	IRELYSYDDDFG (Hep3)				
NS	CPGDRGQRRLFSKIEGPC (MM-2)	[341]	Prostate Cancer	PC-3	FliTrx
NS	NVVRQ (TMTP1)	[342]	Prostate Cancer	PC-3M-1E8	FliTrx
NS	VECYLIRDNLCIY	[343]	Breast cancer	ZR-75-1	CPX
NS	EWCGIVRVGYCLGGGKK (PepC3)	[344]	Breast tumor	MDA-MB-231, MCF-7, and T47-D	CPX
NS	CGGRRLGGC	[345]	Murine squamous carcinoma	SCC VII	FliTrx
NS	WFCSWYGGDTCVQ	[346]	Lung cancer	A549	CPX fluorescence-library

[a]Cysteine residues that form disulfide bonds are indicated in bold and italic. NS: not specified. CPX: circularly permuted outer membrane protein OmpX.

Table 6

Tumor-targeting peptides identified from mRNA-display peptide library.

Receptor	Sequence	Ref	Note
AKT2	*YILVRNRLLRVD *CG (Pakti-L1)	[347]	Inhibited Akt2 with IC_{50} of 100 nM, and exhibited 10- and 25-fold selectivity over Akt1 and Akt3, respectively
IL-6	NQQLIEEIIQILHKIFEIL (CA11)	[89]	RA07 prevented the L-6/IL-6R complex from binding to gp130
	INTLLSEINSILLDIISLL (RA07)		
VEGFR	*FVVVSTDPWVNGLYID C (L1)	[90]	Inhibited HUVEC tube formation. Inhibit VEGF-induced HUVEC growth with IC_{50} of 60 nM
SIRT2	*YSNFRIK(Tfa)RYSNSS *C (S2iL8)	[348]	Bound and inhibited SIRT2 with IC_{50} of 3.8 nM and exhibited 10- and 100-fold selectivity over SIRT1 and SIRT3, respectively

*An *N*-terminal chloroacetyl-L-tyrosine or chloroacetyl-,L-phenylalanine and a cysteine are cyclized with a thioether bond. Tfa: trifluoroacetyl.

Table 7

Tumor-targeting peptides identified by OBOC combinatorial library methods.

Receptor	Sequence[a]	Ref	Cancer type	Cancer cell line used for screening	*In vivo* Imaging	Therapy
α3β1 integrin	***c***dG-Phe(3,5-diF)-G-Hyp-N***c***R (LXY30)	[134]	Glioblastoma	U-87 MG	[134]	–
	cNGQGEQ***c*** (pA)	[349]	NSCLC	A549	–	–
	cDGLGDD***c***	[350]	Ovarian cancer	CaOV-3, ES-2, SKOV-3, OVCAR-3	–	–
	cdG-HCit-GPQ***c*** (OA02)	[131]	Ovarian cancer	CaOV-3, ES-2, SKOV-3	[3,131]	[121]
	cNGRGEQ***c***	[351]	NSCLC	A549	–	–
	cdGLG-Hyp-N***c*** (LXY1)	[132]	Glioblastoma	U-87 MG	[132]	–
	cdG-Tyr(3-NO_2)-G-Hyp-N***c*** (LXY3)	[133]	Breast cancer	MDA-MB-231	[133]	–
	kmviywkag (RZ-3)	[352]	Prostate cancer	DU145	–	–
α3β1/α6β1 integrin	kikmviswkg (HYD-1)	[352]	Prostate cancer	DU145	–	[353]
α6β1 integrin	LNIVSVNGRH (RU-1)[b]	[354]	Prostate cancer	DU145	–	–
NS	QMARIPKRLARH	[355]	Prostate cancer	LNCap	–	–
IgM kappa	wGeyvmvnG	[356]	Murine lymphoma	WEHI-231	–	–
APN	YVEYHLC (AP-1)	[162]	Liver cancer	HepG2	[162]	–
α4β1 integrin	***c***-Nle-D-Nle-T-Hyp-r***c*** (pM2)	[357]	NSCLC	H1650	–	–
	LTGpLDI	[145]	Leukemia	Jurkat	–	–
	cLDYWDc, ***c***WDLDHH***c***, sppLDIn, eapLDId, fypLDFf, FSIpLDI, QSYpLDF	[3]	Lymphoma	Raji	–	–
	"LLP2A" peptidomimetic and a series of analogues [115,358]	[115,358]	Leukemia Lymphoma	Jurkat Molt-4, Raji	[115,149–151,153]	–
	c-Nle-DWEE***c***, ***c***-Nle-DVDE***c***, ***c***-Nle-D-Chg-YM***c***, ***c***SD-Nle-D-Chg-***c***	[3,350]	Ovarian cancer	ES-2	–	–
	yminp-Nle-DIdnhh					
	vswap-Nle-DIgspd					
	vqgp-Nle-DIafvl					
	vgnvp-Nle-DIgqea					
	wdinp-Nle-DIgsfn					
	wsrip-Nle-DIqeps					
	cLDI-Chg-Hyp-Y***c***	[3,350]	Ovarian cancer	SKOV-3	–	–

Receptor	Sequence[a]	Ref	Cancer type	Cancer cell line used for screening	*In vivo* Imaging	Therapy
	c-Nle-D-Chg-NDF***c***					
	c-Nle-D-Nle-PhgD***c***					
	cDEL-Nle-EW***c***					
NS	***c***QDGRMGF***c*** (PLZ4)	[359]	Bladder cancer	5637	[359,360]	[361]
αvβ3 integrin	***c***GRGDd***vc*** (LXW7)	[155]	Glioblastoma	U-87 MG	[155]	–
	cGRGDdv***c*** (LXW7)	[155]	Melanoma	A375M	[155]	–
	cGRGDd-nal1-***c*** (LXW64)	[108]	Glioblastoma	U-87 MG	[108]	–
CD21	YILIHRN (B1), PTLDPLP (B2), LVLLTRE (B3)	[163]	–	–	–	–

NS: not specified. NSCLC: non-small cell lung cancer.

[a] D-Cysteine residues that form disulfide bonds are indicated in bold and italic.

[b] Original sequence has an uncertain amino acid at the c-terminal of RU-1. When omitted, the peptide retained binding to DU 145 cells in FACS and plate adhesion assay.