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Ligand-directed Cancer Gene Therapy to Angiogenic Vasculature

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ABSTRACT

Gene therapy strategies in cancer have remained an active area of preclinical and clinical research. One of the current limitations to successful trials is the relative transduction efficiency to produce a therapeutic effect. While intratumoral injections are the mainstay of many treatment regimens to date, this approach is hindered by hydrostatic pressures within the tumor and is not always applicable to all tumor subtypes. Vascular-targeting strategies introduce an alternative method to deliver vectors with higher local concentrations and minimization

of systemic toxicity. Moreover, therapeutic targeting of angiogenic vasculature often leads to enhanced bystander effects, improving efficacy. While identification of functional and systemically accessible molecular targets is challenging, approaches, such as *in vivo* phage display and phage-based viral delivery vectors, provide a platform upon which vascular targeting of vectors may become a viable and translational approach. © 2009, Elsevier Inc.

I. INTRODUCTION

Cancer is a heterogeneous disease marked by aberrant cellular growth. It remains one of the leading causes of mortality in the United States and in the last several years has shown increases in incidence (National Center for Health Statistics and Centers for Disease Control and Prevention, 2006). While improvements have been made in standard treatment regimens for solid tumors, including gamma-knife surgery and radiation- and/or chemotherapy, the survival rates vary widely both between tumor types and between individual patients. For example, in the case of pancreatic tumors, the median survival is less than 6 months, despite aggressive standard therapies (Greenlee *et al.*, 2000), whereas other tumors can have overall survival rates of greater than 70% in 5 years, such as prostate cancer (National Center for Health Statistics and Centers for Disease Control and Prevention, 2006). In addition, resistance to radiation- and/or chemotherapy as well as metastatic spread for advanced tumors further complicate treatment and disease prognosis. Therefore, a need remains for newer alternative therapies that would be applicable to many, if not all, solid tumor types and that would have efficacy in a setting of advanced tumor development where genetic or epigenetic alterations in tumor cells enhance resistance.

Through early advances in molecular biology that enabled scientists to sequence and clone genes, the field of gene therapy emerged with a rationale to treat disease by replacing, manipulating, or supplementing nonfunctional genes. Numerous basic and preclinical studies lead to the first clinical trial in 1989 in which Rosenberg *et al.* (1990) used *ex vivo* gene therapy with retroviruses to treat metastatic melanoma. Enthusiasm for gene therapy strategies in cancer remains high, as nearly two-thirds of all current clinical gene therapy trials are directed against cancer (Edelstein *et al.*, 2004).

The emergent data from clinical gene therapy trials have brought to light the contribution of numerous variables for successful end results. Noted factors include gene target regulation, cell transduction efficiency, duration of gene expression, vector stability, and allowing for readministration. To optimize these factors, and thereby minimize variability, the choice of vector delivery system remains crucial. The most widely used vector remains adenovirus, with recent increased use of adeno-associated virus (AAV) and nonviral delivery systems. However, use of these approaches often necessitates intratumoral or

local injection and attempts to deliver these vectors systemically have met with poor results. One of the basic tenets of systemic targeting is that the first cellular layer a circulating agent would encounter is the endothelial lining of blood vessels. The introduction of vascular targeting to gene-delivery vehicles could permit higher local concentrations for transduction, increase exposure, and minimize systemic toxicity. This review focuses on therapeutic concepts for targeted cancer gene therapy, vectors suitable for site-directed delivery, and methods to identify suitable receptors for ligand-directed delivery.

II. THERAPEUTIC CONCEPTS IN CANCER GENE THERAPY

The complexity of the tissue and tumor microenvironment permits a number of different targeting strategies toward different cell types relevant for therapy. The abundant genetic abnormalities in tumor cells present a clear target for genetic manipulation. In addition, introduction of genes into genetically stable cellular components in the tumor, such as the stroma and endothelial cells of blood vessels, provides an alternative strategy for delivery. Another approach involves stimulation of the immune system for tumor growth inhibition. The advantages and disadvantages of these methods along with current concepts for target genes are further explored in the following sections.

A. Immunomodulation

Intense study in the area of immunology over the last decade has made cancer immunobiology one of the more promising and dominant approaches in cancer gene therapy (Blankenstein et al., 1996). The goal is to stimulate a host response against the tumor by enhancing or inducing the native immune system using direct vaccination and immunization of tumor antigens.

To enhance the immunogenicity of tumors, transfection of an individual's tumor cells and autologous vaccination have emerged as successful methods for gene delivery. The tumor cells transfected with a number of candidate genes for use in this type of treatment are genes expressing costimulators of T-cell activation (e.g., CD80, CD86, and CD40) (Vesosky and Hurwitz, 2003); cytokines (e.g., interleukin-2 (IL-2), IL-3, IL-4, IL-6, IL-7, IL-10, IL-12, granulo-cyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor (TNF), and interferon-γ) to facilitate differentiation and/or activation of effector cells (Qian et al., 2006); allogeneic MHC class I proteins (e.g., transfection of HLA-B7 into the tumors of HLA-B7-negative patients) (Nabel et al., 1993); or syngeneic MHC class II proteins whose expression enables the tumor cell to present antigens to T-lymphocytes and stimulates activation of T-helper cells (Hock et al., 1995).

The introduction of these genes permits activation and targeting of the tumor cells for elimination. These strategies have led to several Phase I/II trials, including in melanoma patients, with autologous vaccination of irradiated, transduced tumor cells, and direction adenoviral delivery of antigens such as MART-1 and GP100 (J.Gene.Med, 2006).

A different approach uses immunization protocols, where gene-delivery vectors express known tumor antigens on the surface of muscle cells, dendritic cells, or T-lymphocytes. These cells in turn stimulate antigen-presenting cells or secondary stimulatory cells for activation of an immune response. An important consideration in this approach is avoidance of sensitization to nontumor cells and antigens by using selectively stimulating antigens, such as those only expressed in embryonic tissue, those protected from immune surveillance (e.g., cancer/testis antigens), or intracellular proteins (Acres *et al.*, 2004; Gunther *et al.*, 2005). Not surprisingly, the type of immunomodulation strategy to use is highly dependent on the goals of the treatment. Selection of the ideal treatment regimen requires consideration of a number of factors:

- *Type of immunity desired*: An antibody-mediated versus a cell-mediated responses require the stimulation of a different subset of T-lymphocytes.
- *Duration of response*: A potent short-term treatment may be desirable for the elimination of residual tumor cells, but if the goal is to prevent metastases, further growth or recurrence, a long-term response needs to be induced.
- *Condition of patients' immune response*: All strategies described above rely on functions of the patients' immune-system. If the patient is immune compromised because of his tumor or chemotherapy/radiation therapy, immunomodulation therapy may not be possible.
- *Tumor antigen*: In order for the immunization protocols with tumor antigens to be successful, the immunizing agent needs to be validated and expression might have to be verified for each individual patient.

These considerations, in addition to the type of cancer selected for treatment, are essential for the success of immunomodulating gene therapy for patients. With increases in understanding of the immune response, particularly in cancer patients, and the molecular mediators influencing activation or suppression, the future success of this gene therapy approach may improve.

B. Prodrug-converting enzymes

The concept of suicide-genes as a treatment modality was introduced nearly 20 years ago and has emerged as standalone treatment modality; gene-directed enzyme prodrug therapy (GDEPT). The concept uses inactive prodrugs that can be converted into active, cytotoxic drugs by enzymatic reactions within cells.

This converting enzyme is the delivered agent to the tumor site and subsequently expressed by cellular machinery. Site-specific delivery of the drug-converting enzyme at the tumor site results in a high local accumulation of cytotoxic drugs, mediating tumor elimination, and little-to-no accumulation of drug elsewhere. Moreover, the localized conversion of cytotoxic drugs also leads to a very potent bystander effect. As such, complete tumor eradication can be achieved with as little as 10% transduction of the tumor mass (Aghi *et al.*, 2000; Rooseboom *et al.*, 2004).

A widely used prototypical example is the herpes simplex virus thymidine kinase (HSV-TK) in combination with ganciclovir. Activation of HSV-TK phosphorylates ganciclovir to generate the toxic species (Eck *et al.*, 1996; Moolten *et al.*, 1990). This treatment strategy has been leveraged in numerous gene therapy trials including direct intratumoral injections in primary brain tumors and intraperitoneal injection for ovarian cancer patients.

Another example is the expression of bacterial cytosine deaminase as the converting enzyme in combination with systemically delivered 5-fluorocytosine (5-FC). Transfected cells convert 5-FC to 5-fluorouracil (5-FU) leading to cytotoxic effects (Crystal *et al.*, 1997; Ohwada *et al.*, 1996). One overriding advantage of these two prototype systems is the use of clinically ready prodrugs to generate a therapeutic effect, thereby streamlining approval for regulatory agencies and avoiding further complications and delays for clinical translation.

C. Tumor suppressor genes and antioncogenes

Although it is established that malignancy is not caused by a single protein or gene, there are dysregulations in several prominent genetic pathways that are very common in cancer. Two common dysregulations are the transcriptional activation of oncogenes or the transcriptional silencing of tumor suppressor genes. Thus, obvious strategies would be to treat tumors with these genetic alterations by replacing or overexpressing silenced suppressor genes or by silencing activated oncogenes. A clear advantage of these therapeutic strategies is the specificity for neoplastic cells, as tumor cells in principle are the cells in which the mutations would occur. Unfortunately, unlike the GDEPT strategies, there would be no bystander effect, necessitating an extremely high gene transfer efficiency within the tumor (e.g., nearly 100% for eradication).

There are a number of classes of tumor suppressor or transcriptionally silenced genes, which include proapoptotic genes (Fas-Ligand, TRAIL, and Bax; Norris *et al.*, 2001) and genes involved in cell-cycle regulation (pRb, p16, and p21; Kang *et al.*, 2002). However, the most widely studied tumor suppressor is p53, or the "guardian of the genome." p53 is mutated or deleted in over half of all human tumors and a single allele loss is often sufficient as a single hit in the two-hit hypothesis for tumorigenesis. Since p53 functions both in the cell cycle and apoptosis, the hypothesis that replacement or overexpression could serve as an

extremely effective therapy (Levine, 1997). Indeed, an injectable recombinant human adenovirus expressing p53 (trademarked as Gendicine[TM]) became the world's first gene therapy product approved by a governmental agency (State Food and Drug Administration of China (SFDA)) for the treatment of cancer. This was a milestone in the field of gene therapy and paves the way for further translational efforts (Peng, 2005).

Silencing activated oncogenes can be achieved using antisense-, ribozyme-, or RNAi-based therapies. Each of these silencing techniques relies on different mechanisms of action, but the net effect is blockage of mRNA translation into protein. In cancer biology, the following classes of genes have been targeted: (1) oncogenes; (2) cell-cycle regulatory genes; (3) drug-resistance genes; (4) angiogenic genes; (5) growth factor receptor genes; and (6) genes in cell signaling pathways (Lebedeva and Stein, 2001; McCaffrey *et al.*, 2002; Scanlon, 2004; Scanlon *et al.*, 1991; Singer *et al.*, 2003; Stein, 2001). These techniques are in early preclinical phases, but have progressed with great enthusiasm.

D. Antiangiogenesis

With work pioneered by the late Judah Folkman, it has become a well-known fact that tumors require a vascular supply to grow beyond a critical size. This realization introduced the field of angiogenesis in cancer biology and brought antiangiogenesis therapy as a viable new strategy to treat the disease. Antiangiogenesis treatments seek to eliminate or inhibit vascular expansion to reduce tumor burden. A number of naturally generated inhibitors of angiogenesis have been studied as a gene therapy modality, including angiostatin and endostatin (Puduvalli, 2004). Alternatively, downregulation of secreted proangiogenic factors, such as VEGF or bFGF, via silencing of hypoxia inducible factor-1 alpha within the tumor have been shown to reduce tumor burden in preclinical models (Folkman, 1990; Nesbit, 2000).

Gene therapy strategies focused on endothelial cells introduce a new cellular target for exploitation and present unique advantages over therapeutic targeting of tumor cells. Despite a population density far less than tumor cells, endothelial cells, in principle, that are transduced with genes acting only within single cells would have an enhanced effect on surrounding tumor cells, akin to the bystander effect. However, this approach also intrinsically poses several unique challenges. The small cellular contribution to the tumor population creates difficulties in delivery efficiency and is likely to be very low in intratumor injections. In addition, antiangiogenic strategies also raise challenges in the selection of gene targets. Tumor cells possess genetic and epigenetic alterations that provide rational targets for intervention; however, endothelial cells lining

tumor blood vessels are largely considered epigenetically stable. Therefore, selection of silencing or inhibitory products would affect normal cells throughout the body if transfected, raising the risk of unwanted side effects.

E. Combination therapies

Therapeutic gene delivery in cancer can also result in enhancement of standard treatment/regimen efficacy. A majority of modern chemotherapies do not discriminate between normal and cancer cells. Cytotoxicity to proliferating normal cells, such as hematopoietic precursor cells, becomes dose-limiting in treatment with chemotherapeutics. Thus, methods to reduce toxicity to normal cells would be a major advance in treatment regimens. For example, bone marrow depletion remains a major side effect of chemotherapy. Transfection of bone marrow cells with multidrug-resistant 1 gene enhances cellular resistance to chemotherapy and allows patients to receive higher doses of conventional agents (Culver, 1996; Huber and Margrath, 1998; Lattime and Gerson, 2001; Mickisch et al., 1992; Templeton and Lasic, 2000).

Another recently suggested approach for synergistic therapy is with the introduction of iodine transporters to the tumor cells by gene delivery. These transporters increase the uptake of radioactive iodine, and this approach demonstrated success in treatment of experimental thyroid tumors (Boelaert and Franklyn, 2003). Expansion to other tumor types has been used preclinically for therapy and imaging purposes.

F. Oncolytic viruses

This strategy makes use of replicating recombinant viruses. The underlying concept is to administer the virus intratumorally after which viral replication will take place in the transduced cells. Infected cells will ultimately be disrupted and viral progeny is released, allowing the spread of infection. It is important to achieve cancer-specific replication to limit viral replication to the site of the tumor (Vecil and Lang, 2003). This can be accomplished by (1) selective cell entry, (2) selective transcription of genes necessary for replication (tumor tissue-specific promoter), or (3) deletion of genes necessary for replication in normal cells but not in tumor cells (e.g., deletion of E1B-gene in ONYX-015).

III. VECTORS FOR LIGAND-DIRECTED GENE DELIVERY

Clinical gene therapy trials have made it clear that success is controlled by several variables, one of the more important being the gene-delivery system. This dictates the type of cell to which the therapeutic genes are transferred, the

expression level of the therapeutic gene and the duration of expression. The fundamental challenge of gene delivery, originates from the fact that DNA has a charged nature, is unstable in biological environments and does not cross biological barriers such as an intact endothelium and cell or nuclear membranes. The addition of targeting ligands that bind to a unique cell-surface receptor, leads to improved and more specific gene transfer to cells expressing the targeted receptor. The challenge is to identify ligands that have a sufficiently high affinity for their targets and to identify cell-surface receptors that are either unique or display increased surface density on the targeted tissue. Another requirement is that the ligand-targeted delivery vehicle gets internalized after recognizing its target receptor. This receptor-mediated endocytosis makes sure the plasmid DNA gets delivered intracellularly.

Gene-delivery systems can be divided into two general categories: biological systems (engineered viruses) and chemical systems (lipid- and polymer-based nanoparticles) (Mah *et al.*, 2002; Thomas and Klibanov, 2003; Walther and Stein, 2000; Zhdanov *et al.*, 2002). Viral gene-delivery systems are genetically engineered nonreplicating viruses capable of infecting cells and delivering their genome containing a therapeutic gene. The viral genome can be integrated into the host genome (retrovirus, lentivirus, and the later stage of AAV), or it can exist as an episome (adenovirus and the early stage of AAV infection) (Bramson and Parks, 2003; Carter, 2003; Pages and Danos, 2003). It is generally recognized that viral vectors are the most effective gene transfer vehicles; however, chemical gene-delivery systems have provided an attractive alternative to viral vectors due to their low immunogenicity, lack of replication risk, and the relative ease to manufacture them on a large scale (Thomas and Klibanov, 2003; Zhdanov *et al.*, 2002). Moreover, the ability to incorporate targeting ligands for specific homing to target tissue with little effect on manufacturing is one of the major advantages of chemical gene-delivery systems (Anwer *et al.*, 2004; Driessen *et al.*, 2008; Wood *et al.*, 2008). Changing viral tropism has been attempted as well (Buning *et al.*, 2003; Krasnykh *et al.*, 1998; Ried *et al.*, 2002; Wickham *et al.*, 1997); however, these modifications involve alteration of viral structural proteins, and it is often problematic to inactivate the endogenous viral ligand–viral receptor interaction and replace it with a new ligand (Roelvink *et al.*, 1999).

Integration of site-specific, systemic targeting of a vector with high gene-delivery profiles would create a powerful system with wide therapeutic and diagnostic application and potentially alleviate the need for invasive procedures. We recently described the development of a new class of hybrid gene-delivery vector incorporating the genetic elements of bacterial and mammalian viruses into a single entity (Hajitou *et al.*, 2006, 2007; Soghomonyan *et al.*, 2007). We exploited the genetic elements of recombinant AAV for improved mammalian cell gene expression with elements affording site-specific targeting

from bacteriophage (phage) creating a novel hybrid virus termed AAVP. In a proof-of-concept study, an AAVP targeted by an RGD-containing motif (arginine-glycine-aspartic acid) homing to alpha-v-integrins was generated carrying the HSVtk gene cassette suitable for imaging and the GDEPT treatment regimen. This vector retained target specificity for alpha-v-integrins mediated by the RGD motif while retaining high transduction efficiency *in vitro*. *In vivo*, the RGD-AAVP mediated strong accumulation within the tumor following systemic administration and strong transgene expression evident 7 days after injection. Furthermore, it was demonstrated that the clinically applicable imaging of [18]FEAU could be integrated to specifically monitor the temporal dynamics and spatial heterogeneity of transgene expression over time by positron emission tomography (PET). Lastly, we observed a robust reduction in tumor burden both in murine mammary tumors in immunocompetent mice as well as in numerous human tumor xenografts in immunocompromised animals following administration of ganciclovir (Hajitou *et al.*, 2006; Soghomonyan *et al.*, 2007). Taken together, these data introduced a novel hybrid vector that may be applied in many disease settings for targeted gene therapy.

In subsequent studies, work with targeted AAVP vectors has expanded with success employing alternative transgenes as well as multiple models of human disease. In soft tissue sarcomas, the clinical standards to determine patient response often correlates poorly with patient outcome. As a proof-of-concept, targeted AAVP vectors carrying the HSV-tk suicide gene were investigated as alternative means to assess tumor response to therapy (Hajitou *et al.*, 2008). Evaluation of transgene expression by PET imaging provided a platform to repeatedly monitor localization and magnitude of gene expression and, thereby, predict the responsiveness to therapy with ganciclovir. Similarly, targeted AAVP vectors delivering an alternative transgene, tumor necrosis factor-alpha (TNFα), have been explored in preclinical models of melanoma and, more recently, in spontaneous cancers in dogs through the Comparative Oncology Trials Consortium at the National Cancer Institute (Paoloni *et al.*, 2009; Tandle *et al.*, 2009). Finally, further study of the mechanism by which AAVP vectors targeted to the vasculature-mediated tumor therapy has implicated a heterotypic bystander killing effect. This endothelial cell–tumor cell interaction is largely mediated through intercellular gap junctions involving connexins 43 and 26 (Trepel *et al.*, 2009).

At present, much of the work involving targeted AAVP vectors has been in models of human disease. However, integration of clinically applicable PET imaging with [18]FEAU and therapy with ganciclovir suggests that rapid translation to patient populations may be imminent. Furthermore, improvements in transgene regulation through developments in tissue-specific promoters may further enhance tissue specificity and improve the therapeutic index for this vector.

IV. LIGANDS FOR TARGETING ANGIOGENIC VASCULATURE

The development of new vasculature occurs during embryonic development, normal physiological processes, and in a number of pathological diseases including most solid tumors. This coordinated, multistage process, termed angiogenesis, involves the local release of growth-promoting factors and subsequent stimulation of endothelial cells lining blood vessels. Activated endothelial cells migrate, proliferate, and invade surrounding tissues, supporting the expansion of tumor cells beyond a critical size (Folkman, 1990; Folkman *et al.*, 1989; Mustonen and Alitalo, 1995). In addition, it is well established that angiogenic endothelial cells lining tumor blood vessels are morphologically and molecularly distinct (Arap and Pasqualini, 2001; Arap *et al.*, 2002; Pasqualini and Arap, 2002; Pasqualini *et al.*, 2001, 2002). The repertoire of cell-surface molecules on angiogenic blood vessels often exist as: (i) new expression of molecules not normally present on quiescent endothelial cells, (ii) elevated levels of proteins normally found at the cell surface, or (iii) rearrangement of cell-surface molecules from luminal or abluminal surfaces. It is this differential expression pattern that suggests an opportunity for site-specific targeting of angiogenic vasculature (Ozawa *et al.*, 2008). The challenge for the field, however, is the identification and validation of systemically accessible molecules with sufficient specificity and expression to mediate targeting. A number of techniques have been applied in this effort. Genomic approaches rely on expression differences of tumor endothelium compared to normal blood vessels. St Croix *et al.* utilized microdissection combined with serial analysis of gene expression (SAGE) to identify several candidate tumor endothelial markers in human colorectal cancers (Saha *et al.*, 2001; St Croix *et al.*, 2000). This work demonstrated the feasibility of genetic analyses of cellular subpopulations, including endothelial cells, and the robust potential to identify targets. Moreover, similar studies have since ensued including generation of expressed sequence tags (ESTs) and analysis of cDNA microarrays. Once identified, the candidates must be validated not only as viable proteins but also must be localized to the cell surface and contribute to systemic targeting. Due to some of these inherent limitations to genetic screens, proteomic screenings often provide greater evidence for relevant and functionally significant targets. Beyond the derivation of protein arrays from cellular homogenates, techniques to directly profile the cell surface of endothelial cells have recently emerged, including *in vivo* screenings with systemically injected biotin derivates or two-dimensional peptide mapping (Roesli *et al.*, 2006a,b; Scheurer *et al.*, 2005). More recently, a report described proof-of-concept analyses *in silico* of bioinformatics-based identification of peptides inhibiting endothelial cell proliferation and migration (Karagiannis and Popel, 2008).

Our group has extensive experience in the identification of accessible targets on angiogenic vasculature using *in vivo* phage display (Kolonin *et al.*, 2001). Phage display is a highly versatile technology that involves genetically

manipulating bacteriophage so that peptides or antibodies can be expressed on their surface (Smith and Petrenko, 1997). This strategy revealed a vascular address system that allows tissue-specific targeting of normal blood vessels and angiogenesis-related targeting of tumor blood vessels. Vascular receptors corresponding to the selected peptides have been identified in blood vessels of normal organs and in tumor blood vessels. Our strategy has shown that it is possible to shed light into selective expression of biologically relevant targets within specialized vascular beds. In the *in vivo* phage display procedure, phage capable of homing into certain organs or tumors following an intravenous injection is recovered from such phage display peptide libraries. The ability of individual peptides to target a tissue can also be analyzed by this method (Pasqualini *et al.*, 2000, 2002). In brief, phage are propagated in pilus-positive bacteria that are not lysed by the phage but rather secrete multiple of copies of phage that display a particular insert. Phage bound to a target molecule can be eluted and then amplified by growing them in host bacteria. Multiple rounds of biopanning can be performed until a population of selective binders is obtained. In addition, for a higher throughput approach and higher stringency, we have also developed an enhanced approach to phage library biopanning *in vivo* by screening a number of organs in parallel (Kolonin *et al.*, 2006b). The amino acid sequence of the recovered peptides is determined by sequencing the DNA corresponding to the insert in the phage genome. Ultimately, this approach allows circulating homing peptides to be detected in an unbiased functional assay, without any preconceived notions about the nature of their target. Aside from their carrier function for targeted gene delivery, the peptides themselves may be used as drug discovery leads for peptidomimetic drugs or for therapeutic modulation of their corresponding receptor(s), given that such receptors can be identified by biochemical or genetic approaches (Pasqualini *et al.*, 2002). Binding properties of the peptide library can also be verified for any human or mouse cell line or tissue (Kolonin *et al.*, 2006a). This biopanning strategy *in vivo* and on intact cells has several advantages. First, as opposed to purified receptors, membrane-bound proteins are more likely to preserve their functional conformation, which can be lost upon purification and immobilization outside the context of intact cells. Second, many cell-surface receptors require the cell membrane environment to function so that homo- or heterodimeric interactions may occur. Third, combinatorial approaches allow the selection of cell membrane ligands in an unbiased functional assay and without any preconceived notions about the nature of the cellular receptor repertoire; thus, unknown receptors can be targeted.

With this and related methodologies, numerous normal murine tissue-specific vascular markers and angiogenesis-related molecules in tumor blood vessels have been identified, even in human patients (Arap *et al.*, 2002). Generally, ligand–receptor pairs identified can be grouped into receptors for angiogenic proteins, adhesion molecules, metabolic receptors, extracellular matrix components, and

stress-response molecules (see Table 4.1). Interestingly, some of the identified markers also serve as viral receptors, such as alpha-v-integrins (receptors for adenovirus; Wickham et al., 1993), CD13/APN (a receptor for coronaviruses; Look et al., 1989; Yeager et al., 1992), and MMP-2 and MMP-9 (shown to be receptors for echoviruses; Pulli et al., 1997). It is tempting to speculate that bacteriophage,

Table 4.1. Validated Cell-Surface Receptors and Homing Motifs Isolated by *In Vivo* Phage Display

Receptor	Localization	Homing motif	References
Receptors for angiogenic proteins			
VEGFR1; Neuropilin-1	ECs	CPQPRPLC	Giordano et al. (2005)
bFGFR	N.D.	MQLPLAT	Maruta et al. (2002)
VEGFR2	N.D.	ATWLPPR	Binetruy-Tournaire et al. (2000)
Adhesion molecules			
αv β3-integrin	ECs, tumor cells	CDCRGDCFC; RGD-containing moieties	Pasqualini et al. (1995, 1997), Temming et al. (2005)
αv β5-integrin	ECs, tumor cells	CMLAGWIPC CWLGEWLGC	Nie et al. (2008)
MCAM/MUC18	ECs, tumor cells	CLFMRLAWC	Staquicini et al. (2008)
VCAM-1	N.D.	VHSPNKK	Joyce et al. (2003), Kelly et al. (2005)
Extracellular matrix components			
CD13	ECs, pericytes	CNGRC	Pasqualini et al. (2000)
Aminopeptidase A	Pericytes, stroma	CPRECESIC	Marchio et al. (2004)
NG2/HMWMAA	Pericytes, tumor	GSL	Burg et al. (1999)
MMP-2/MMP-9	ECs, tumor cells	CTTHWGFTLC	Koivunen et al. (1999)
MDP	ECs (lung)	GFE	Rajotte et al. (1998)
Stress-response molecules			
GRP78	Tumor cells	WIFPWIQL WDLAWMFRLPVG	Arap et al. (2004)
HSP90	Tumor cells	CVPELGHEC	Vidal et al. (2004)
Miscellaneous			
IL-11R	ECs, tumor cells	CGRRAGGSC	Arap et al. (2002), Cardo-Vila et al. (2008)
CRKL	Tumor cells	YRCTLNSPFF-WEDMTHECHA	Mintz et al. (2009)
Prohibitin	ECs on WAT	CKGGRAKDC	Kolonin et al. (2004)

VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; MCAM, melanoma a cell adhesion molecule; EC, endothelial cells; HMWMAA, high molecular weight melanoma-associated antigen; MMP, matrix metalloproteinase; CRKL, chicken tumor no. 10 regulator of kinase-like protein; HSP, heat shock protein; WAT, white adipose tissue; VCAM, vascular cell adhesion molecule.

which is a class of prokaryotic viruses, could use the same cellular receptors of eukaryotic viruses given a specific targeting peptide moiety. While the natural host of bacteriophage and eukaryotic virus is vastly different, the structure of the phage capsid protein provides good evidence that bacteriophage share ancestry with animal viruses. More than an evolutionary biology footnote, these findings do suggest that the receptors isolated by *in vivo* phage display will have cell internalization capability, a key feature if one wishes to utilize peptide motifs as gene therapy carriers targeted to specific cell subpopulations.

V. CONCLUSION

One of the hallmark events in cancer progression is angiogenesis. In this chapter we have described how the unique characteristics of angiogenic tumor vasculature can be exploited to deliver genes specifically and efficiently. We explored various therapeutic gene therapy strategies and methods to uncover vascular ZIP-codes of proliferating endothelium have been described. The ligand–receptor pairs discovered by such technologies can be used to target gene-delivery vehicles. We have also highlighted a new hybrid gene-delivery vector (AAVP) which has shown antitumoral efficacy in multiple animal models and tumor subtypes. In conclusion, vascular-targeting strategies for cancer gene therapy may become a new treatment paradigm to improve and enhance current therapeutic protocols.

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