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Ligand-Directed AAVP to Deliver a Therapeutic Transgene to Neuroendocrine Tumors of the Pancreas

Tracey L. Smith

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LIGAND-DIRECTED AAVP TO DELIVER A THERAPEUTIC TRANSGENE TO NEUROENDOCRINE TUMORS OF THE PANCREAS

by

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LIGAND-DIRECTED AAVP TO DELIVER A THERAPEUTIC TRANSGENE TO NEUROENDOCRINE TUMORS OF THE PANCREAS

A

DISSERTATION

Presented to the Faculty of
The University of Texas
Health Science Center at Houston
and
The University of Texas
M. D. Anderson Cancer Center
Graduate School of Biomedical Sciences
in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

by
Tracey Lynn Smith, B.S.
Houston, Texas
August 2013
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ACKNOWLEDGMENTS

I am especially indebted to Dr. Renata Pasqualini and Dr. Wadih Arap, who have been consistently supportive of my education and career development over the years. I would also like to thank the members of my GSBS committees, and, specifically, my Supervisory Committee, for their support in this endeavor.

Additionally, I am grateful to all of those with whom I have had the pleasure to work during this experience. Each of the members of the Arap/Pasqualini Laboratory have provided me extensive personal and professional guidance and taught me a great deal about both scientific research and life in general.

Nobody has been more important to me in the pursuit of this project than the members of my family. Specifically, I would like to thank my mom, whose love and support continue through all of my pursuits; Larry and Linda, for the guidance, encouragement, and food; and my little brother, Ryan, for becoming less annoying over time.

Finally, the financial support of the 2012 Caring for Carcinoid Foundation-American Association for Cancer Research (AACR) award for carcinoid tumor and pancreatic neuroendocrine tumor research was critical for the completion of this project.
Human pancreatic neuroendocrine tumors are rare tumors that form in the endocrine pancreas. Patients with these tumors have limited therapeutic options, and curative intervention is limited to surgical resection. These tumors do, however, consistently express somatostatin receptor type 2 (SSTR2) making them vulnerable to somatostatin analogs. Exploiting cell surface receptors overexpressed in tumors is a common avenue for ligand-directed delivery of imaging or therapeutic agents to tumors. In this regard, identification of novel ligand/receptor pairs with combinatorial peptide libraries has produced multiple candidates for clinical translation. Alternatively, here, we introduce a hybrid vector of adeno-associated virus and phage (AAVP) displaying a known, rationally chosen, biologically active peptide for the delivery of tumor necrosis factor (TNF). Our ligand, octreotide, is a somatostatin analog with specific affinity for SSTR2 that is currently used clinically for both imaging studies and the relief of symptoms associated with pancreatic neuroendocrine tumors. When displayed in AAVP, octreotide mediates selective internalization of the viral particles after systemic administration. We validated the internalization and transduction capabilities of the octreotide-targeted AAVP in a neuroendocrine tumor cell line expressing SSTR2. Additionally, we confirmed AAVP homing and TNF expression in vivo in a transgenic mouse model of pancreatic neuroendocrine tumor development that mimics human disease. With further investigation, therapeutic gene delivery using the octreotide/SSTR2 ligand/receptor pair could represent a viable clinical alternative for patients lacking adequate treatment options.
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### ABBREVIATIONS

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<tr>
<td>AAVP</td>
<td>Adeno-associated virus and phage</td>
</tr>
<tr>
<td>MCS</td>
<td>Multiple cloning site</td>
</tr>
<tr>
<td>MEN1</td>
<td>Multiple endocrine neoplasia type 1</td>
</tr>
<tr>
<td>NET</td>
<td>Neuroendocrine tumor</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>SRS</td>
<td>Somatostatin receptor scintigraphy</td>
</tr>
<tr>
<td>SSTR</td>
<td>Somatostatin receptor</td>
</tr>
<tr>
<td>SSTR2</td>
<td>Somatostatin receptor type 2</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TU</td>
<td>Transducing unit(s)</td>
</tr>
<tr>
<td>VIP</td>
<td>Vasoactive intestinal peptide</td>
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The nature of bacteriophage (phage) makes it amenable to the discovery and characterization of ligand/receptor pairs, which has been described thoroughly over the past three decades. The originally described purpose of combinatorial phage libraries as tools for characterizing antibody epitopes [1-10] has expanded to include describing ligand/receptor pairs in individual proteins [11-20], cell lines [21-29], normal vasculature [30-35], as well as tumors in vivo [32, 36, 37]. Natural progression of the field of phage mapping and targeting raises the question: can the genetically modifiable nature of phage be exploited for targeted drug delivery using a known ligand/receptor pair? Furthermore, which ligand/receptor pair can be used for realization of this hypothesis? As described further in the following sections, there are many reasons for choosing the octreotide/somatostatin receptor type 2 (SSTR2) ligand/receptor pair as the first candidate for study. Briefly, these reasons include the known and repeatedly observed overexpression of SSTR2 in neuroendocrine tumors (NETs) broadly and pancreatic NETs specifically, the specificity of octreotide for SSTR2, an unmet clinical need for patient treatment options beyond the currently available, and the potential of octreotide-targeted imaging studies that are already standard diagnostic practice as a potential clinical bio-marker of treatment susceptibility.

Brazeau’s discovery of somatostatin in 1973 [38] and the ensuing investigation identified the therapeutic properties of somatostatin analogs in tumors overexpressing somatostatin receptors (SSTRs). This opened a new avenue for clinical intervention in pancreatic NETs previously limited to surgical resection. Somatostatin is a peptide hormone secreted from the endocrine pancreas to regulate glucose metabolism. Somatostatin is also secreted along the central nervous system and digestive tract, to regulate the production and secretion of pituitary and gastrointestinal hormones, respectively [39]. Tumors arising from these neuroendocrine cells often express one SSTR receptor family member, commonly SSTR2 [40], providing an avenue for ligand-directed imaging and therapeutic opportunities [41-49]. The half-life of circulating somatostatin is less than 3 minutes [50], eliminating its utility as a targeting agent and necessitating the development of synthetic analogs for clinical applications [51]. One such synthetic somatostatin analog, octreotide (amino acid
sequence FCFWKTCT), has a specific affinity for SSTR2 [52] and an increased half-life in circulation [52, 53], ensuring its clinical utility.

Our strategy to selectively target SSTR2 employs an AAVP hybrid vector that combines genetic elements from adeno-associated virus (AAV) and bacteriophage (phage). Incorporating the octreotide peptide sequence into the phage region for display on the pIII viral coat protein enables internalization of the AAVP particles in tumor cells expressing SSTR2, specifically. An octreotide-targeted AAVP designed to incorporate tumor necrosis factor (TNF) into the cellular DNA, termed Oct-AAVP-TNF, acts as a therapeutic reporter gene, and, after systemic internalization, mediates expression of an apoptotic agent within the vulnerable vasculature of the pancreatic NETs without toxicity to normal organs.

The category of human pancreatic NETs includes tumor manifesting in the course of multiple endocrine neoplasia type 1 (MEN1) syndrome. To evaluate the activity of our octreotide-targeted AAVP, we will use a Men1 transgenic mouse model that mimics tumor formation and disease progression observed in human MEN1 syndrome. Elimination of the Men1 tumor suppressor gene in the entirety of the pancreas results in tumor development in the endocrine pancreas but no pathological abnormalities evident in the exocrine tissue, similar to what is found in human patients [54], providing a preclinical model for studying our AAVP in vivo in the context of the complicated tumor microenvironment of pancreatic NETs.

The expression of cell-surface receptors provides an avenue for targeted drug delivery specifically to the tumor eliminating toxicity to normal tissues, a goal in cancer treatment. Biotherapy with somatostatin analogs, particularly octreotide, is already used clinically for relief of symptoms associated with hormone hypersecretion in functioning tumors. Additionally, radiolabeled octreotide is also used to diagnose, localize, and stage pancreatic NETs. Exploiting the known, biologically active, and rationally chosen octreotide/SSTR2 ligand/receptor pair for the delivery of the TNF gene represents a viable alternative for patients lacking treatment options. This led us to hypothesize that displaying the octreotide peptide in the AAVP gene delivery system would recapitulate the specific binding attributes of the parental peptide, enabling ligand-directed delivery of a therapeutic agent to pancreatic NETs expressing SSTR2. The following sections will establish the rationale for the selection of the octreotide/SSTR2 ligand/receptor pair in greater detail and
clarify the current clinical utility of such an agent, provide an analysis of the suitability of AAVP as our gene delivery agent, and describe the Men1 transgenic mouse model and refine its applicability as our preclinical mouse model. Following these sections, the experimental protocols used to prepare and evaluate the octreotide-targeted AAVP-TNF will be described. The validation of Oct-AAVP-TNF activity in vitro and in vivo will be presented in the results section, confirming construction of the hybrid vector as well as specific homing and gene delivery. Finally, the conclusions drawn from our results, the further experimental plans for the octreotide-targeted AAVP, and the potential broader applications of both the Oct-AAVP-TNF and the untapped opportunities for the incorporation of biologically active peptides into the phage system will be presented as an opportunity for further investigation.
BACKGROUND

PANCREAS ANATOMY

The pancreas is located in the epigastric region of the abdomen. Necessarily for its digestive functions, it the head of the pancreas sits in the curve of the duodenum and extends to rest behind the stomach. The pancreas can be divided into two functional components: exocrine and endocrine. The exocrine pancreas comprises the vast majority, more than 95 percent, of total pancreas volume and is defined by the acini glands that secrete digestive enzymes to the small intestine to facilitate digestion. The endocrine pancreas is defined by the production and secretion of hormones into the bloodstream for actions throughout the body. Small cell populations, termed the islets of Langerhans, distributed throughout the pancreas, designate the endocrine pancreas [55].

The composition of cells in the human islets is heterogeneous and incredibly variable from islet to islet within a single pancreas and between multiple pancreases [56-67]. The combined effects of the islet cells and the hormones they produce are responsible for regulating glucose metabolism [68]. The large population of beta cells is responsible for insulin production. The alpha cells produce glucagon [69, 70], and the small population of delta cells is responsible for somatostatin production [71-75]. Additional cell types are also present in small numbers in the human islets. These cells include the pancreatic polypeptide (PP) -producing cells [76, 77] and the ghrelin [78] -producing epsilon cells [79-82] and make up less than five percent of the total islet cellular population [56, 61].

Analysis of organogenesis reveals both the exocrine and endocrine pancreas is derived from a common progenitor cell [83-85]. Transcription factors in the gut during embryogenesis activate the necessary signaling pathways for cellular differentiation. In addition to analysis of human fetal pancreas development [86-91], time course and transcription factor expression during pancreatic development has been studied in numerous model organisms. These mechanisms are tightly regulated to ensure the correct formation of the pancreas [83, 85, 90, 92-109]. Specifically, in pancreatic differentiation, the pancreatic and duodenum homeobox 1 (PDX1; previously GSF, IPF1, IUF1, IDX-1, MODY4, PDX-1,
and STF-1) transcription factor is expressed in mice at embryonic day 8.5 (E8.5) [110-112], which coincides with total pancreas derivation from gut epithelium at E9.5 [100, 113-116]. This early time-point, along with further analyses, denote PDX1 expression essential for all epithelial cells of the pancreas, both exocrine and endocrine in function [111-113, 117-124]. The importance of PDX1 expression is further revealed in knockout mice wherein mice lacking PDX1 were born without a pancreas [114, 125, 126]. Loss of PDX1 has also been indicated as detrimental to pancreas development in humans [127]. Critical roles have also been identified multiple additional proteins, including those of the neurogenin 3 (NEUROG3; previously ngn3, NGN-3, Atoh5, Math4B, and bHLHa7) [95, 113, 128-141], notch family members [128, 129, 142-156], sonic hedgehog (SHH; previously TPT, HHG1, HLP3, HPE3, SMMCI, TPTPS, and MCOPCB5) [157-164], and pancreas specific transcription factor 1a (PTF1A; previously bHLHa29 and PTF1-p48) [118, 165-174] signaling pathways, that act in a delicate balance during organogenesis for cellular differentiation and lineage determination [175-177]. Upon pancreatic differentiation from progenitor cells in the gut, cells disperse through the developing pancreatic tissue and proliferate to form the islets as clusters located throughout the pancreas [85, 89, 91, 105, 115, 178, 179]. Simultaneous to organogenesis and cellular differentiation, blood vessel formation in the budding pancreas also encourages growth and development [174, 180-187].

The pancreas, necessitated by its role as an endocrine organ, is highly vascularized, allowing for the dissemination of hormones and other neural transmitters via the bloodstream [63, 65, 184, 188-193]. Pancreatic vascularization is tightly regulated [194] and becomes evident in mice around E14 [85, 105, 186] and islet vascularization begins appearing at E15 [105]. Pancreatic capillaries in general are highly permeable [195, 196], with noticeable increases in both vascularization [180, 181, 190, 197-199] and permeability in islet blood vessels [200]. Capillaries of the islets also have a marked increase in both diameter [180, 190, 199, 201-204] and the number of fenestrations present along the endothelium [181, 204, 205]. Islet vasculature formation is also structured to allow for variability in blood flow in response to glucose [206-209] and other hormonal stimulation [209-214]. Multiple angiogenic factors present in islet tissue, including vascular endothelial growth factor (VEGF) family members and their receptors, could facilitate this discrepancy [180, 181, 183, 194, 215-220]. In addition to morphological differences, blood flow to the
islets constitutes five to fifteen percent of total pancreatic blood volume [197, 207, 221, 222], which is remarkable because islet cell population is only around two percent of total pancreas volume [197, 221, 223-225].

**Tumors Originating in the Pancreas**

The duality of the pancreas is further manifested in pancreatic tumors. Both the exocrine and endocrine pancreas can give rise to tumors, but the resulting tumors are markedly different. The vast majority, in excess of eighty percent, of tumors arising from the exocrine pancreas are pancreatic ductal adenocarcinomas [226-236], currently representing the fourth leading cause of cancer-related mortality in the United States [237, 238]. The overall five-year survival rate of patients diagnosed with pancreatic adenocarcinoma is less than five percent [226, 236-246]. These tumors are difficult to treat with more than eighty percent of pancreatic adenocarcinomas not resectable at diagnosis [234, 237, 242, 243, 247-251]. Survival for patients with surgically resectable tumors only increases to around fifteen percent after five years [239, 241, 245, 248-268]. The unfavorable prognosis for patients diagnosed with pancreatic ductal adenocarcinoma is exacerbated by the frequency of this tumor type.

Alternatively, tumors arising from the endocrine pancreas make up less than five percent of all pancreatic tumor diagnoses [226, 230, 236, 269-271]. Tumors of the endocrine pancreas have a generally better prognosis than those of the exocrine pancreas, with a five-year survival rate of from about 40 to 60 percent [226, 236, 270, 272-280]. However, this proportion remains moderately less than the overall survival at five years for all cancer sites combined of greater than 65 percent [281]. Median survival is also extended from four months for patients with exocrine tumors of the pancreas to more than two years for patients with endocrine tumors [232]. Not coincidentally, as opposed to pancreatic adenocarcinomas, which are considered stroma-rich and atypically avascular tumors [282-287], tumors of the endocrine pancreas are consistently vascularized [215, 288-293].

Endocrine tumors of the pancreas initiate an angiogenic cascade to enable the generation of new blood vessels corresponding to disease formation and progression in islet
cell tumors [215, 217, 290, 292, 294-305]. The new vascular network is random and
disordered [291, 292, 297, 304, 306, 307], in agreement with observations of other
malignancies [306, 308-313]. Tumor vascularization and angiogenesis are necessary for
tumor development and sustainment [302, 308, 314-317], but tumors are consistently
evolving to circumvent decreases to their blood supply and the corresponding limitations in
oxygenation of the tumor cells and microenvironment while diminishing the efficacy of
some therapeutic options [318-325]. The discrepancy in vascularization between tumors of
exocrine and endocrine origin is significant but insufficient to describe the variability found
in pancreatic malignancies.

NEUROENDOCRINE TUMORS OF THE PANCREAS

Tumors originating in the endocrine pancreas have a variety of designations, including
“neuroendocrine tumors (NETs) of the pancreas” for the much broader association and
classification of tumors arising from the many endocrine organs; “gastroenteropancreatic
(GEP) tumors” for the class of tumors originating from the gastrointestinal (GI) system,
stomach, and pancreas; the historical “carcinoid tumor”; and the more descriptive pancreatic
designations “pancreatic endocrine tumors” and “islet cell tumors” [326-328]. These are rare
neoplasms with incidences reported historically ranging from two to twelve individuals of
every million [236, 269-271, 277, 327, 329-339]. The number of tumors identified at
autopsy is occasionally higher, however, indicating the possibility of a greater incidence of
asymptomatic pancreatic NETs [339-341].

Histological classification of pancreatic NETs includes three categories. Well-
differentiated NETs have either benign or low-grade malignancy potential, while poorly
differentiated tumors are considered a high-grade malignancy [342-344]. Additionally, there
are two broad categories generally used to describe pancreatic NETs: functioning and non-
functioning tumors [328, 345, 346]. Table 1 summarizes the various tumor types and their
clinical characteristics. Functioning tumors produce excess hormones corresponding to the
islet cell type the tumor originates from [336, 345, 347, 348]. Additionally, endocrine
tumors of the pancreas can secrete hormones from other cell types along the gastrointestinal
## Table 1. Tumor types and clinical characteristics of pancreatic NETs [269, 326, 334, 337, 339, 352, 367, 386, 387]

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>Percent of pancreatic NET diagnoses (%)</th>
<th>Malignancy (%)</th>
<th>Syndrome‡</th>
<th>Clinical symptom(s)§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-functioning</td>
<td>15 - 80</td>
<td>&gt; 80</td>
<td>None</td>
<td>Tumor bulk symptoms (jaundice, weight loss, abdominal pain, abdominal mass)</td>
</tr>
<tr>
<td>Insulinoma</td>
<td>32 - 60</td>
<td>≤ 10</td>
<td>Whipple’s triad</td>
<td>Weakness, sweating, dizziness, disorientation, seizure</td>
</tr>
<tr>
<td>Gastrinoma</td>
<td>5 - 30</td>
<td>&gt; 60</td>
<td>Zollinger-Ellison syndrome</td>
<td>Peptic ulcer, diarrhea, bleeding, heartburn</td>
</tr>
<tr>
<td>Glucagonoma</td>
<td>1 - 7</td>
<td>&gt; 70</td>
<td>Glucagonoma syndrome</td>
<td>Rash, diabetes, cachexia</td>
</tr>
<tr>
<td>Somatostatinoma</td>
<td>&lt; 1 - 4</td>
<td>&gt; 70</td>
<td>Somatostatinoma syndrome</td>
<td>Steatorrhea, diabetes, gallstones, diarrhea</td>
</tr>
<tr>
<td>VIPoma</td>
<td>&lt; 1 - 3</td>
<td>&gt; 50</td>
<td>Verner-Morrison (or WDHA) syndrome</td>
<td>Watery diarrhea, hypokalemia, achlorydria</td>
</tr>
<tr>
<td>Carcinoid (pancreatic)</td>
<td>&lt; 1</td>
<td>&gt; 60</td>
<td>Carcinoid syndrome</td>
<td>Flushing, diarrhea, cramps, cardiovascular abnormalities</td>
</tr>
<tr>
<td>PPoma</td>
<td>&lt; 1 - 2</td>
<td>&gt; 80</td>
<td>None</td>
<td>Abdominal pain, diarrhea</td>
</tr>
</tbody>
</table>

* [269, 273, 274, 327, 338, 339, 348-362]
† [334, 351, 355, 363-370]
‡ [352, 371-380]
§ [289, 334, 355, 367, 381-385]
(GI) tract, including gastrin, serotonin, and vasoactive intestinal peptide (VIP) leading to speculation of a common precursor or stem cell population [361, 370, 388-392]. The hormones secreted from tumors forming from these various cell types is then used to designate the type of functioning tumor [329, 337, 393, 394]. The significance of functional status is evident in the clinical presentation of the different tumors. Excess hormones circulating in the blood can produce clinical symptoms that can lead to a diagnosis of the presence of a specific malignancy [345, 384, 395-399]. Alternately, non-functioning tumors do not generate excess hormones and, therefore, remain clinically silent until the tumor mass itself becomes a burden or the mass is identified during an unrelated or routine imaging procedure [278, 349, 355, 368, 381, 400-403]. Symptoms identified during clinical presentation associated with non-functioning tumors include abdominal pain, weight loss, fatigue, jaundice, and nausea [289, 359, 381, 404].

The various functioning tumors are indicated by evidence of a pancreatic mass in addition to digestive system or neurological symptoms based on the nature and activity of the hormone that is over produced in that tumor. The hormone output of these tumors can be so significant that symptoms are present while the tumor itself is too small even to be detected by diagnostic imaging techniques [405-407]. Diagnosis of functioning tumors after a clinical presentation of symptoms requires a measurement of hormone levels in the blood [367, 399, 408].

Insulinomas are the most commonly diagnosed functioning pancreatic NET, the majority of which are classified as benign tumors [365, 409]. Insulinomas present clinically with symptoms of hypoglycemia because excess circulating insulin leads to sequestering of glucose and a corresponding neurological impairment in function indicated by confusion, disorientation, sweating, and, potentially, loss of consciousness [365, 409-415]. Diagnosing insulinomas requires an analysis of the component diagnostic factors of Whipple’s triad: plasma glucose level less than 2.5 mmol/L (45 mg/dL), neuroglyopenia, and symptomatic relief following glucose administration [380, 416]. Additional criteria have been established, including the evaluation of plasma insulin and the connecting peptide (C-peptide), another metabolite of the production of insulin from proinsulin [417], levels [365, 418]. A fasting test of up to 72 hours can detect abnormal concentrations of both glucose and insulin to support the insulinoma diagnosis in up to 100 percent of patients, and is considered the
clinical gold standard for diagnosis [413, 414, 418-421].

Gastrin-secreting tumors [422-424] are the second most common functioning tumor [348]. Gastrinomas typically originate in the duodenum but are also found as primary tumors of the pancreas [375, 425-439]. The clinical presentation of gastrinomas typically encompasses the symptoms associated with Zollinger-Ellison syndrome [440, 441] due to excess circulating gastrin: hyperchlorhydria, peptic ulcer disease, diarrhea, and abdominal pain or cramps [375, 425, 438, 439, 442-445]. Fasting gastrin levels, serum gastrin evaluation following administration of secretin or calcium, and gastric acid pH are diagnostic tests for gastrinoma diagnosis [383, 426, 427, 446-451]. Malignancy is common in gastrinomas [436, 439, 452, 453], and about half of patients have lymph node or liver metastases at diagnosis [348, 426, 427, 429, 430, 438, 441, 454-457]. Presence or absence of liver metastases is the major pathological criterion for survival estimates in these patients [382, 430, 434, 455, 458, 459]. Metastasis formation has been correlated with site of the primary tumor, with gastrinomas originating in the pancreas behaving more aggressively, resulting in poorer prognosis [425, 432, 434, 436].

Glucagonomas arise from the alpha cells of the islets and secrete excess glucagon resulting in various symptoms including weight loss, a migrating rash, diabetes, and diarrhea [360, 371, 374, 377, 379, 460-464]. Diagnostic criterion requires a fasting blood test for glucagon levels [367, 371, 379, 461, 462, 465]. Prognosis with these tumors is dependent on the presence or absence of metastases at diagnosis [466]. Liver metastases are commonly found at diagnosis [360, 462, 463, 467], significantly decreasing five-year survival odds [374, 379, 466].

Somatostatinomas are exceedingly rare [358, 378, 468-470]. Clinical symptoms associated with this tumor type are associated with the inhibitory effects of somatostatin on other hormones and include hyperglycemia, diabetes mellitus, diarrhea, weight loss, and the formation of gallstones, and a diagnosis is possible with evidence of elevated somatostatin levels in patients [376, 468, 470-472]. The presence of metastases at diagnosis decreases the five-year survival rate dramatically [376], and metastases of the lymph nodes or liver are present in half of patients with somatostatinomas [358, 366, 367, 468].

Vasoactive intestinal peptide (VIP) is produced in the central nervous system as well as the pancreas and intestine, peripherally, and functions in motility and absorption in the
small intestine [473, 474]. About eighty-five percent of VIPomas occur in the pancreas [367, 373]. These tumors are malignant in half of the cases and are typically diagnosed as advanced disease with lymph node or liver metastases present at diagnosis [372, 373]. Clinical diagnosis of a VIPoma is based on patients presenting with severe diarrhea [372, 467, 473] and elevated levels of circulating VIP in the blood [367, 475, 476]. This tumor is associated with Verner-Morrison syndrome [477, 478], or WDHA syndrome, an acronym indicating the presence of watery diarrhea, hypokalemia, and achlorydria [477-481].

Serotonin-secreting functional tumors, historically termed carcinoids, arise from enterochromaffin (EC) cells and are rarely found as primary pancreatic lesions [363, 482-485] but have been increasing in incidence over time [486]. These tumors are typically at an advanced stage at diagnosis with a rate of metastasis around 70 percent [363, 482, 483, 485, 487] and the likelihood of metastatic aggressiveness increasing with an apparently corresponding increase in primary tumor size [394]. Carcinoid tumors secreting serotonin present with a constellation of symptoms that include flushing episodes, diarrhea, cramps, and cardiac abnormalities [487, 488] collectively defined as carcinoid syndrome [489-491]. A test for 5-hydroxyindoleacetic acid, a metabolite of serotonin, levels in urine is demonstrative of a carcinoid [363, 487].

Tumors secreting pancreatic polypeptide (PPomas) do not generally result in clinical symptoms associated with hormone production [492, 493]. Therefore, these tumors are generally found incidentally or due to the effects of tumor mass or metastases causing symptoms [278], resulting in their occasional classification as non-functioning tumors.

Comparing patient prognoses reveals a historically conflicted discrepancy in survival probability among patients with functioning NETs versus those with non-functioning tumors. Significant clinical indications of poor prognosis and decreased survival for patients with non-functioning tumors include poor tumor differentiation, lymph node or liver metastases at diagnosis, and increased mitotic index [404, 494-496]. The 5-year survival rate for patients with non-functioning tumors ranges from around 30 to 70 percent [327, 353, 355, 361, 402, 403, 497], with at least a third of patients with metastatic disease at diagnosis [272, 278, 279, 327, 353, 355, 381, 402, 404, 498-500]. Some studies indicate an increase in long-term survival when diagnosed with a functioning tumor compared to a non-functioning tumor [273, 277, 329, 348, 353, 357, 466, 497, 501-504]. This can be justified by the lag
before diagnosis that occurs with non-functioning tumors lacking the clinically relevant symptomatology found in functioning tumors [289, 353, 355, 381, 402, 492, 499]. However, though identified as a significant predictor of prognosis at diagnosis [329], functional status of pancreatic NETs has been contested as clinically relevant historically [272, 275, 354, 505, 506]. This could be skewed by multiple sources, including sample size [354, 505-507], the prognostic variability among non-functioning tumors [496], or the distinction between overall survival and disease-free survival [504]. Generally, though the tumor biology remains unchanged between the groups [289], the percent of patients with non-functioning tumors diagnosed with advanced disease, either infiltration of regional lymph nodes or metastases, is greater than what is found in patients presenting with functioning tumors [348, 353, 355, 368, 499, 501]. Numerous studies reveal an earlier diagnosis and better overall prognosis for patients related to tumor stage at diagnosis [272, 354, 362, 466, 505, 506]. Moreover, overall survival drops precipitously with advanced disease at diagnosis independent of tumor functional status [270, 272, 273, 329, 357, 466, 508].

Malignant pancreatic NETs most often metastasize to lymph nodes and the liver, but bone metastases and lung are also found in some patients [272, 374, 375, 509]. Stage of disease at diagnosis and aggressiveness of the tumor are indicators for prognosis [329]. The presence or absence of hepatic metastases is significant clinically, because prognosis is dramatically reduced in patients with disease advanced to the liver [270, 272-275, 278, 279, 354, 404, 466, 483, 492, 497, 501, 502, 506-508, 510, 511]. With no evidence of hepatic metastases, survival rates are greater than seventy percent at ten years. However, long-term survival drops precipitously when liver metastases are found concurrent with the primary tumor [327, 354, 404, 466, 483]. Additionally, the presence of bone metastases is also indicative of a poor prognosis with a reduction in patient survival [459, 512]. Less is understood regarding the significance of positive lymph node metastases at diagnosis, though the majority of studies, but not all, indicate infiltration of lymph nodes is an indication of poorer prognosis overall [270, 272, 278, 327, 359, 404, 434, 466, 494, 506, 507, 510, 513-515].

In addition to tumor stage and functional status, prognosis of all pancreatic NETs is also affected by tumor size, differentiation, mitotic index, degree of necrosis, location within the pancreas, age of the patient at diagnosis, treatment susceptibility, and the presence or
absence of a genetic syndrome associated with tumor formation [270, 273, 274, 339, 421, 458, 466, 502, 506, 516-520]. The most common genetic syndrome found in NETs of the pancreas is multiple endocrine neoplasia type 1 [392, 521, 522].

**Multiple Endocrine Neoplasia Type 1**

Pancreatic NETs can be sporadic or associated with an inherited disorder. Genetic diseases that have a pancreatic component include multiple endocrine neoplasia type 1 (MEN1) syndrome, von Hippel-Lindau (VHL) disease, and neurofibromatosis type 1 (NF-1) [521-525]. VHL syndrome involves tumors in multiple organs including manifesting as cysts or adenomas of the exocrine pancreas or non-functioning endocrine tumors in 15 to 25 percent of patients [526-533], while NF-1 syndrome can result in somatostatinomas or insulinomas of the pancreas in rare cases [366, 376, 534-537]. MEN1, however, has a much more common frequency of endocrine tumor formation in the pancreas, with up to ten percent of all pancreatic NETs associated with MEN1 and over half of MEN1 patients diagnosed with a pancreatic NET [538-542].

The constellation of symptoms and tumors first described by Wermer in 1954 eventually came to be identified as MEN1 [543, 544]. MEN1 syndrome is an autosomal dominant disease that results in tumors of the parathyroid, pituitary, foregut, adrenocortical glands, as well as the endocrine pancreas [521, 538, 541, 542, 545-549]. Classically presenting MEN1 patients have tumors in at least two of three common sites, typically the endocrine pancreas, the pituitary, or the parathyroid [538, 539, 541, 549-553]. The prevalence of MEN1 syndrome ranges from two to 20 individuals of every 100,000 [539].

MEN1 syndrome results from the loss of *MEN1* tumor suppressor gene function following Knudson’s two-hit hypothesis [554-557]. The *MEN1* gene is located on chromosome 11q13 and encodes the protein menin from ten exons [558-564]. Menin, a nuclear protein [565-567], is expressed ubiquitously [568-571] and acts a tumor suppressor with roles in DNA repair [572-574] as well as cell proliferation and transcription regulation [575-585]. Studies of familial MEN1 disease reveal germline mutations in up to 90 percent of cases, with additional mutations probable outside the studied region [538, 550, 551, 586-
The mutations, consisting of nonsense and missense mutations as well as insertions and deletions, occur in multiple exons and typically alter the amino acid sequence of menin, resulting in loss of protein function [586-589, 593-597]. Loss of heterozygosity and epigenetic studies reveal alternative mechanisms of silencing the MEN1 gene in these patients [389, 540, 558, 562, 598-605]. Differential expression analysis reveals a complete lack on menin expression in MEN1 tumors, while sporadic pancreatic NETs and tissue from non-tumor associated endocrine and exocrine pancreas retain menin expression [567], confirming loss of protein function in the MEN1 tumors.

Multiple pancreatic NETs are associated with MEN1 syndrome [553]. MEN1 syndrome can result in numerous small tumors, functioning and non-functioning, located throughout the pancreas [425, 551, 606-611]. The frequency of tumor types in MEN1 patients is widely variable with non-functioning tumors, insulinomas, and gastrinomas the most commonly occurring, while somatostatinomas, glucagonomas, VIPomas, and carcinoid tumors are found less often in these patients [538-541, 545, 547, 552, 592, 607, 608, 612-616]. About five percent of total insulinoma and twenty-five percent of gastrinoma diagnoses are related to MEN1 syndrome [375, 418, 425-427, 436, 442, 445, 617-619]. Interestingly, MEN1 associated tumors can vary in both number and size compared to the same tumor type as a sporadic tumor. Noticeably distinct from sporadic insulinomas that present as solitary tumors, insulinomas associated with MEN1 syndrome occur as multicentric tumors, and, commonly, are linked to disease recurrence over time [369, 418, 539, 620-622]. Gastrinomas arising in MEN1 patients typically localize to the duodenum, while pancreatic gastrinomas associated with MEN1 syndrome are occasionally found [439, 608, 617, 623-625], but these tumors, like insulinomas in MEN1 syndrome, often occur as multiple, small tumors rather than isolated single tumors typical of sporadic cases [425, 439, 599, 610, 617, 625, 626]. Up to half of MEN1-associated gastrinomas are found as metastatic tumors [425, 429, 607, 617] corresponding with a poor prognosis [458]. In addition to insulinomas and gastrinomas, VIPomas are occasionally found in patients with MEN1 syndrome, while other functioning pancreatic NETs are exceedingly rare in association with MEN1 disease [606]. Alternatively, non-functioning tumors occur in about half of MEN1 patients [609].

Malignant pancreatic NETs, and the associated symptoms, are commonly the cause of
death in patients with MEN1 syndrome [546, 613, 627-629]. To combat this, primary tumors identified in MEN1 kindreds under surveillance are resected to limit, or delay, formation of hepatic metastases [630]. Additionally, the early age at which tumors occur in MEN1 patients requires diligence in imaging studies and clinical assessment of circulating hormones to identify primary tumors [541, 610, 631]. Genetic testing for Men1 mutations can also identify at-risk MEN1 patients requiring more stringent or frequent biochemical or imaging analyses than otherwise warranted [632]. This is a long-term requirement as recurrence or further disease manifestation is also common in MEN1 patients [538, 632-634]. In addition to the familial MEN1 syndrome, loss of heterozygosity, mutation, or other loss of function of the MEN1 gene has been found in up to 40 percent of sporadic NETs of the pancreas [562, 594, 635-644], totaling more than a thousand different mutations in MEN1 identified in both sporadic and familial cases of pancreatic NETs [356, 540, 556, 560, 586, 588, 592, 594, 595, 597, 615, 636-640, 642-647].

**Somatostatin and Somatostatin Analogs**

Consistent with other tumors with a hormonal component, NETs, and pancreatic NETs specifically, overexpress receptors related to hormonal homeostasis and regulation. Tumors express these receptors inconsistently and variably, but the majority of pancreatic NETs overexpress somatostatin receptors (SSTRs) generally, and somatostatin receptor type 2 (SSTR2) specifically [41-49, 51, 648-650].

SSTRs are G-protein coupled receptor family members distributed throughout the gastrointestinal tract and the nervous system [49, 651-655]. The five known SSTRs (SSTR1 through SSTR5) are distributed in variable concentrations throughout the body [656-666]. SSTR2, however, is consistently found in around 80 percent of pancreatic NETs [41, 45, 650, 667-673], making it susceptible to ligand-directed therapeutic agents [674, 675]. Tumor-type specific analysis of SSTR2, reveals that greater than eighty percent of gastrinomas, glucagonomas, and somatostatinomas, in addition to half of insulinomas, are positive for SSTR2 expression, characterized as both dense and homogeneous in tumors positive for expression [45, 457, 649, 669, 673, 676-678]. This could be due to the variable
activity of the SSTR subtypes mediating different physiological activity. Specifically, as opposed to other pancreatic peptide hormones, SSTR5, and not SSTR2, mediates insulin activity in the pancreas [679-683].

Somatostatin, first identified as a powerful inhibitor of various hormones, was initially named accordingly and has been termed growth hormone-inhibiting hormone (GHIH), somatotropin release-inhibiting factor (SRIF), or somatotropin release-inhibiting hormone (SRIH) historically [38, 684]. Physiological activities of somatostatin include inhibitory actions against insulin and glucagon from the pancreatic islets [685-700], growth hormone (GH) and thyroid stimulating hormone (TSH) in the pituitary gland [38, 695-697, 701-705], and various other gastrointestinal hormones including gastrin, secretin, motilin, and vasoactive intestinal peptide (VIP) [39, 686, 699, 706, 707]. In addition to physiological activities of somatostatin, regulatory roles in pathological conditions have also been described [39, 700].

Somatostatin is produced in islets of the pancreas, stomach, and intestines as well as the central nervous system, including the hypothalamus [71, 75, 700, 705, 708, 709]. There are two known forms of somatostatin, a 28 amino acid peptide and a 14 amino acid peptide, derived from a somatostatin pro-hormone [710-716], in response to glucose, lipids, amino acids and circulating hormone levels [39, 717, 718]. Somatostatin also inhibits cellular proliferation, demonstrating further utility as an oncologic agent [719, 720]. Somatostatin binds all of the SSTRs with affinities in the nanomolar range [721-723], initiating signal transduction cascades through multiple pathways depending on the cell type expressing the receptors. Binding the G-protein coupled receptors of the neurons initiates potassium (K\(^+\)) channel activity and decreases calcium (Ca\(^{2+}\)) channel function, keeping the channel closed to hormones exiting the cells [724-727]. Receptor internalization upon ligand binding occurs with SSTRs dependent on the length of exposure to the ligands in circulation as well as the total ligand concentration [728-733].

Somatostatin administration to various species, including human, reveals minimal toxicity, and inhibitory effects observable within the fifteen minutes following administration, supporting clinical applications [734]. There are at least five cleavage sites within the length of the 14 amino acids of somatostatin [735], and, as a result, endogenous somatostatin has a circulating half-life of around 2 minutes [50, 736], making it ineffective
for clinical applications and requiring synthetic analogs be used instead [51, 737, 738]. Studies identified the beta-turn region encoded by the four amino acid sequence Phe-Trp-Lys-Thr (FWKT) as the minimal region necessary for biologic activity [739-741], encouraging the development of numerous peptide analogs of somatostatin with this knowledge in mind. Compounds have been synthesized with varying modifications and amino acid substitutions outside of this four amino acid region and analyzed for their ability to recapitulate biological activity and physiological inhibitory functions [52, 727, 739-752].

Peptide analogs are designed to resist circulating proteases. In the case of somatostatin analogs specifically, this allows the manufactured analogs to have an increased half-life in circulation while mimicking the pharmacological activity of somatostatin itself, providing a clinically relevant avenue for tumor targeting [753]. Octreotide (amino acid sequence FCFWKCTCT) is one such synthetic somatostatin analog [52]. Octreotide is a cyclic octapeptide with a 90-minute half-life in circulation [52, 53]. This peptide, originally termed SMS 201-995, is marketed clinically in the salt form octreotide acetate, and is identified as Sandostatin. Octreotide binds preferentially to SSTR2, with binding to SSTR3 AND SSTR5 observed to a lesser extent [656, 722, 727, 749, 754-756], encouraging its use in the treatment of pancreatic NETs consistently overexpressing SSTR2 [735, 757]. A long acting form of octreotide is released into the circulation over a couple of weeks, rather than hours, following a single intra-muscular injection [758-760], allowing much fewer administrations than the original formulation.

The octreotide peptide has been thoroughly evaluated for the ability to recapitulate the biological activity of the native somatostatin peptide [53, 735]. Effective inhibition of growth hormone, insulin, and glucagon secretion in rodent, non-human primates, and humans was observed early on in the evaluation of the peptide for clinical utility and encouraged speculation that octreotide would be effective in the treatment of disorders associated with hormone deregulation clinically [52, 212, 761-770]. Administration of octreotide also dramatically reduces islet blood flow specifically, while total blood flow to the pancreas remains unchanged [212], tangentially supporting the physiological inhibitory properties of somatostatin and its analogs by limiting the accessibility of circulating hormones, and to the alpha and beta cells of the islets, and, therefore, limiting the corresponding stimulation and hormonal release in response. Oncological applications of
somatostatin analogs were quickly identified [763, 771-782], and early efficacy and toxicity trials revealed clinical efficacy with limited toxicity and minimal side effects in patients [774, 783-786] including those with pancreatic NETs [763, 765, 785, 787-790].

**IMAGING NEUROENDOCRINE TUMORS OF THE PANCREAS**

Definitive tumor detection, localization, and staging for all pancreatic NETs, functioning and non-functioning, requires a combination of imaging techniques with varying specificity and sensitivity [791-795], including ultrasound, computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET), and somatostatin receptor scintigraphy (SRS). CT and ultrasound can be useful for tumors larger than two centimeters in diameter. There are greater limitations on tumors with a diameter less than five millimeters, however, limiting the utility of these imaging techniques for some functioning tumors. The typically small size of these tumors makes localization difficult and supports with use of multiple imaging studies for definitive assessment of tumors [796-798].

Ultrasound imaging, including transabdominal and endoscopic ultrasonography, vary in sensitivity, but both techniques are generally able to detect tumors greater than three centimeters in size [796-799]. Endoscopic ultrasounds possess greater sensitivity and can detect small tumors in most cases [293, 631, 800-814]. Endoscopic ultrasonography can also determine the status of lymph node metastases [798, 808], and combining needle biopsy with endoscopic ultrasound enables tumor localization and staging [804, 813, 815]. Limitations to ultrasound imaging for the evaluation of pancreatic NET are due to tumor location in the pancreas and the size of the tumor mass [810, 816].

CT is critical for pre-operative tumor localization with its ability to distinguish various soft tissues providing precise anatomical localization of the tumor within various organs. Therefore, CT imaging is widely used for detection of pancreatic NETs [817, 818]. Its sensitivity is variable among pancreatic NET subtypes. Insulinomas and gastrinomas, for example, have a detection rate of around 60 to 80 percent with CT in multiple studies [405, 410, 416, 418, 810, 819-824]. CT sensitivity is increased for large tumors and hypervascular tumors, both of which allow accumulation of the contrast agent within the tumor for visual enhancement [793, 825-827]. Success with CT is enhanced with combination with another
imaging modality [823, 828]. For example, combining ultrasound and CT increases sensitivity significantly [810, 826].

MRI is incredibly sensitive for identifying single and multiple primary pancreatic NETs as well as secondary tumors of the lymph node and liver [406, 818, 821, 829-834]. MRI for the detection of liver metastases from multiple NET primary sites, including pancreas, reveals a sensitivity greater than eighty percent, with an emphasis on the ability to detect primary tumors and metastases that are small in size [831-833, 835-837].

PET imaging uses radiolabeled tracers to evaluate metabolism and cellular uptake in tumors, and, therefore, is beneficial for detection of primary and metastatic pancreatic NETs [834-842]. Due to the nature of cells of the endocrine system and their facilitation of amine precursor uptake and decarboxylation [843-846], alternative tracers, including fluorine-18 (18F)- or carbon-11 (11C)-labeled L-3,4-dihydroxyphenylalanine (L-DOPA) and 11C-labeled 5-hydroxy-L-tryptophan (5-HTP) are superior to traditional 2-(18F)-fluoro-2-deoxy-D-glucose (18F-FDG) to evaluate uptake and metabolic activity for use as a radiotracer for imaging functioning NETs of various organs [840, 844, 847-851], including the pancreas [828, 838-841, 852, 853]. Nonfunctioning, highly proliferative, and poorly differentiated tumors, however, can retain a more traditional metabolism supporting identification with 18F-FDG PET scans [854-858]. In addition, PET imaging using labeled somatostatin analogs, including octreotide, is also effective for localizing pancreatic NETs with superior sensitivity for both primary and secondary tumors sites [857, 859-866]. Size, tumor differentiation status, and functional status of tumors determine the applicability of PET imaging studies, with the majority of functioning tumors especially susceptible to PET imaging [842, 847, 852, 865]. PET can also be used to monitor tumor response to treatment over time, especially in functional tumors [840, 853, 859].

Radioactive octreotide is also used clinically for both diagnosing and localizing somatostatin-sensitive tumors [867-874]. This procedure, called somatostatin receptor scintigraphy (SRS) and marketed as the OctreoScan, allows clinicians to visualize radiolabeled octreotide accumulated in tumors to identify both primary or metastatic tumor sites overexpressing somatostatin receptors [484, 672, 794, 809, 856, 871, 875-880]. SRS utilizes octreotide or another somatostatin analog conjugated to a radioisotope, commonly indium-111 (111In), technetium-99m (99mTc), or gallium-67 (67Ga), to identify octreotide-
avid tumors after a scintigraphy scan with both high sensitivity and specificity [672, 794, 864, 875, 876, 881, 882].

Because both primary and metastatic tumor sites express SSTR2, SRS can localize primary and secondary tumor lesions (Table 2) [648, 677, 792, 823, 835, 847, 856, 869, 877, 883, 892-897]. Insulinomas, compared to other pancreatic NETs, express low levels of SSTR2, making SRS less effective for diagnostic and localization studies, requiring the use of other imaging technologies [386, 835, 861, 897, 898]. However, as SSTR2 expression levels vary between tumors and patients and increase in malignant or progressing insulinomas, SRS cannot be omitted as a potential imaging tool for use in patients with insulinomas [45, 365, 649, 672, 899-905]. Alternatively, the remaining pancreatic NET

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>SRS Localization (% positive)</th>
<th>Metastatic Sites Positive</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatic neuroendocrine tumor (combined)</td>
<td>74 - 92</td>
<td>Lymph node, liver, bone, soft tissue</td>
<td>[828, 856, 864, 875, 876, 881, 882]</td>
</tr>
<tr>
<td>Non-functioning tumor</td>
<td>56 - 87</td>
<td>Lymph node, liver</td>
<td>[667, 796, 823]</td>
</tr>
<tr>
<td>Insulinoma</td>
<td>13 - 83</td>
<td>Lymph node, liver</td>
<td>[672, 796, 802, 809, 823, 847]</td>
</tr>
<tr>
<td>Gastrinoma</td>
<td>61 - 100</td>
<td>Lymph node, liver, bone</td>
<td>[667, 672, 796, 809, 823, 883-885]</td>
</tr>
<tr>
<td>Glucagonoma</td>
<td>95 - 100</td>
<td>Lymph node, liver</td>
<td>[360, 667, 672]</td>
</tr>
<tr>
<td>VIPoma</td>
<td>100</td>
<td>Lymph node, liver</td>
<td>[667]</td>
</tr>
<tr>
<td>Carcinoid</td>
<td>50 - 91</td>
<td>Lymph node, liver, bone</td>
<td>[823, 847, 848]</td>
</tr>
<tr>
<td>Other functioning tumors</td>
<td>57</td>
<td>Lymph node, liver</td>
<td>[823]</td>
</tr>
</tbody>
</table>

Table 2. Effectiveness of SRS for tumor localization in pancreatic NETs
types express SSTR2 in the vast majority of cases, allowing utilization of SRS for localization of primary and metastatic tumor sites associated with these tumors, with successful localization correlated with the size of the primary tumor [883, 885, 906]. SRS in combination with at least one other diagnostic imaging technique is recommended to fully appreciate the extent of disease [386, 672, 792, 816, 869, 876, 884, 893, 907, 908]. Common sites of metastases for pancreatic NETs include lymph nodes, liver, and bone [374, 375, 379, 509]. Liver metastases larger than one or two centimeters [835, 856] and bone metastases [830, 856, 864, 885, 909] are visible with SRS. Use of single photon emission computed tomography (SPECT) increases the sensitivity of SRS [835, 870, 904, 910], while CT or MRI provide additional anatomical information regarding tumor localization.

Successful localization of tumors varies greatly among different tumor types. [677]. The presence of multiple or small tumors, as is sometimes found in MEN1 patients, also leads to difficult definitive localization with SRS [911]. Additionally, correctly discerning non-functioning pancreatic NETs from pancreatic tumors of exocrine origin is possible using SRS, further expanding the diagnostic utility of SRS [381]. Because SRS sensitivity corresponds to SSTR2 expression in tumors, a positive SRS scan indicates octreotide-avid tumors expressing SSTR2. These tumors are potentially susceptible to treatment with somatostatin analogs, including octreotide, making a positive SRS a clinical biomarker [648, 868, 912].

**CLINICAL INTERVENTION**

Pancreatic NETs typically require a twofold approach in the clinic. In addition to a primary curative objective for all patients, relief of symptoms associated with hormone hypersecretion is a secondary clinical goal for patients with functioning tumors [269, 486, 913, 914]. Surgical resection remains the only potentially curative option currently available for patients [508, 915-918], but its application is dependent on tumor size, invasiveness, location in the pancreas, stage at diagnosis, and whether an inherited syndrome is present [334, 466, 493, 507, 916, 917, 919-923]. The surgical approach towards pancreatic NETs can compromise enucleation, pancreatic resection, and total pancreatectomy for tumors
isolated to the pancreas, with tumor de-bulking, lymph node resection, liver resection, and even liver transplantation indicated for advanced disease [269, 327].

Enucleation of the tumor is recommended for small tumors that are isolated from the main pancreatic duct [412, 924]. This technique requires concurrent ultrasound imaging to locate and guide the surgeon to the tumor. The tumor is removed from the pancreatic tissue and any blood vessels associated with the tumor are cut and sutured to limit bleeding [467]. This is the most common surgical procedure for removing small, benign insulinomas [365, 369, 413, 418, 467, 924-926] and other small, resectable tumors [429, 430, 630]. Patients presenting with large or multiple tumors generally require resection of the pancreas or even the more aggressive total pancreatectomy [504, 620]. The traditional pancreateoduodenectomy, or Whipple procedure [927], is indicated for aggressive tumors located in the head of the pancreas that have invaded into the duodenum or metastasized to the lymph nodes [503, 928, 929]. This surgical procedure has been refined to protect the pylorus, the section of the duodenum that emerges from the stomach, called a pylorus-preserving pancreateoduodenectomy [930, 931], that is indicated for some patients with aggressive or invasive tumors in the head of the pancreas [612, 615, 929, 930, 932]. For tumors located in the tail of the pancreas a distal pancreatectomy is indicated [429, 924]. Patients with multiple tumors, highly aggressive tumors, or MEN1 syndrome sometimes require a more aggressive surgical approach including total pancreatectomy [610, 612, 615, 621, 803, 911, 922, 933, 934]. Though uncommon, malignant insulinomas also require a more aggressive surgical intervention compared to the benign tumors that make up the majority of diagnoses [364, 416, 935, 936]. The length of disease-free survival after surgical pancreatic resection varies widely among patients, but surgical intervention remains the only potential curative approach to tumor management [241, 272, 289, 362, 404, 434, 454, 503, 929, 937].

Metastatic disease necessitates greater surgical intervention. Resection of liver metastases, in addition to primary tumors, is indicated for better overall prognosis and quality of life for patients [272, 279, 349, 402, 498, 935, 938-954]. Limitations of liver resection are associated with the degree of tumor dissemination and the number of lobes containing metastases [459, 951, 955-958]. Ablation therapies, including chemoembolization and radiofrequency ablation, have also been evaluated for their
effectiveness in decreasing tumor volume in patients with metastatic disease that is not suitable for surgical resection [381, 952, 959-964]. These treatments attempt to either destroy the tumor tissue or reduce vascularity in tumors, especially metastases in the liver, with radiofrequency ablation, cryotherapy, or injecting alcohol directly into the tumor when possible [959, 962]. Patients with diffuse hepatic metastases require a palliative clinical approach utilizing tumor de-bulking surgery, chemotherapy, radiotherapy, and peptide biotherapy for symptomatic relief and to decrease the tumor burden [421, 935, 942, 965-973]. Alternatively, reports of liver transplantation have been documented with varying results [948, 949, 951, 974-992]. The consensus seems to be that liver transplantation for patients with un-resectable liver metastases is a viable option, but limited donor organs requires careful assessment of the patient and tumor and overall caution with proceeding [948, 977, 983-986, 988-991, 993-995].

Since curative surgery is not always possible, some patients require alternative or supplemental treatment to suppress tumor growth or relieve symptoms manifesting from functioning tumor hypersecretion. The course of treatment is dependent on tumor aggressiveness and can vary from a “wait and see” approach for non-growing or slow-growing tumors to aggressive chemotherapy and radiotherapy treatments. Additionally, biotherapy with somatostatin analogs is recommended as a single-agent therapy or in combination with other agents to manage symptoms associated with functioning tumors [336, 996].

Somatostatin analogs, and specifically octreotide, are useful for treating symptoms associated with functioning pancreatic NETs due to their ability to both inhibit hormone secretion and stabilize tumor growth [51, 759, 771, 915, 997-1004]. Biotherapy with somatostatin analogs is particularly useful clinically because of the limited, and generally minimal, side effects associated with therapy as well as an increase in overall patient quality of life in regards to symptomatic relief of hormone hypersecretion [785, 788, 789, 997-1000, 1005-1017]. The clinical significance of octreotide and other somatostatin analogs as antiproliferative therapeutics are variable, but these agents are potentially effective in a subset of patients with advanced or progressing disease at diagnosis [996, 998, 1002, 1004, 1005, 1016, 1018-1024]. Isolated and sporadic occurrences of effective tumor shrinkage in these reports could be due to apoptosis induction in tumor cells specifically with
somatostatin analog therapy at high doses [723, 1003, 1025-1031], although others show no such benefit associated with somatostatin analog treatment related to tumor response.

Somatostatin analogs mimic the biological activity of somatostatin itself, limiting their utility in the treatment of benign insulinomas, but enabling symptomatic relief in other tumor types. Somatostatin is considered an inhibitory hormone, suppressing the release of gastrointestinal hormones including glucagon, gastrin, and VIP, supporting its effectiveness in the relief of symptoms associated with tumors secreting the corresponding hormones. However, as glucagon acts to lower blood glucose levels, somatostatin analogs can exacerbate neuroglucopenia in insulinoma patients. Accordingly, insulinomas have a variable response to octreotide biotherapy dependent on somatostatin receptor expression in the tumors [901, 1032], but alternative therapeutic options, including the utility of diazoxide, known to inhibit insulin secretion [1033], have been studied in these patients [1034, 1035].

Sandostatin, the brand name of the octreotide salt and long-acting formulations distributed by Novartis Pharmaceuticals, has been approved by the United States Food and Drug Administration (FDA) for the treatment of watery diarrhea associated with VIPomas [481] and flushing episodes related to carcinoid syndrome [758, 1036]. It is also used off-label for treatment of symptoms associated with other functioning tumors, including erythema and anorexia from glucagonomas [462] as well as chemotherapy-induced and other pathologic diarrhea [1037, 1038]. Symptomatic relief of hormone secretion in response to somatostatin analog therapy has been observed in patients with functioning tumors, but tumor growth inhibition has been suggested but not definitively supported by studies in patients [723, 759, 998-1000, 1004, 1039-1044]. Side effects associated with somatostatin analog therapy typically lessen overtime and include gastrointestinal discomfort, abdominal pain, steatorrhea, and nausea [51, 788, 970, 1009, 1045]. Somatostatin analog therapy is also used in conjunction with surgical procedures, with an octreotide regimen initiated in patients prior to and following surgery to limit the possibility of a hormonal surge in response to surgery creating a metabolic crisis [51, 467, 962].

The usefulness of chemotherapy is dependent on individual tumor characteristics and the effectiveness of alternative therapies [915, 962, 1046, 1047]. Chemotherapeutics in pancreatic NETs are used clinically in a couple of tumor types. Etoposide and cisplatin are used to treat some fast growing, poorly differentiated tumors [962, 1048-1051], while
streptozotocin alone or in combination with either doxorubicin or 5-fluorouracil have been effective in treating some well differentiated tumors [936, 1052-1055]. However, tumors of endocrine origin are generally resistant to chemotherapy, and variable responses to treatment are seen in tumors with similar histological profiles [962, 1046, 1056-1058]. Specifically, some malignant insulinomas and VIPomas are responsive to chemotherapy [936, 1052, 1053, 1059-1061] while other tumor types have not had significant clinical responses to chemotherapy studies [421, 962, 1046, 1062]. However, the variability of response rates among different agents and tumor types indicates chemotherapy cannot be excluded as a potential treatment option for any tumor type [1056, 1063]. Additionally, symptoms and side effects commonly associated with chemotherapy are consistently observed in these trials and should be weighed in consideration of patient quality of life concerns [1046, 1048, 1055, 1056, 1062].

Interferon alpha is effective for the treatment of pancreatic NETs, and specifically a viable therapeutic option for tumors negative in an SRS scan indicating a lack of SSTR2 expression for peptide-targeting biotherapy [1060, 1064-1066]. Significant side effects and a decreased overall quality of life are occasionally observed with interferon alpha therapy [1060, 1066].

Targeted delivery of radiation directly to tumors is also being studied. This technique, called peptide receptor radionuclide therapy (PRRT), requires conjugation of a radioisotope to somatostatin analogs [1067] to deliver radiation directly to octreotide-avid tumor cells [870, 1068, 1069]. Once within the cells, radionuclides are capable of delivering a much higher dose of radiation than traditional external beam radiation. Studies using this strategy, specifically yttrium-90 ($^{90}$Y) and lutetium-177 ($^{177}$Lu) radionuclides conjugated to octreotide or another somatostatin analog called lanreotide, have been evaluated for efficacy and toxicity in patients [1068, 1070-1082]. The few studies available have shown disease regression and increases in both progression-free survival and overall survival in patients treated with PRRT [905, 965, 1068, 1071, 1076, 1080, 1083, 1084]. However, an extensive toxicity profile with side effects to bone marrow, kidney, and liver were observed, requiring dosage manipulations, co-injections with protective amino acid or gelatin solutions, and monitoring physiologic function for changes after administration [960, 1068, 1069, 1071-1073, 1077, 1078, 1080, 1083, 1085-1090].
Novel clinical biomarkers and their corresponding derivative drug candidates are being identified and evaluated for anti-tumor activity in NETs [1091-1101]. Additionally, agents that function as inhibitors against receptors common in tumors, including agents active against markers of angiogenesis and tumor progression, are also being evaluated clinically for their utility as therapeutic agents in pancreatic NETs in patient trials [486, 918, 1102-1106]. For example, the efficacy of everolimus, an inhibitor of mTOR activity previously evaluated for the treatment of renal cell carcinoma [1107-1109], was shown in large, multi-center clinical trials for the treatment of pancreatic and other NETs [1110-1113]. Recently, the FDA approved everolimus, marketed as Afinitor, for the treatment of advanced pancreatic NETs. Everolimus is capable of suppressing the activity of the mammalian target of rapamycin (mTOR) protein, and, therefore, eliminating the cell growth and corresponding tumor progression associated with activation of the mTOR signaling pathway [676, 1114-1118]. Furthermore, another agent, sunitinib, has also been studied in recent clinical trials for efficacy in patients with advanced disease [1102, 1119-1121]. Studies with this drug, known to inhibit receptor tyrosine kinases, including vascular endothelial growth factor (VEGF) family receptors, associated with tumor angiogenesis and cell proliferation [1122, 1123], reveal effectiveness as an anti-tumor therapy [1119], with limited side effects observed in patients [1120]. FDA approval for sunitinib for the treatment of progressive disease and well-differentiated pancreatic NETs came just two weeks after the approval of everolimus for the same patient population, indicating the interest in, and the importance of, identifying therapeutic agents for this patient population with limited treatment options (Table 3).

Additionally combinatorial approaches have been studied using somatostatin analogs with other agents including interferon-alpha [881, 882, 1040, 1066, 1124-1126], chemotherapy [1127, 1128], and mTOR inhibitors [1110, 1111, 1113]. Effectiveness of combinatorial octreotide and interferon alpha is variable and affected by tumor status at the initiation of treatment as well as tumor susceptibility to the agents [882, 1124]. However, direct comparisons between combinatorial therapies and octreotide monotherapy reveal a significant disparity in patient quality of life with increased frequency and severity of

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1 http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm254350.htm
2 http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm256237.htm
adverse symptoms associated with the non-octreotide agent in the combination that is not observed in the octreotide treatment group alone [1111, 1124]. This side effect profile was confirmed with alternative somatostatin analog combinatorial therapy trials [1040].

Table 3. FDA approved drugs for the treatment of pancreatic NETs

<table>
<thead>
<tr>
<th>Drug (Brand Name)</th>
<th>Type</th>
<th>Year Approved by FDA</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptozocin (Zanosar)</td>
<td>Chemotherapy</td>
<td>1982</td>
<td>Metastatic pancreatic NET</td>
</tr>
<tr>
<td>Octreotide (Sandostatin)</td>
<td>Biotherapy (somatostatin analog)</td>
<td>1998</td>
<td>VIPoma Carcinoid syndrome</td>
</tr>
<tr>
<td>Everolimus (Afinitor)</td>
<td>mTOR inhibitor</td>
<td>2011</td>
<td>Unresectable, locally advanced, or metastatic pancreatic NET</td>
</tr>
<tr>
<td>Sunitinib (Sutent)</td>
<td>Receptor tyrosine kinase inhibitor</td>
<td>2011</td>
<td>Unresectable, locally advanced, or metastatic pancreatic NET</td>
</tr>
</tbody>
</table>

3 http://www.fda.gov/
RATIONALE

With the goal of identifying a candidate biologically active peptide for evaluation in a novel manner in the phage display system, the octreotide/SSTR2 ligand/receptor appears to be appropriate. As described in the previous section, SSTR2 is known to be overexpressed in pancreatic NETs, providing a candidate receptor to exploit on the cell surface. The somatostatin analog octreotide has a specific affinity for SSTR2 and is already used clinically with much regard for its utility, confirming its appropriateness for use as our ligand. Finally, although pancreatic NETs are very rare, there are very limited therapeutic options for these tumors, indicating an unmet clinical need for continued investigation into potentially useful therapeutic agents in this tumor type.

The profile of receptors displayed on the cell surface of NETs provides a basis for molecular targeting of these tumors specifically. SSTR2 expression is already used clinically for pancreatic NET localization and tumor staging. SRS imaging with somatostatin analog targeted delivery of radioisotopes for imaging is a clinical diagnostic standard for tumors of neuroendocrine origin [856, 1129]. Displaying a somatostatin analog capable of targeting SSTR2 in a phage construct should allow us to recapitulate the binding profile observed in the parent peptide. Furthermore, by utilizing an adeno-associated virus and phage hybrid (AAVP), the SSTR2-targeting AAVP should be able to deliver a therapeutic reporter gene directly to the tumor cells for activity.

AAVP is one example of a viral gene-delivery system. Driessen et al. describe requirements for a successful targeted gene-delivery system [1130]. The subset of these requirements regarding cellular transduction capabilities and transgene expression can be met by the use of adeno-associated virus (AAV) with its utility as a gene transfer vehicle [1131]. Additionally, vector stability has long been proven for the phage viral particle. The other requirements are based on the receptor profile expression and appropriate ligand selection or identification. The necessity of receptor expression that is both accessible for systemic targeting and specific for target cells is consistent with selective targeting approaches, generally, and targeted cancer treatment, specifically [1132]. In the case of pancreatic NETs, SSTR2 expression is found in around 80 percent of tumors, described
thoroughly in the previous section, which is already being exploited for clinical studies with SRS imaging standard for clinical diagnosis and staging of disease. The last general necessity for a successful ligand-directed gene-delivery system is appropriate ligand selection. The octreotide peptide ligand was rationally chosen as a well-studied and reasonable ligand for targeted delivery to NETs of the pancreas to address an under-met need in patients with these tumors. Furthermore, continued advocacy for further clinical applications directed by or in combination with octreotide or other somatostatin analogs indicates a clinical interest in the identification of alternative approaches using these peptides [727, 735, 754, 1133, 1134].

Any gene-delivery system must be carefully designed for optimal therapeutic benefit. Incorporating a targeting ligand for display on the viral particle of AAVP allows cells expressing receptors of interest to be specifically targeted. Historically, the use of phage libraries required displaying peptides within a specific conformational constraint in the gene for the pII coat protein, either linear peptides (Xₙ) up to about 30 amino acids, cyclic peptides with a conformation of CXₙC, or other engineered amino acid formations and consensus motifs [12]. These libraries are then administered to receptors of interest, monoclonal antibodies, isolated cells or cell lines, in vivo to normal or tumor-bearing animals, or even patients and then recovered and evaluated to determine which peptide ligand bound what receptor. After identification of a ligand/receptor pair, this new knowledge can then be translated into generation of peptides conjugated to clinical agents, or targeted imaging or cytotoxic gene therapy agents. To further understand the activity of these ligand/receptor pairs, new information is gained concerning receptor localization or receptor expression patterns in normal and tumor vasculature to further expand the human vascular map.

However, to date, no studies have shown that peptides with a known biological activity could function in a phage system. Typically, peptide analogs, as is the case with somatostatin, are evaluated to determine binding affinity and the ability to evade protease activity in circulation, thereby extending their circulating half-life and increasing their utility as therapeutic agents. Tumor cells overexpressing specific receptors have been exploited for targeted drug delivery, but none of these approaches involve a gene therapy approach for therapeutic drug delivery with a biologically active peptide known to have specific
functionality within the tumor type. Therefore, generation of an AAVP targeted with a known peptide modifying the traditional laboratory protocol [1135], but rather than library production, incorporating a sequence encoding a specific amino acid sequence into the pIII gene for display on the viral coat protein, should produce a viral particle capable of mimicking the activity of the parental peptide and provide the means for targeted delivery of a therapeutic gene specifically to the corresponding receptor of this peptide. Specifically, exploiting the known overexpression of a receptor on tumor cells by incorporating targeting an AAVP particle with the corresponding peptide ligand should effectively enable tumor targeting without first identifying candidate ligand/receptor pairs. In our study, generation of an AAVP targeted by the octreotide peptide should allow for specific binding and localization to tumor cells overexpressing SSTR2 in vitro and in vivo. This octreotide-targeted AAVP engineered to deliver the gene for tumor necrosis factor (TNF) should enable transduction of tumor cells, integration of the TNF gene into cellular DNA, and translation of TNF for activity within the tumor itself. In this case, TNF acts as a reporter gene, measureable by protein- and RNA-specific quantification techniques.

To evaluate AAVP constructs targeting SSTR2 and delivering TNF, we will use the Men1 transgenic mouse model. This conditional knockout model forms functioning tumors of the endocrine pancreas, recapitulating human disease development and progression. Upon successful completion of these studies, we will have generated a pancreatic NET cell-specific gene therapy vector with an origin in a known ligand/receptor pair. This AAVP will be specific for pancreatic NETs with confirmed SSTR2 expression and octreotide avidity and will be studied using a well defined and relevant preclinical transgenic mouse model. Validation of our AAVP could yield clinical opportunities that could be incorporated into the current, established clinical repertoire available to patients with pancreatic NETs, providing a new possibility for pancreatic NET management or treatment.

**Adeno-Associated Virus and Phage (AAVP)**

Phage has long been regarded as an incredible tool for discovering new ligand/receptor pairs and mapping the mouse [30, 31, 35, 1136-1141] and human vasculatures [1142, 1143],
in addition to tumor-specific vasculatures [11, 14, 20, 36, 298, 1144-1148]. Phage also provides an opportunity to evaluate discrete alterations in a specific model of disease, such as the pre-metastatic niche and organotropism in mouse models of tumor metastases [1149, 1150], and DNA damage repair pathways found in the tumor microenvironment [1151].

The clinical usefulness of phage itself is debatable. For research, phage display is a technology that is easily modifiable and relatively cheap, but is a powerful tool for evaluating ligand/receptor interactions for targeted drug delivery [1152-1155]. After administration of a phage, the bound phage particles can then be recovered and evaluated to determine the ligand and its candidate receptor [30, 1138, 1156]. This tested and true functional activity has allowed a prolific expansion of the understanding of normal and tumor vasculature, even in humans, having been studied in terminal patients on life-support [1142, 1143]. Additionally, the use of phage historically as an antibiotic agent is providing an argument for a resurgent advocacy of the utility of phage as a clinical counter measure to overcome bacterial resistance [1157, 1158], further supporting the appropriateness of using a phage-based therapy approach in patients. Specifically, the maintenance of a single, complete, known genome consistently confirmed and maintained, should overcome the disinclination or avoidance of regulatory agencies to approve the native phage as “druggable” for the treatment of human diseases [1157]. Evaluation and characterization of ligand/receptor pairs identified in phage library screenings is an effective avenue for drug development [1140, 1159, 1160]. In addition to indentifying small peptidomimetics that can function as drugs [1161, 1162], conjugating peptide mimetics of identified ligands to various therapeutic agents allows for targeted drug delivery to the known receptors displayed in the tumor or disease state. For example, peptide ligands have been prepared for delivery of chemotherapeutics [14, 36], proapoptotic domains [22, 1136, 1140, 1159, 1163-1165], cytokines [1166-1168], and genes [1169, 1170] as anti-cancer agents in addition to targeted delivery of fluorophores [1171-1173] for imaging.

AAVP is a ligand-directed viral particle capable of targeted drug delivery formed from the combination of two unrelated genetic systems [1174-1176]. AAVP increases the utility of phage alone as a targeting agent by creating a hybrid viral genome of adeno-associated virus (AAV) and phage to enable cellular transduction and reporter gene expression. By combining the most advantageous components of both prokaryotic phage and eukaryotic
AAV, the beneficial aspects of each can be utilized in the new particle [1131]. Phage is a consistently effective targeting agent as it is easily modifiable for the introduction of targeting peptides in the pIII coat protein [6]. Additionally, the generation of peptides requiring a specific conformational constraint, as is the case with the cyclization of the octreotide peptide sequence, displayed in the pIII minor coat protein is possible, ensuring recapitulation of the functional activity of the native peptide [1177]. However, phage alone is unable to efficiently deliver a gene to mammalian cells [1178]. Its activity is restricted to receptor binding and induction of any receptor-mediated signaling pathways. AAV, alternatively, has no native targeting capabilities and is not easily altered for such ligand-directed mechanisms [1179]. However, the transduction capabilities of AAV are unquestionable with well-described mechanisms of transgene delivery and cellular transduction [1180, 1181]. The limitations of both phage and AAV are overcome by combining the ligand-directed targeting capabilities of prokaryotic phage and the superior transduction capabilities of eukaryotic AAV. The resulting hybrid vector, AAVP, is capable of specific homing and mammalian cell transduction for transgene delivery [1174-1176, 1182-1184]. Inclusion of AAV inverted terminal repeat (ITR) sequences enables integration of the gene into the mammalian genome [1174, 1175, 1185], resulting in DNA transcription and transgene expression [1186]. The use of AAVP for cancer cell targeting could allow for increased integration of AAV into the cellular genome due to increased replication activity of tumor cells [1187], enabling incorporation of the AAV sequence into the frequent double-strand breaks in the DNA [1186]. The targeting capabilities and engineered tropism for cells specifically expressing a receptor of interest allows for gene expression with limited amounts of AAVP circulating after systemic administration, increasing efficacy with minimal toxicity [1131].

AAVP is an attractive vehicle for research and therapeutic investigations because it is relatively inexpensive to produce, but can be processed with high yields in *Escherichia coli* (*E. coli*) host bacteria without the addition of trans-acting factors or helper viruses. Moreover, there is no AAV capsid formation, eliminating any native AAV tropism to mammalian cells [1175]. Homing to tissue- and tumor-specific receptors is possible due to the inclusion of peptide ligands for display on the pIII coat protein of the phage-based portion of the AAVP particle [1175]. AAVP viral particles are generated as non-replicating
viruses that can infect mammalian cells and incorporate the TNF gene cassette into the cell genome for transgene expression. Systemic targeting with phage and AAVP viral particles is consistently effective for binding receptors displayed on normal and tumor endothelium, and directly targeting tumor cells expressing specific receptors has also been shown [1148, 1163, 1188]. This capability is possible because the vascular network of tumors is generally disordered and leaky [306, 308-313]. This permeability allows for extravasation of circulating ligands into the tumor microenvironment [1132]. Our goal is to take advantage of this characteristic tumor pathophysiology in pancreatic NETs. As described in the previous section, the angiogenic processes in NETs of the pancreas expand the already well-vascularized and fenestrated vessel network of the islets necessary for endocrine function and hormone distribution.

Clinical translation of the octreotide-targeted AAVP-TNF particles could provide alternative options for cancer patients lacking adequate therapeutic choices. Numerous molecular biology, molecular-genetic imaging, and tumor-targeting studies in various preclinical models [1175, 1182, 1184, 1189-1191] indicate our ligand-directed AAVP-based system is an obvious candidate for therapeutic applications in pancreatic NETs.

**Tumor Necrosis Factor and Apoptosis**

Tumor necrosis factor (TNF; previously cachexin, or cachectin) is a cytokine with variable physiologic and pathologic functions [1192-1194]. TNF binding tumor necrosis factor receptor type 1 (TNF-R1; also called cluster of differentiation 120 (CD120)), results in recruitment of a number of proteins to the receptor complex for signaling induction. The first proteins assembled include members of the death domain protein family. These include tumor necrosis factor receptor type 1-associated death domain protein (TRADD) [1195] and Fas-associated protein with death domain (FADD) proteins. Interaction of receptor-interacting serine/threonine-protein kinase 1 (RIPK1) or TNF receptor-associated factor 2 (TRAF2) with TRADD leads to activation of signal transduction pathways [1196, 1197]. RIPK1 is responsible for activation of inhibitor of nuclear factor kappa-B kinase (IKK) proteins, which, in turn, phosphorylates NF-kappa-B inhibitor alpha (NFKBIA). Because
NFkBIA is responsible for the sequestration of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in the cytoplasm, upon phosphorylation NF-κB is released from NFkBIA, enabling translocation of NF-κB to the nucleus for transcription activation [1198, 1199]. TRAF2 mediates apoptosis induction along with members of the inhibitor of apoptosis protein (IAP) family by blocking caspase activation directly [1200, 1201]. Additionally, TRAF2 activates the mitogen-activated protein (MAP) kinase pathway, leading to c-Jun N-terminal kinase (JNK) signaling and, eventually, formation of the activator protein 1 (AP-1) transcription factor with roles in differentiation, proliferation, and apoptosis. Alternatively, FADD activation is primarily responsible for the induction of apoptosis. Caspase-8 recruitment upon FADD activation signals apoptosis induction mediated through downstream caspase processing and activation [1202-1204].

The variable functions of TNF-R1 activation are mediated by internal balance and regulatory mechanisms. For example, inhibition of IAP family member proteins circumvents the anti-apoptotic pathway of TNF-R1 induced by IAP activity [1205, 1206]. Eliminating the IAP-mediated resistance to apoptosis results in loss of NF-κB activity and the activation of the FADD/caspase signaling of the TNF-R1 death domain [1207, 1208]. Preclinical trials with a version of AAVP-TNF targeted by the RGD peptide [36, 1144, 1209] specific for integrins expressed on tumor endothelium have shown significant anti-tumor responses, including significant tumor regression observed in spontaneous tumors in dogs [1189, 1191]. Inclusion of a small molecule peptide mimic of the second mitochondria-derived activator of caspases (Smac; also referred to as DIABLO) protein that functions as an inhibitor of X-chromosome linked IAP (XIAP) and cellular IAP (cIAP1 and cIAP2) proteins, increased the efficacy of RGD-targeted AAVP-TNF in a synergistic effect [1184].

Antitumor effects of TNF require targeted or local delivery rather than systemic administration [1210, 1211]. However, TNF is well studied as a therapeutic agent for tumors. Direct administration of TNF to the tumor itself, through isolated perfusion to the organ or tumor specifically results in effective tumor response and limited systemic toxicity [1192, 1212]. Furthermore, evidence of SSTR2 activation inhibiting the anti-apoptotic mechanism of TNF and upregulating TNF receptor expression [1213] potentially provides an inherent pro-apoptotic environment to increase efficacy of an octreotide-targeted AAVP-TNF.
In addition to the benefits mentioned previously, inclusion of TNF as the gene delivered in our AAVP is due to two main factors. The first is that, as opposed to previous AAVP constructs delivering a gene suitable for imaging studies [1174, 1175, 1182, 1190], pancreatic NETs are currently imaged clinically with SRS and PET imaging of octreotide-targeted radiopharmaceuticals, diminishing the necessity of incorporating a gene with imaging capabilities. Secondly, because this study attempts to exploit a known ligand/receptor pair for targeted gene delivery, previous preclinical studies with an AAVP-TNF targeted by an integrin binding sequence provide a framework for both understanding experimental nuances and a somewhat comparable baseline to evaluate targeting efficiency [1184, 1189, 1191]. The octreotide amino acid sequence displayed in the AAVP maintains the cleavage sites identified for the native somatostatin peptide within and immediately surrounding the four critical amino acids of the beta turn region [735]. Effective homing of the octreotide-targeted AAVP-TNF both in vitro and in vivo requires the pIII coat protein of the phage virus to provide a protective conformational configuration to avoid proteases and maintain integrity of the targeting peptide [1214]. Evaluating a novel targeting design in concert with a previously untested therapeutic gene increases the number of variables changed and eliminates the ability to evaluate any downstream effects observed.

**The Men1 Transgenic Mouse Model**

Shen *et al.* [54] describe the transgenic mouse model displaying the characteristics of MEN1 syndrome in humans that we used as our pre-clinical model for *in vivo* studies. This model evolved from a series of investigational mutations in the *Men1* gene in mice in an attempt to recapitulate human MEN1 disease. As described in a previous section, mutations in the *MEN1* tumor suppressor gene are inherited in an autosomal dominant pattern in patients with the familial MEN1 syndrome, and these patients develop tumors in two or more endocrine organs, often including NETs of the pancreas, through loss of *MEN1* gene function [540, 1215]. Several iterations of mouse models of Men1 disease have been generated. For example, homozygous deletion of *Men1* in embryonic stem cells results in embryonic lethality [1216, 1217]. Heterozygous mice containing one wild-type *Men1* allele
and one mutant Men1 allele produce a variety of endocrine tumors, similar to what occurs in MEN1 patients. Tumors form in the pancreatic islets, thyroid, parathyroids, adrenal glands, and pituitary, all revealing loss of the wild-type Men1 allele [1216-1218]. Attempts to induce endocrine tumors of specific origin have also produced several models of Men1 tumors in individual organs using conditional knockout models [1219]. Deletion of Men1 in the parathyroid glands results in hyperparathyroidism and neoplasia formation in the parathyroid [1220]. Models of Men1 inactivation in individual islet cell types have attempted to produce specific functioning tumors. Beta cell-specific deletion of Men1 generated mice with insulinomas [1221-1223]. Inactivation of Men1 in the pancreatic alpha cells also resulted formation of both glucagonomas and insulinomas [1224, 1225]. Additional manifestations of individual Men1 tumors are continuing to be described, while investigators are also evaluating the molecular mechanism of Men1 activity [1226, 1227]. Temporal manipulation of Men1 expression reveals an increase in islet cell proliferation after loss of menin expression, confirming Men1 activity in cell cycle regulation [1228].

To study the effect of Men1 deletion from the whole pancreas, a mutant Men1 allele was put under control of the Pdx1 promoter to generate a conditional knockout (termed Pdx1-Cre;Men1 f/f) with Men1 deleted in only pancreatic tissue, recapitulating the characteristics of human MEN1 disease manifestation in the pancreas [54]. At the level of Pdx1 expression, which occurs very early in gut derivation and pancreatic organogenesis (described in the Background Section), the resulting loss of Men1 in these mice affects the entirety of the pancreas. The Pdx1-Cre;Men1 f/f mouse model represents a conditional knockout of Men1 tumor suppressor gene loss using the Cre-loxP system [54]. MEN1 in humans and Men1 in mice are highly conserved in both nucleotide and amino acid sequences [568-570, 1215, 1229], supporting the development of mouse models of this human disease. Pdx1-Cre;Men1 f/f mice are generated by crossing mice engineered to incorporate loxP sites at both terminal regions of the Men1 gene with mice expressing Cre recombinase under control of the Pdx1 promoter. As described in the Background Section, Pdx1 expression at such an early time point in pancreatic development provides an opportunity to eliminate Men1 throughout the entirety of the pancreas. The resulting mice do show homozygous deletion of Men1 in the entirety of the pancreas, and pancreas development itself is unaffected by a lack of menin during embryogenesis [54]. However, by
three months age, mice show increases in endocrine cell proliferation, islet vascular density, and structural abnormalities in islet blood vessels. These mice develop functioning tumors of the endocrine pancreas with no evidence of exocrine pathology or abnormality. Hyperplastic islets are evident at 5 to 6 months of age, and functioning insulinomas, and a corresponding increase in vascularization, consistently form by 10 to 12 months of age [54], following the progression described for tumorigenesis in hereditary NETs including MEN1 syndrome [269]. Additionally, the Pdx1-Cre;Men1 f/f transgenic mice show increased levels of VEGF expression [54], corresponding to the VEGF levels observed in other models of islet cell tumorigenesis as well as human tumor progression [215, 291, 298, 317, 1230]. This observation, combined with the high levels of vascularization observed in these mice [54], support the ability of the tumor microenvironment to maintain necessary levels of oxygenation and enable recruitment of nutrients and matrix proteins for tumor growth [296, 317, 318, 1231]. The Men1 transgenic mice also have a significantly reduced lifespan compared to normal and control animals, demonstrating further similarities to MEN1 patients [54].

Structural organization varies minimally in rodent and human islets, most likely in response to glucose requirement of variable diets. The general islet construction is similar in these two groups, with the five endocrine cell types present in both but with different distributions of the cell type frequencies and altered orientation within the islets [1232]. Beyond the similarities in islet architecture, utility of the Men1 mouse model for targeted delivery of our AAVP requires escape from the vasculature and accessibility to the tumor cells. The Men1 conditional knockout model shows increased vascularity and an abnormal vasculature with blood vessel dilation and tortuosity evident in the islet cell tumors [54]. SSTR expression analysis confirms high levels of SSTR2, in addition to SSTR1, SSTR3, and SSTR5, evident throughout the tumor of the Men1 transgenic mice, consistent with what is observed in human tumors [1233]. Therefore, this mouse transgenic mouse model is a well-defined preclinical model for studying our AAVP in the context of the complicated tumor microenvironment in vivo. Since this Men1 mouse model mimics human MEN1 syndrome, and produces pancreatic NETs representative of patient tumors in both vascularization and SSTR2 expression, our findings in this preclinical model could be translatable to human disease.
MATERIALS AND METHODS

CLONING

Materials

Oligonucleotides and primers for polymerase chain reaction (PCR; Table 4) were purchased from Sigma-Aldrich (St. Louis, Missouri). Restriction enzymes, specifically AvrII (BlnI), BamHI, BglII, BssHII, EcoRI, HindIII, MluI, PstI, SfiI, and XbaI, were purchased from Roche Applied Science (Indianapolis, Indiana) or New England BioLabs (Ipswich, Massachusetts). T4 DNA Ligase was purchased from Invitrogen (Carlsbad, California), and the Rapid DNA Ligation Kit was purchased from Roche Applied Science. XL1-Blue MR supercompetent cells were purchased from Agilent Technologies (Santa Clara, California), DH10B and DH5α competent cells were obtained from Invitrogen, and K91Kan E. coli and the electrocompotent E. coli strain MC1061 are propagated and maintained within the laboratory. The pBluescript II KS (-) phagemid vector was purchased from Agilent Technologies. The pcDNA3.1 (+) plasmid containing the CMV promoter was acquired from Invitrogen. The filamentous phage display vectors fUSE5 [2] (GenBank accession: AF218364) and fMCS1 (GenBank accession: AF218733) are distributed by Dr. George Smith (University of Missouri; Columbia, Missouri). SOC medium was purchased from Invitrogen. Phosphate buffered saline (PBS; pH 7.4), Luria Bertani (LB) broth, LB-streptomycin (100 µg/mL), LB-ampicillin (100 µg/mL), LB-kanamycin (100 µg/mL), LB-tetracycline (40 µg/mL), and LB-kanamycin/tetracycline (100 µg/mL kanamycin and 40 µg/mL tetracycline) plates are produced and distributed by the University of Texas M. D. Anderson Cancer Center Media Facility (Houston, Texas). For PCR experiments, GoTaq DNA polymerase and its associated reaction buffer were purchased from Promega (Madison, Wisconsin), deoxyribonucleotide triphosphates (dNTPs) were acquired from Fisher Scientific (Fair Lawn, New Jersey), and dimethyl sulphoxide (DMSO) was purchased from Sigma-Aldrich. Agarose was obtained from IscBioExpress (Kaysville, Utah) and E-Gel precast agarose gels (0.8%, 2% and 4% agarose) were purchased from Invitrogen for electrophoresis. Nucleotide removal, gel extraction, PCR purification, and plasmid miniprep
Table 4. PCR primers and oligonucleotides for cloning bacteriophage constructs

<table>
<thead>
<tr>
<th>Primer Set</th>
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<th>DNA Sequence</th>
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<td>BamHI</td>
</tr>
<tr>
<td></td>
<td>FCFWKCTCT Peptide</td>
<td>CACTCGGCCGACCGGGCTTTCTGCTTTCGAAACCTGCACCAGGGGCGCTGGGGCCGAA</td>
<td>BglII</td>
</tr>
</tbody>
</table>
and midiprep kits were purchased from Qiagen (Valencia, California).

**AAVP construction and preparation – Polymerase chain reaction**

Several steps during cloning and production of the targeted AAVP-TNF required amplification of a DNA fragment by PCR. Cloning pBKS/AAV required PCR amplification of the polyadenylation signal sequence and the 3’ ITR using primer set number 1 (**Table 4**). The PCR reaction mix utilized up to 50 nanograms (ng) of template DNA and required use of 7.5 percent DMSO in the reaction mix for proper amplification. In addition, the PCR mix contained one microliter of each primer (10 picomole per microliter (pmol/µl)), 3 microliters of a stock dNTP mixture at a concentration of 2.5 millimolar (mM) for each dNTP, a GoTaq reaction buffer volume of 20 percent, and up to 2.5 units of GoTaq polymerase. The optimized PCR thermocycler conditions required an initial denaturation at 95 °C for two minutes followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 60 °C for 30 seconds, and extension at 72 °C for one minute and then a final extension of 10 minutes at 72 °C. The PCR product was purified using the QIAquick PCR Purification Kit.

Replacement of the original CMV promoter from pAAV-MCS-RR with the CMV promoter from the pcDNA3.1 plasmid required amplification of the CMV promoter DNA sequence with PCR primer set number 2 (**Table 4**). The reaction mix for this PCR utilized 40 ng of template DNA in addition to one microliter of each primer from a stock concentration of 10 pmol/µl, two microliters of dNTPs from a stock concentration of 2.5 mM for each dNTP, a reaction buffer volume of 10 percent, and 0.5 microliters of *Taq* DNA polymerase in a total reaction volume of 20 microliters. In addition to an initial denaturation at 94 °C for 2 minutes and a final extension period of 10 minutes at 72 °C, amplification of the PCR product required 30 cycles of denaturation at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds, and extension at 72 °C for 1 minute.

Preparing the TNF gene for integration into fAAV utilized PCR primer set number 4 (**Table 4**). Two microliters of plasmid DNA containing the TNF DNA was added to 0.5 microliters dNTPs, 1 microliter of each primer from a stock concentration of 10 pmol/µl, 0.3 microliters of *Taq* DNA polymerase, and 4 microliters of GoTaq buffer in a 20 microliter reaction volume. The thermocycler settings were an initial denaturation at 95°C for 3 minutes; 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 60 °C for 60
seconds, and extension at 72 °C for 60 seconds; and a final 3 minutes at 72 °C. This PCR protocol was also used to confirm the status of the TNF gene after ligation and throughout the generation and expansion of the resulting AAVP particles.

Incorporating the octreotide amino acid sequence (FCFWKTCT) into the pIII gene retained from fUSE5 required primer set number 5 (Table 4) to convert the oligonucleotide encoding the peptide sequence to double-stranded DNA. The PCR master mix components and PCR settings are described in detail by Christianson et al. [1135]. The oligonucleotide was resuspended to a stock concentration of 100 pmol/µl. Conversion of the single stranded template DNA to double stranded DNA required 0.5 microliters of the stock oligonucleotide suspension in addition to 1 microliter of each primer from a stock concentration of 10 pmol/µl, 0.5 microliters of the dNTP mix, 0.4 microliters of DMSO, 0.3 microliters of Taq DNA polymerase, and a GoTaq reaction buffer volume of 20 percent in a reaction volume of 20 microliters. The reaction conditions for the thermocycler were: 94 °C for 4 minutes; 30 cycles of 94 °C for 30 seconds, 60 °C for 30 seconds, 72 °C for 30 seconds; and 72 °C for 5 minutes.

To confirm the insert incorporation into the pIII gene of fUSE5, primer set number 6 (Table 4) was used. The PCR master mix for this reaction required a total volume of 20 microliters. This total included 4 microliters of GoTaq reaction buffer, 0.65 microliters of 10 mM dNTP mix, 0.65 microliters of 25 mM magnesium chloride (MgCl₂), 1 microliter of each primer from a stock solution of 10 pmol/µl, and 0.3 microliters of GoTaq polymerase. Additionally, to distinguish AAVP constructs from traditional phage particles, a small region (134 base pairs) of the TNF gene in fAAV-TNF and subsequent AAVP-TNF products were amplified simultaneously with the addition of 1 microliter of each primer (10 pmol/µl) from primer set number 7 (Table 4) in the same PCR conditions. The thermocycler conditions for this PCR required 3 minutes at 94 °C; 35 cycles of 10 seconds at 94 °C, 30 seconds at 60 °C, and 60 seconds at 72 °C; and a final 5 minutes at 72 °C. The reverse primer for the pIII insert into fUSE5 was used for sequencing reactions.

**AAVP construction and preparation – Annealing oligonucleotides**

To expand the distance between the BglII and PstI recognition sequences in the multiple cloning site (MCS) of fUSE5/MCS1, a pair of oligonucleotides was designed for
incorporation into the XhoI and HindIII restriction enzyme sites in the MCS of fUSE5/MCS1. DNA oligonucleotides encompassing the region were prepared (primer set number 3, Table 4). These sequences contained recognition sites for XhoI, BglII, NotI, AvrII, PstI, and HindIII, and were designed with “sticky ends” incorporated for ligation into the XhoI and HindIII sites of fUSE5/MCS1 without digestion. To prepare for the ligation reaction, the oligonucleotides were resuspended in water to prepare a 100 pmol/µl stock solution for each oligonucleotide. Ten microliters from each stock solution were allowed to anneal in a tube suspended in water heated to 95 °C for about 5 minutes, and then cooled with the water to room temperature for further use. The double-stranded DNA insert was then ligated into the fUSE5/MCS1 plasmid DNA digested with XhoI and HindIII to form the fUSE5/MCS plasmid (described below).

**AAVP construction and preparation – Digestion, ligation, and plasmid preparation**

Cloning steps throughout the generation of the targeted AAVP-TNF required restriction enzyme digestion and ligation of the digested DNA. Briefly, all digestions were incubated in the recommended buffers and indicated temperatures according to manufacturer instructions. Restriction enzyme digested DNA was purified by either oligonucleotide and enzyme removal with the QIAquick Nucleotide Removal Kit or run in an agarose gel and the appropriate sized band excised and subjected to gel purification with the QIAquick Gel Extraction Kit. Purified digested DNA was ligated with either T4 DNA Ligase in its buffer or the Rapid DNA Ligation Kit with a vector to insert ratio of one to three. The ligation products were introduced to competent cells as the protocols described and the transformation mixture plated on LB plates with appropriate antibiotics. Single colonies were picked and suspended in a 10 percent glycerol solution for storage and further analysis. Colonies verified to contain the appropriate conformation were grown in small cultures, and the plasmid DNA recovered from overnight cultures using the QIAprep Spin Kits.

To prepare the AAV components, the pAAV-MCS-RR and pBKS plasmids were grown in cultures of LB media and the appropriate antibiotics overnight (ampicillin; 100 µg/mL). The following day, plasmid DNA was recovered using the Qiagen Miniprep Kit. Double digestions with EcoRI and PstI were incubated in the recommended buffer.
(SuRE/Cut buffer H, Roche) at 37 °C according to manufacturer instructions. The digestion product for the plasmid digestions were run on an agarose gel and the appropriate bands excised and DNA recovered using the QIAquick Gel Extraction Kit. The DNA was ligated overnight with T4 DNA ligase and its appropriate ligation buffer using the protocol recommended by the manufacturer with a vector to insert ratio of one to three. The ligation reaction was used to transform XL1-Blue MR supercompetent cells according to manufacturer guidelines. Single colonies were recovered from the LB-ampicillin (100 µg/mL) plates following the transformation for analysis. Small volume cultures were grown overnight in LB-ampicillin (100 µg/mL) and plasmid DNA recovered using the Qiagen Spin Miniprep Kit. Plasmid DNA was analyzed for appropriate size, and restriction mapping using PstI and EcoRI restriction enzymes confirmed the incorporation of the insert DNA and generation of pBKS/5CMV. This intermediate vector was then used to generate pBKS/AAV.

The insert containing the polyadenylation signal and 3’ ITR (described in the section on PCR) and pBKS/5CMV vector DNA were digested with EcoRI and HindIII in the appropriate buffer (NEBuffer 2, New England Biolabs) at 37 °C per manufacturer instruction. The digestion products were gel purified and ligated as described above. The ligation reaction was used to transform XL1-Blue MR supercompetent cells per manufacturer instructions. Single colonies were picked and evaluated for incorporation and the correct configuration. Small cultures were grown overnight and plasmid DNA recovered with the Qiagen Spin Miniprep Kit. Incorporation of the insert was confirmed with PCR using primer set number 1 (Table 4) and DNA sequencing, and restriction enzyme mapping confirmed the size and orientation of the resulting pBKS/AAV* plasmid.

Replacement of the CMV promoter required amplification of the CMV promoter region from the pcDNA3.1 vector using primer set number 2 (described above). Purified insert DNA was initially incubated with the AvrII restriction enzyme in NEBuffer 2 (New England Biolabs) for 1 hour at 37 °C, then the BssHII enzyme was added and the digestion was transferred to 50 °C for an additional hour to complete the digestion. Vector DNA was digested in a double digest with both AvrII and MluI enzymes in the appropriate buffer (SuRE/Cut buffer H, Roche) at 37 °C for 1 hour. The digestion products were run on an agarose gel, the DNA extracted, and ligated using T4 DNA Ligase, per manufacturer.
instructions. Ligated DNA was used to transform XL1-Blue MR supercompetent cells according to the protocol provided by the manufacturer. Subsequent colonies were used to grow small cultures, and the plasmid DNA recovered. Restriction enzyme mapping with PstI and EcoRI enzymes was used to confirm the incorporation and generation of pBKS/AAV.

Preparation of the phage component required the reorganization of the fUSE5 and fMCS1 filamentous phage genomes. Plasmid DNA from both phage vectors was used for a double digestion with XbaI and BamHI restriction enzymes and the appropriate buffer (SuRE/Cut buffer A, Roche) at 37 °C. The digestion products were run on an agarose gel, and the appropriate sized bands excised with a blade for DNA recovery. T4 DNA ligase was used to ligate the digested DNA, according to manufacturer guidelines, to prepare fUSE5/MCS1. The ligated DNA was used to transform competent cells, and stock E. coli was prepared for further use. To replace the MCS, a large culture of fUSE5/MCS1 was grown overnight, and plasmid DNA was recovered using the Qiagen Plasmid Midiprep Kit. About 500 nanograms (ng) of plasmid DNA was double digested with XhoI and HindIII restriction enzymes and the appropriate digestion buffer (SuRE/Cut buffer B, Roche) according to manufacturer instructions at 37 °C. Digested DNA was run in an agarose gel and the appropriately sized band excised and gel purified using the Qiagen QIAquick Gel Extraction Kit. A ligation reaction was performed using purified digested fUSE5/MCS1 DNA and the annealed multiple cloning site insert DNA described above (in the Annealing oligonucleotides section) using T4 DNA ligase and its recommended buffer according to manufacturer instructions. Individual colonies were picked and evaluated and grown in small cultures overnight. Plasmid DNA was recovered with the Qiagen Spin Miniprep Kit and evaluated for incorporation of the insert sequence using restriction enzyme mapping to confirm formation of fUSE5/MCS.

Combining the AAV and phage components to generate the fAAV construct required double digestion of both plasmids with enzymes that form compatible ends upon digestion. Plasmid DNA from fUSE5/MCS was digested with BglII and PstI in the appropriate buffer (SuRe/Cut buffer H, Roche), while DNA from pBKS/AAV was digested with BamHI and SbfI in a buffer suitable for both restriction enzymes (NEBuffer 4, New England Biolabs), for 2 hours at 37 °C. The digestion products were run on an agarose gel, the appropriate bands excised, and digested DNA purified. The two digestion products were ligated with T4
DNA ligase, per manufacturer instructions, and the ligated DNA used to transform XL1-Blue MR supercompetent cells according to the protocol provided. The resulting colonies were evaluated for integration using restriction enzyme mapping to identify individual clones with the correct conformation of the fAAV plasmid.

The PCR product encompassing the TNF DNA sequence and containing XbaI and BamHI restriction enzyme recognition sequences was purified using the PCR Purification kit per manufacturer instructions. Four microliters of the recovered DNA was digested with XbaI and BamHI restriction enzymes in the recommended buffer (SuRe/Cut buffer A, Roche) overnight at 37 °C. Simultaneously, fAAV plasmid DNA was digested with BlnI and BglII restriction enzymes and NEBuffer 3 (New England Biolabs) overnight at 37 °C. The following day, the digestion products were cleaned with the Nucleotide Removal Kit, and the digested DNA ligated using the Rapid DNA Ligation Kit according to manufacturer instructions. The ligated DNA was heat-shocked into XL1 Blue MR supercompetent cells as described in the manufacturer protocol. The small culture was plated on LB-tetracycline (40 µg/mL) plates and incubated overnight at 37 °C. Individual colonies were picked with toothpicks and suspended in a 10 percent glycerol solution for validation by PCR using the primers for the TNF DNA (primer set number 4; Table 4). Small cultures were grown overnight, and the fAAV/TNF plasmid DNA was recovered the next day using the Qiagen Miniprep Kit.

Integration of the targeting sequence into the pIII gene of fAAV/TNF followed the traditional laboratory protocol for generation of phage libraries in fUSE5 [1135]. After amplification with PCR, the insert DNA was digested with the BglII enzyme as described by the manufacturer. The fAAV-TNF vector DNA was digested per manufacturer instructions with the SfiI restriction enzyme to remove the 14 base pair stuffer DNA in the pIII gene-encoding region. The digestion products were purified using the QIAquick Nucleotide Removal Kit to remove the digestion contaminants. The purified digestion products for the octreotide insert and fAAV-TNF vector were then ligated using the Rapid DNA Ligation Kit as described by the manufacturer. A vector to insert ratio of one to three was effective for successful ligation. The ligation product was introduced into XL1-Blue MR supercompetent cells using the transformation protocol provided by the manufacturer. One hundred microliters of the resultant transformation mixture was plated on LB-tetracycline plates and
incubated overnight at 37 °C. Individual colonies were recovered from the plates and suspended in a 10 percent glycerol stock. These colonies were subjected to PCR amplification of the pIII region using the primer set number 6 (Table 4) and sequenced to determine correct incorporation of the insert. Colonies with the correct conformation for Oct-AAVP-TNF were grown in small cultures overnight in LB-tetracycline (20 µg/mL), and plasmid DNA was then recovered from the cultures using the QIAprep Spin Miniprep Kit.

**AAVP construction and preparation – Particle amplification**

One microliter of the miniprep DNA for Oct-AAVP-TNF was electroporated into the electrocompetent *E. coli* strain MC1061. The transformed bacteria was added to one milliliter of SOC medium, shaken for one hour at about 225 revolutions per minute (RPM) at 37 °C, plated on LB-tetracycline plates, and incubated overnight at 37 °C. Single colonies were picked the following day and transferred to a 10 percent glycerol solution for storage.

To produce the original stock AAVP particles, a small volume of the colony suspension was amplified for phage particle production using the protocol described by Hajitou *et al.* [1174]. Briefly, two microliters of the colony suspension was transferred to 500 milliliters of LB-tetracycline (20 µg/mL) and grown overnight at 37 °C. After centrifugation to remove the bacterial debris, AAVP particles were purified from the resultant culture supernatant by precipitation with a polyethylene glycol (PEG) and sodium chloride (NaCl) solution, resuspended in PBS (pH 7.4), and repeatedly centrifuged to remove any remaining debris [1135].

A small volume (from two to five microliters) of the purified supernatant containing AAVP particles in suspension was then used to infect 500 microliters of K91Kan *E. coli* for amplification and phage particle production at a higher titer for experimental use. The bacterial infection was transferred to 500 milliliters of LB-kanamycin/tetracycline (100 µg/mL kanamycin and 20 µg/mL tetracycline), grown overnight, and phage particles recovered the following day [3, 1135, 1174]. Sequencing the insert sequence in fUSE5 using primer set number 6 (Table 4) throughout production and subsequent amplification of the phage particles was critical for ensuring the integrity of the phage preparation. Insertless control AAVP constructs derived from the empty fd-tet vector were amplified in parallel with the octreotide-targeted AAVP-TNF vector for experimental use.
**AAVP construction and preparation – Titration**

The Oct-AAVP-TNF suspension was titrated in parallel with insertless control AAVP by bacterial infection with K91Kan *E. coli* to determine the number of transducing units (TU). To do this, a serial dilution of the AAVP suspension was prepared, and the dilutions ($10^7$ to $10^9$) were infected with K91Kan *E. coli* for 30 minutes at room temperature. The infection was plated on LB-kanamycin/tetracycline (100 µg/mL kanamycin and 40 µg/mL tetracycline) plates and incubated overnight at 37 °C. Colonies were counted the following day and titers determined [1234]. Titers were analyzed over time and prior to individual experiments as necessary to ensure correct input and maintain experimental integrity.

**Generation of bacteriophage constructs**

Simultaneous to generation of the AAVP-TNF constructs described above, octreotide-targeted and insertless (fd-tet) [1235] control phage were also generated using the traditional laboratory protocol [1135], modified for incorporation of a known targeting sequence to provide an estimate of binding efficacy. The first step in generating the phage constructs was to convert the single stranded DNA oligonucleotide to double stranded DNA using PCR. The PCR master mix components and thermocycler settings are described above and detailed by Christianson *et al.* [1135]. Generation of the octreotide-targeted phage required conversion of the single-stranded oligonucleotide encoding the FCFWKTC insert (Table 4) to double-stranded DNA using primer set number 5 (Table 4).

After amplification with PCR, the octreotide insert DNA was digested with the BglII enzyme as described by the manufacturer. The fUSE5 vector was digested per manufacturer instructions with the SfiI restriction enzyme to remove the 14 base pair stuffer DNA in the pIII gene-encoding region. The digestion products were purified using the QIAquick Nucleotidase Removal Kit to remove the digestion contaminants and extraneous DNA. The purified digestion products for the inserts and fUSE5 vector were then ligated using the Rapid DNA Ligation Kit as described by the manufacturer in a vector to insert ratio of one to three.

The ligation product was introduced into XL1-Blue MR supercompetent cells using the transformation protocol provided by the manufacturer. One hundred microliters of the
resultant transformation mixture was plated on LB-tetracycline plates and incubated overnight at 37 °C for colony growth. Individual colonies were picked from the plates using toothpicks and suspended in a 10 percent glycerol stock. These colonies were subjected to PCR amplification of the pIII region using primer set number 6 (Table 4) and sequenced to determine correct incorporation of the insert. Colonies with the correct conformation were grown in small cultures overnight in LB-tetracycline (20 µg/mL), and plasmid DNA was recovered from the cultures using the QIAprep Spin Miniprep Kit. One microliter of the recovered miniprep DNA was electroporated into the electrocompetent E. coli strain MC1061. The transformed bacteria was added to one milliliter of SOC medium, shaken at 37 °C, plated on LB-tetracycline (40 µg/mL) plates, and incubated overnight at 37 °C. Single colonies were picked the following day and transferred to a 10 percent glycerol solution for long-term storage at -20 °C.

To produce the original stock phage particles, an individual colony was amplified for phage particle production using the protocol described by Hajitou et al. [1174]. Briefly, two microliters of the colony suspension is transferred to 500 milliliters of LB-tetracycline (20 µg/mL) and grown overnight at 37 °C. Phage particles were then purified from the resultant culture after centrifugation to remove the bacteria and precipitation with a PEG/NaCl solution [1135]. A small volume (from two to five microliters) of the stock phage was then used to infect 500 microliters of K91Kan E. coli for amplification and phage particle production at a higher titer for experimental use. The bacterial infection was transferred to 500 milliliters of LB-kanamycin/tetracycline (100 µg/mL kanamycin and 20 µg/mL tetracycline), grown overnight, and phage particles recovered the following day [1135, 1174]. Sequencing the insert sequence in fUSE5 using primer set number 6 (Table 4) throughout production and subsequent amplification of the phage particles ensured the integrity of the phage preparation over time.

The octreotide phage was titrated in parallel with insertless control (fd-tet) phage by bacterial infection with K91Kan E. coli to determine the number of transducing units. To do this, serial dilution of the phage suspensions were prepared, and the dilutions infected with K91Kan E. coli for 30 minutes at room temperature. The infection was then plated on LB-kanamycin/tetracycline (100 µg/mL kanamycin and 40 µg/mL tetracycline) plates and incubated overnight at 37 °C. Colonies were counted the following day and titers
determined. Titers were analyzed over time and prior to individual experiments as necessary to ensure correct input and ensure experimental integrity.

**Receptor Binding and Inhibition Assays**

**Binding assay**

Octreotide-targeted or insertless control phage and AAVP-TNF constructs were evaluated for the ability to bind SSTRs (SSTR1, SSTR2, SSTR3, and SSTR5), GST, and BSA immobilized on microtiter wells, as described previously [19, 21]. SSTR1 (catalog number H00006751-P01), SSTR2 (catalog number H00006752-P01), SSTR3 (catalog number H00006753-P01), and SSTR5 (catalog number H00006755-P01) recombinant proteins and GST epitope tag protein (catalog number P0001) were purchased from Novus Biologicals (Littleton, Colorado). Fifty nanograms of each SSTR or control protein was used to coat duplicate wells for each phage or AAVP. After allowing the proteins to adhere overnight, the wells are washed once with PBS (pH 7.4) and then a 3 percent bovine serum albumin (BSA; Sigma-Aldrich) solution in PBS was used to block non-specific binding during a 1 hour incubation at room temperature. The blocking buffer was removed and $10^9$ transducing units (TU) of either targeted or control phage or AAVP was added to each well and left at room temperature for about 2 hours. Unbound particles were then removed in 15 to 20 washes with PBS, while bound AAVP was recovered by bacterial infection for one hour with the addition of 100 microliters of K91Kan E. coli ($\text{OD}_{600} = 0.180 - 0.200$). The infection was transferred to 10 milliliters LB-kanamycin/tetracycline (100 $\mu$g/mL kanamycin and 20 $\mu$g/mL tetracycline) for 30 minutes and then 10 and 100 microliters were plated in duplicate on LB-kanamycin/tetracycline plates (100 $\mu$g/mL kanamycin and 40 $\mu$g/mL tetracycline). The plates were incubated at 37 °C overnight, and the colonies counted the following day.

**Inhibition assay**

Competitive inhibition with synthetic octreotide peptide was also evaluated for a dose-dependent effect, as described previously [19, 21]. Octreotide-targeted or control (fd-
tet) phage were evaluated for the ability to bind SSTR2, GST, and BSA immobilized on a 96-well plate after the addition of synthetic peptide. Briefly, after allowing the protein to adhere to microtiter wells overnight, the wells are washed once with PBS and blocked with 3 percent BSA as described for the binding assay above. Increasing concentrations of synthetic octreotide acetate peptide (catalog number H-5972; Bachem) were added to the adhered SSTR2 and control proteins for 30 minutes, followed by incubation with $10^9$ TU of octreotide or insertless control phage, washing, phage recovery, and quantification.

**CELL CULTURE**

The NCI-H727 (ATCC number CRL-5815) neuroendocrine tumor cell line was purchased from the American Type Culture Collection (ATCC; Manassas, Virginia). NCI-H727 cells were grown in RPMI 1640 medium containing L-glutamine supplemented with 10 percent fetal bovine serum (FBS), penicillin (100 units/mL), and streptomycin (100 µg/mL) at 37 °C in 5 percent carbon dioxide (CO$_2$). Prior to use, cells were detached with 5mM ethylenediaminetetraacetic acid (EDTA) in PBS and counted using the Bio-Rad TC10 Automated Cell Counter.

**FLOW CYTOMETRY ANALYSIS OF SSTR2 EXPRESSION**

Primary monoclonal antibodies against human SSTR2 (catalog number MAB4224) or mouse IgG isotype control (catalog number MAB0031) were purchased from R&D Systems (Minneapolis, MN). Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG secondary antibody (catalog number 115-095-146) was acquired from Jackson ImmunoResearch Laboratories (West Grove, Pennsylvania). NCI-H727 cells ($3 \times 10^5$ cells per tube) were incubated with a primary antibody against human SSTR2 or isotype control at a concentration of 25 µg/mL for one hour on ice in the dark. Cells were washed three times, using centrifugation at 350 xg to pellet the cells followed by resuspension in ice cold PBS. The FITC-conjugated goat anti-mouse secondary antibody was diluted in PBS (1:150),
and the cell pellet after the third wash was resuspended in 150 µL of the secondary antibody solution for 20 minutes on ice in the dark. The cells were washed three times, as before, and resuspended in 150 µL ice cold PBS. Flow cytometry analysis was performed immediately with the BD FACSCanto II flow cytometer. Analysis of FITC expression from the cells was performed with FlowJo (version 8.8.7) software.

**BINDING AND RAPID ANALYSIS OF SELECTIVE INTERACTIVE LIGANDS (BRASIL) CELL BINDING ASSAY**

Bovine serum albumin (BSA), dibutyl phthalate, and cyclohexane were purchased from Sigma-Aldrich. The protocol for BRASIL cell binding analysis has been described [21, 1135]. Following detachment with 5 mM EDTA, NCI-H727 cell suspensions were spun down at 500 xg (relative centrifugal force, RCF) for 5 minutes to pellet the cells and then resuspended in 1 percent bovine serum albumin in minimum essential medium (BSA/MEM) at 10⁶ cells per milliliter and kept on ice. Aliquots of the cell suspension were incubated with 10 µL of a 1 mM stock solution of synthetic somatostatin-14 peptide (catalog number H-1490; Bachem) on ice with occasional flicking to disperse the cell/peptide mixture. After 30 minutes, 10⁹ TU targeted or insertless control AAVP-TNF was added to the tubes and the suspension was incubated on ice for two hours. Following this incubation, the unbound AAVP was separated from the cells through a differential centrifugation technique. First, 200 µL of a 9:1 (v:v) mixture of dibutyl phthalate:cyclohexane (termed BRASIL oil) was added to microcentrifuge tubes with attached caps and a volume of 0.4 mL (VWR Scientific Products; West Chester, Pennsylvania). Then, 100 µL of the cell suspension was added to each tube, and the tubes were spun at 10,000 xg for 10 minutes. The cell pellets were carefully removed using microcentrifuge cutters (Bel-Art Products; Pequannock, New Jersey) over a 50 mL tube to allow the tips containing the cell pellets to fall into the large tube, and extraneous oil was removed from the tip by pipetting. To recover the bound AAVP, 200 µL K91kan *E. coli* (OD₆₀₀ = 0.180 - 0.200) was added to the tip and pipetted up and down to mix thoroughly. After an incubation of 1 hour, 20 mL LB-kan/tet (100 µg/mL kanamycin and 20 µg/mL tetracycline) was added to each 50 mL tube. Ten microliters and
100 µL of the LB suspension were plated in triplicate on LB-kan/tet plates (100 µg/mL kanamycin and 40 µg/mL tetracycline) and incubated overnight at 37 °C. The following day, colonies were counted to determine binding efficiency in the varying conditions.

**AAVP Internalization Assay**

Rabbit anti-fd bacteriophage antibody (catalog number B7786) was purchased from Sigma-Aldrich. Normal rabbit immunoglobulin G (IgG; catalog number 011-000-003), Cy3-conjugated goat anti-rabbit IgG secondary antibody (catalog number 111-165-045), and normal goat serum (catalog number 005-000-121) were purchased from Jackson ImmunoResearch Laboratories. To evaluate AAVP internalization, 5 x 10^4 cells were seeded per well in Nunc Lab-Tek II 8-well glass chamber slides (catalog number 154941; Fisher Scientific) and allowed to attach overnight at 37 °C in 5 percent CO_2_. The following day, 30 percent FBS supplemented minimal essential medium (FBS/MEM) was used to block non-specific binding for 1 hour at 37 °C. The cells were then incubated with 10^9 TU octreotide or control AAVP-TNF in 2 percent FBS/MEM for 2 or 24 hours. Negative control wells were incubated with 2 percent FBS/MEM alone. At each indicated time point, the AAVP suspension or medium was aspirated from each well, and the cells were washed five times with 10 percent BSA in PBS (BSA/PBS). The cells were then washed three times with glycine buffer (20 mM glycine, 150 mM NaCl; pH 2.3). This washing step required 3 minutes of slight shaking for each wash to remove bound phage particles from the cell surface. The cells were then washed three times with PBS and fixed with a 15 minute incubation in freshly prepared 4 percent paraformaldehyde in PBS. Following fixation, the cells were again washed three times with PBS, and one set of wells for each experimental condition was permeabilized with 0.2 percent Triton X-100 (catalog number T9284; Sigma-Aldrich) for five minutes. The cells were then washed five times with PBS and blocked with 5 percent normal goat serum in 1 percent BSA/PBS for 30 minutes. Immediately following the blocking, anti-bacteriophage antibody (1:500 dilution in 1 percent BSA/PBS) was added. Non-immune anti-rabbit IgG (1:1000 dilution in 1 percent BSA/PBS) was prepared for one set of wells as a negative control. After incubation for 1 hour, the wells were washed five
times with 1 percent BSA/PBS, and the secondary antibody (Cy3-conjugated goat anti-rabbit IgG in a 1:300 dilution in 1 percent BSA/PBS) was added to each well and the slides kept in the dark for 30 minutes. Following this incubation, the wells were washed five times with 1 percent BSA/PBS and fixed with fresh paraformaldehyde (4 percent in PBS) for 15 minutes. The cells were washed twice more with PBS and the slides mounted. Vectashield mounting medium with DAPI (4′, 6-diamidino-2-phenylindole; Vector Laboratories; Burlingame, California) was added to each well and a cover slip was secured in place with Cytoseal mounting medium to maintain integrity. The slides were allowed to set in the dark overnight at 4 °C prior to visualization. AAVP internalization into NCI-H727 cells was visualized with a fluorescence microscope.

**Transgene Expression In Vitro**

TNF gene expression analyses were carried out *in vitro* as described previously [1174, 1191]. Cells were seeded at a concentration of $5 \times 10^4$ cells per well in a 24-well plate in 0.5 mL complete medium and incubated overnight at 37 °C in 5 percent CO$_2$. Three wells were prepared for each experimental condition: targeted AAVP, insertless control AAVP, and medium alone without AAVP. When the cells reached about 50 percent confluency, $5 \times 10^{10}$ transducing units of octreotide-targeted or insertless control AAVP-TNF was added in 200 µL RPMI 1640 medium alone without FBS to ensure transduction efficiency [1174]. The plate was tilted every 15 minutes for 4 hours, after which 0.3 mL of complete medium was added to the wells for a total of 0.5 mL. The cells were incubated at 37 °C in 5 percent CO$_2$ for 10 days. Every 2 days, the medium was refreshed for the cells and the culture supernatant collected. The collected supernatant was immediately transferred to -20 °C for storage until the levels of secreted TNF in the liquid could be measured to determine gene expression levels in the transduced cells.

Culture supernatant was evaluated using an enzyme-linked immunosorbent assay (ELISA) kit for human TNF [1184, 1191]. This ELISA kit (catalog number KHC3012, Invitrogen) uses anti-human TNF monoclonal antibody coated wells to evaluate samples in a sandwich ELISA compared to standards. The manufacturer instructions and assay protocol
were followed. To determine the sample values, first a scatter plot was prepared in a Microsoft Excel spreadsheet plotting the values of the logarithm to base 10 (log) of the standard concentration values against the log of the average optical densities (OD) for each standard value after subtraction of the value for the blank well. A linear trendline was established in the graph and the equation of the line determined from the software. To evaluate the samples, first, the value of the blank well was subtracted from all OD values for the plate. Then, the log of each of the values was incorporated into the linear equation and the value of “x” was found for each value, and the inverse log was then calculated for each sample to determine the value of TNF in each sample.

**Animal Care**

Pdx1-Cre;Men1 f/f transgenic mice were maintained in accordance with governing standards and all experiments were conducted according to protocols approved for institutional compliance.

**Measurement of Serum Insulin Levels**

Serum insulin was measured as described previously [54, 1233]. Following an 18 hour fast, blood was collected via retro-orbital bleeding using 0.5 mol/L EDTA as an anticoagulant for analysis with the Ultrasensitive Mouse Insulin ELISA kit (Mercodia, Inc.; Uppsala, Sweden), according to the manufacturer’s instructions.

**AAVP Homing In Men1 Transgenic Mice**

Three 12-month-old Men1 mice (Pdx1-Cre;Men1 f/f) with known tumors (based on measurement of serum insulin levels as described above) were used per experiment. Control animals were analyzed in parallel. Octreotide-targeted AAVP-TNF \(10^{11} \text{ TU}\) was
administered intravenously into the tail vein of Men1 or control mice under anesthesia. After the AAVP was allowed to circulate for 4 days, the mice were sacrificed and organs and tissues of interest were recovered for analyses. Resected pancreas, including any tumors, and normal tissues (brain, heart, lung, spleen, liver, skeletal muscle, and kidney) were flash-frozen and stored at -80°C. Small sections of each tissue sample were formalin-fixed and paraffin-embedded for immunofluorescent analysis. Peripheral blood was also collected from all animals at days 0 and 4. To evaluate AAVP homing, the paraffin sections (5 μM) were analyzed by dual immunofluorescence. The sections were incubated with 5 percent normal goat serum in 2.5 percent BSA/PBS for 1 hour at room temperature to block non-specific binding, followed by incubation with rabbit anti-phage (1:1000 dilution; Sigma-Aldrich) and guinea pig anti-swine insulin (1:500 dilution; DAKO) primary antibodies overnight. After washing, the slides were incubated with goat anti-rabbit Alexa Fluor 488 and goat anti-guinea pig Alexa Fluor 647 (both 1:200 dilutions; Invitrogen) for 45 minutes in the dark. The slides were then mounted in Vectashield mounting medium with DAPI (Vector Laboratories) and allowed to set. Images were taken using a fluorescence microscope with an attached camera [1184, 1189, 1191, 1233].

**TNF Expression In Vivo**

*Gene expression assay – ELISA*

TNF expression in vivo was evaluated by ELISA, as described above and in previous reports [1184, 1191]. Total cell lysates from frozen pancreas including any tumors, control tissues (brain, heart, lung, spleen, skeletal muscle, liver, and kidney), and peripheral blood recovered before and after treatment were prepared in lysis buffer (50 mM Tris-HCl, pH 7.4; 140 mM NaCl; 0.1% sodium dodecyl sulfate; 1% NP-40; and 0.5% sodium deoxycholate) containing a protease inhibitor cocktail (Roche). The lysates were cleared by centrifugation (13,000 rpm for 10 minutes), and protein was quantified with a protein assay reagent (BioRad; Hercules, California). 100 micrograms of total protein was assayed for the presence of human TNF using ELISA (catalog number KHC3012, Invitrogen) and quantified as described above.
**Gene expression assay – Real-time RT-PCR**

Human TNF messenger RNA (mRNA) was measured by real-time reverse transcription (RT) PCR with primer and probe sequences specific for the human TNF sequence present in octreotide-targeted and insertless control AAVP-TNF (sense primer: 5’-TTCAGCTCTGCATCGTTTTG-3’, antisense primer: 5’-CTCAGCTTGAGGGTTTGC-TACA-3’, and probe 5’-FAM-TTCTCTTGGCGTCAGATCATCTTCTCGAAC-TAMARA-3’) [1184, 1189]. Total RNA was extracted from the frozen tissues (pancreas with any tumor, brain, heart, lung, spleen, liver, muscle, and kidney) as well as tissues recovered from laser capture microdissection (described below) with Trizon (Invitrogen) and the RNeasy Total RNA Kit (Qiagen). cDNA was first generated from RNA, and quantitative RT-PCR was performed with a Gene Amp 7500 Sequence Detector (Applied Biosystems/Invitrogen). Measurements of fluorescent signal intensity after standardization with an 18S ribosomal RNA (rRNA) internal control allowed quantification.

**Laser Capture Microdissection**

Three 5 µM thick paraffin sections were used for laser capture microdissection (LCM) with the Leica LCM Microsystem (Buffalo Grove, Illinois). Excised tissue was transferred to RNA lysis buffer solution for RNA extraction and analysis (described above).

**Statistical Analysis**

Statistical analyses were performed using the functions of the Microsoft Excel software. Error bars indicate the standard deviation of the mean. Significance was determined between indicated groups using an unpaired, two-tailed Student’s t test. p values less than 0.05 were considered statistically significant and are indicated in the charts with the following symbols: *, p < 0.05; **, p < 0.01; ***, p < 0.001.
RESULTS

To evaluate the hypothesis that octreotide displayed in the AAVP vector could recapitulate the binding activity of the native peptide and allow targeted gene delivery specifically to NET cells expressing SSTR2 required construction of an octreotide-targeted AAVP with TNF as a therapeutic reporter gene followed by evaluation of activity in vitro and in vivo. First, the construction strategy for generation of the Oct-AAVP-TNF will be described.

CLONING STRATEGY FOR AAVP

Generation of the octreotide-targeted AAVP gene delivery vector containing TNF required multiple steps and intermediate vectors. Both AAV and phage components were engineered to ensure correct orientation during construction and proper function of the resultant viral particles [1174, 1175]. Initially, the AAV components were prepared. The 5′ and 3′ inverted terminal repeats (ITRs), the cytomegalovirus (CMV) promoter gene cassette, and the polyadenylation (poly(A)) signal of AAV were recovered from a derivative of the pAAV-MCS plasmid, called pAAV-MCS-RR. The pAAV-MCS-RR plasmid had a new multiple cloning site (MCS) inserted between the PstI and EcoRI restriction enzyme sites of a pAAV-MCS commercial plasmid that provided additional cloning opportunities for later modifications.

To prepare the AAV-based vector controlled by CMV promoter (pBKS/AAV), the AAV components from pAAV-MCS-RR were transferred to the pBluescript cloning vector (pBKS: pBluescript II KS (-)) in a two-step cloning process. Generating the pBKS/AAV plasmid containing both 5′ and 3′ ITRs for AAV genome integration and the strong and ubiquitous CMV promoter, first required production of the intermediate pBKS/5CMV plasmid (4.3 kilobase (kb)). To do this, the pAAV-MCS-RR plasmid was digested with PstI and EcoRI restriction enzymes, and the resulting 1.3 kb DNA fragment carrying the 5′ ITR and the CMV promoter was subcloned into the PstI and EcoRI sites of pBKS (Figure 1).
Next, the fragment from pAAV-MCS-RR containing the poly(A) tail and the 3’ ITR (0.7 kb) was amplified using primer set number 1 (Table 4). These primers maintain the PstI and PvuII sites of the 3’ end for further use while incorporating a HindIII cut site. The newly created HindIII site and the EcoRI site maintained from the 5’ end were used to clone the
fragment into pBKS/5CMV. The resulting plasmid, pBKS/AAV* (5.0 kb), contains the 5′ ITR and 3′ ITR at both terminal regions of the CMV promoter gene cassette as well as the polyadenylation signal (**Figure 1**). Additionally, to ensure maximal gene expression levels were achieved, the original CMV promoter from pAAV-MCS-RR was replaced with another CMV promoter from the pcDNA3.1 (+) plasmid. The CMV promoter DNA was recovered from the plasmid using PCR primer set number 2 (**Table 4**). These primers were designed to incorporate restriction enzyme recognition sequences for BssHII and AvrII enzymes on the 5′ and 3′ terminal regions, respectively, for cloning into MluI and AvrII sites of the original pBKS/AAV* DNA (**Figure 1**). The pcDNA3.1 CMV promoter includes the complete enhancer region for CMV activity but does not contain the intron within the promoter, decreasing the size of the promoter region, while ensuring the transduction capabilities of pBKS/AAV.

The fUSE5 phage display vector has two SfiI restriction enzyme recognition sequences in the pIII gene for displaying peptides on the viral coat protein. However, fUSE5 does not contain an easily accessible sequence for integration of the large AAV sequence necessary to generate AAVP. However, another phage vector of the same lineage, fMCS1, does possess a MCS region in an untranslated region of the phage genome for cloning applications. The cloning strategy for the phage component of the AAVP vector, therefore, entailed combining the necessary elements of these two filamentous phage vectors to create a new phage display vector containing both the SfiI sites in the pIII coat protein from the fUSE5 vector and the multiple cloning site from the fMCS1 vector for cloning. The filamentous phage display vector fUSE5 was cut with BamHI and XbaI, and the resulting 3.9 kb DNA fragment was subcloned into the BamHI and XbaI sites of the fd-tet-based filamentous phage display vector, fMCS1, possible because of their shared lineage. The resulting phage genome containing the elements from both fUSE5 and fMCS1, designated fUSE5/MCS1 (8.9 kb), is shown in **Figure 2**. Additionally, although the MCS sequence from fMCS1 contains both the BglII and PstI restriction enzyme cut sites necessary for the incorporation of the AAV components into the phage genome, the recognition sites for these enzymes overlap in the original MCS. To overcome this potentially limiting factor, a stuffer DNA fragment was engineered for incorporation into the XhoI and HindIII sites of the MCS to extend the distance between the BglII and PstI recognition sites to ensure complete
digestion and increase ligation efficiency. Forward and reverse DNA primers encompassing the region were prepared for incorporation into the original multiple cloning site (Figure 2).

To clone the AAVP vector (fAAV), the AAV components from pBKS/AAV and the phage display vector fUSE5/MCS were combined. The fAAV phage display vector was generated by cloning the 1.8 kb fragment resulting from pBKS/AAV digestion with BamHI and SbfI into the BglII and PstI sites of the fUSE5/MCS vector. The resulting AAVP vector (fAAV; 10.7 kb) contains the 5’ and 3’ ITRs, CMV promoter, and polyadenylation signal of AAV and the filamentous phage display vector with two SfiI cut sites in the pIII gene for
incorporation of the targeting peptide ligand on the pIII coat protein (Figure 3). This “empty” AAVP vector is easily modifiable for integration of various genes suitable for imaging and therapeutic applications into the AvrII and BglII restriction enzyme sites of the MCS of the AAV region as well as incorporation of countless targeting peptides into the SfiI sites for cloning into the pIII gene for display on the pIII coat protein.

Construction of the AAVP for targeted delivery of TNF first required integration of the TNF gene cassette into the MCS remaining in the AAV segment of the fAAV vector. Oligonucleotides were designed for the amplification of the TNF DNA sequence as well as addition of restriction enzyme sites specific for XbaI in the sense primer and BamHI in the antisense primer (primer set number 2; Table 4). The plasmid containing TNF (pG1SiTNF, [1236]) was subjected to PCR amplification using primer set number 2 (Table 4) to amplify the TNF sequence. After digestion, the 0.54 kb DNA was subcloned into AvrII and BglII

Figure 3. Cloning scheme for generation of an AAVP vector (fAAV) for targeted gene delivery. The BamHI and SbfI digested fragment (1.8 kb) from pBKS/AAV was incorporated into the BglII and PstI restriction enzyme sites of the fUSE5/MCS phage display vector to create an “empty” AAVP vector, fAAV (10.7 kb), for targeted gene delivery.
sites of fAAV. The resulting AAVP vector (fAAV-TNF; 11.2 kb) contained the TNF gene cassette under control of the CMV promoter with two SfiI cut sites in the pIII gene of the phage display vector for incorporation of the targeting peptide ligand (Figure 4A).

To generate the octreotide-targeting AAVP for delivery of TNF (Oct-AAVP-TNF), an oligonucleotide was designed encoding the octreotide peptide sequence (FCFWKTCT) as well as BglII restriction enzyme sites (5′ - GCCNNNNNGGC - 3′) at both terminal regions (Table 4). This oligonucleotide was subjected to PCR amplification using primer set number 3 (Table 4) to generate a double-stranded DNA sequence from the single-stranded oligonucleotide. The resulting DNA was digested with BglII and then subcloned into the SfiI sites of fAAV-TNF, maintained from fUSE5, to create Oct-AAVP-TNF (11.2 kb; Figure 4B). The five variable nucleotides in the BglII recognition sequence restrict the ligation into the fAAV-TNF in only the appropriate orientation. The inclusion of cysteine residues in the pIII gene, when in multiples of two, produces viable viral particles with disulfide bond formation between the residues [1237]. The combination of these factors supported the correct formation of the octreotide peptide.

**Octreotide Displayed in a Bacteriophage System Binds Specifically to SSTR2**

Concurrent with the construction of the octreotide-targeted AAVP designed to deliver TNF, an octreotide-targeted phage was generated to determine binding affinity for SSTR2 and evaluate the feasibility of a phage construct targeted by a biologically active peptide, which had not been previously reported. Specific binding with the octreotide-targeted phage was evaluated in a binding assay against commercially available recombinant SSTR proteins immobilized on a microtiter plate. The recombinant proteins were tagged with glutathione-S-transferase (GST), so in addition to the experimental BSA control, immobilized GST was also used as a negative control. Octreotide-targeted phage bound specifically to SSTR2, with no observable binding above background levels evident for SSTR1, SSTR3, or SSTR5. Insertless control phage (fd-tet) revealed no binding to any SSTR or control proteins (Figure 5A).
Figure 4. Cloning strategy for construction of octreotide-targeted AAVP delivering TNF. (A) The TNF gene cassette was incorporated into the AvrII and BglII sites of the fAAV MCS to generate an AAVP vector containing TNF under control of the CMV promoter (fAAV-TNF; 11.2 kb). (B) The DNA sequence encoding the octreotide peptide sequence and BglII restriction enzyme sites at both terminal regions was subcloned into the SfiI sites in the pIII gene for display on the pIII coat protein, generating the targeted AAVP for delivery of TNF to SSTR2 (Oct-AAVP-TNF; 11.2 kb).
Figure 5. Octreotide-targeted phage binds SSTR2 specifically. (A) Recombinant SSTR proteins, GST, or BSA was used to coat duplicate wells of a microtiter plate. Octreotide-targeted or insertless phage was incubated with the immobilized proteins, and bound phage was recovered with bacterial infection and quantified. (B) Octreotide phage binding was inhibited with increasing concentrations of synthetic octreotide peptide. Recombinant SSTR2 protein was coated on wells of a microtiter plate. Prior to the addition of octreotide-targeted or insertless control phage, increasing concentrations of peptide were added to duplicate wells for each condition. Bound phage was recovered by bacterial infection and quantified. Values indicated are results from a representative example from three experiments. Error bars indicate standard deviation of the mean. The symbol *** indicates a value of $p < 0.001$. 
To confirm binding specificity, octreotide phage binding to SSTR2 was evaluated after administration of increasing concentrations of synthetic octreotide peptide. Binding was inhibited in a dose-dependent manner (Figure 5B). Calculations revealed the half maximal inhibitory concentration (IC$_{50}$) to be about 25 nM. These experiments with octreotide-targeted phage confirmed recapitulation of the binding activity of the native octreotide peptide with specificity for SSTR2 and encouraged continued construction of the octreotide-targeted AAVP.

Upon completion of the octreotide-targeted AAVP-TNF (Oct-AAVP-TNF), binding specificity of the AAVP was confirmed in a binding assay identical to the assay previously used for the analysis of phage binding. Again, binding was observed for SSTR2 alone, while SSTR1, SSTR3, SSTR5 and the control proteins, GST and BSA, showed no binding. Insertless control AAVP-TNF revealed no binding to any SSTR or control proteins (Figure 6). In addition to the sequencing analysis of the targeted AAVP construct, the results of the binding assay confirm the correct incorporation and orientation of the octreotide peptide.

![Figure 6](image-url)

**Figure 6. Octreotide-targeted AAVP-TNF binds SSTR2 specifically.** Recombinant SSTR proteins tagged with GST, GST alone, or BSA were used to coat duplicate wells of a microtiter plate. Octreotide-targeted or insertless control AAVP-TNF was incubated with the immobilized proteins, and bound phage was recovered with bacterial infection and quantified. Values indicated are results from a representative example of three experiments. The Error bars indicate standard deviation of the mean. symbol *** indicates a value of $p < 0.001$. 


**Oct-AAVP-TNF Activity in a Cell Line Expressing SSTR2**

To evaluate Oct-AAVP-TNF activity in vitro, the NCI-H727 NET cell line expressing SSTR2 was used [1118, 1238]. SSTR2 expression on the cell surface was confirmed in the NCI-H727 cell line using fluorescence-activated cell sorting (FACS) flow cytometry analyses with an anti-human SSTR2 antibody. After incubation with the anti-SSTR2 or isogenic control antibody, FITC-conjugated secondary antibody was added for fluorescence sorting with a flow cytometer. Compared to the isogenic control antibody, the H727 cells exhibited increased FITC expression corresponding to an increase in anti-SSTR2 antibody binding (Figure 7). Gating the software parameters for analysis of single, live cells allowed evaluation of a similar cell population in the various experimental and control

![Histogram](image)

**Figure 7. NCI-H727 cells express SSTR2.** NCI-H727 cells were incubated with an antibody specific for human SSTR2 or isotype-matched control (mouse IgG) monoclonal antibody followed by a FITC-labeled goat anti-mouse secondary antibody. This histogram plot shows the intensity of FITC expression determined by flow cytometry. Cells from each experimental group were gated for inclusion of single, live cells from the total cell population. The level of FITC expression (x-axis) was compared for the SSTR2 antibody experimental group and controls in the same plot. There is a dramatic shift in the anti-SSTR2 population (red) compared to controls.
conditions, and incubating the cell suspension on ice limited receptor internalization and allowed evaluation of SSTR2 expression on the cell surface.

AAVP functionality requires first binding and then internalization into the cells expressing the receptor of interest to mediate AAV integration into the cellular genome and transgene expression. Both of these cellular activities were evaluated in the NCI-H727 cell line expressing SSTR2. Biopanning and rapid analysis of selective interacting ligands (BRASIL) cell binding studies allowed evaluation of AAVP binding to receptors displayed on the cell surface. Using the NCI-H727 cell line, we evaluated binding of targeted and control AAVP-TNF in the presence or absence of synthetic somatostatin peptide. Binding with the octreotide-targeted AAVP was significantly increased compared to the insertless control AAVP. Incubating the NCI-H727 cells with synthetic somatostatin peptide prior to the addition of AAVP to the cell suspension eliminated Oct-AAVP-TNF binding to NCI-H727 cells (Figure 8).

We next evaluated the ability of Oct-AAVP-TNF to internalize into cells using the NCI-H727 cell line. Octreotide-targeted, but not insertless control, AAVP-TNF was found to internalize into the cells at both two and 24 hours (Figure 9). Analysis of secondary antibody alone and an IgG control revealed no fluorescence, indicating no non-specific AAVP binding. Lack of immunofluorescence in the non-permeabilized cells confirmed that multiple washes with a glycine buffer were sufficient to eliminate bound AAVP from the cell surface and indicated the fluorescence in the permeabilized cells was due to internalized AAVP alone. Fluorescence was observed in the cytoplasm of the majority of cells after administration of Oct-AAVP-TNF, with increasing accumulation observed at the later time point. In addition to the clathrin-mediated endocytosis AAVP undergoes upon receptor binding [1183, 1186], SSTR internalization also occurs due to exposure to circulating ligands, especially at high concentrations [728, 729, 732, 733, 1239, 1240]. The dual modes of internalization could be responsible for the large percentage of cells with some evidence of fluorescence indicating the presence of the AAVP particles.

After confirmation of receptor binding and internalization, the NCI-H727 cells were evaluated in an experiment to confirm the transduction capabilities of the AAV component of the Oct-AAVP-TNF. Because TNF is a secreted protein, incubating the cells with either octreotide-targeted or insertless control AAVP allowed for an analysis of TNF expression
over time. Cell culture medium collected every two days was analyzed at the conclusion of the 10 day experiment to reveal increased TNF expression over time in the octreotide-targeted, but not insertless control, samples (Figure 10). The level of TNF expression, analyzed with an ELISA for anti-human TNF, steadily increased over the 10 days in the octreotide wells, while no increase was observed in the wells incubated with insertless control AAVP-TNF. This was consistent with transgene expression observed in previous studies [1174, 1175, 1183]. No cytotoxic effects were evident in the cells during the study, and the cells remained confluent at the end of the ten day study, confirming previous reports that indicate a supplemental or synergistic agent must be added to the cells for an observable cytotoxic effect [1241-1243]. This is not necessarily the case in vivo where the cytotoxic activity of TNF is observed in endothelial cells of the tumor vasculature [1210, 1244].

Figure 8. Oct-AAVP-TNF binds NCI-H727 cells. Octreotide-targeted, but not insertless control, AAVP-TNF was able to bind NCI-H272 cells, and the addition of synthetic somatostatin peptide eliminated AAVP binding. After incubation of the cells with the AAVP in the presence of absence of synthetic somatostatin peptide, the cells and any bound AAVP were separated from unbound AAVP in the aqueous suspension with a single differential centrifugation through an organic phase to pellet in the tube. Bacterial infection of the cell pellet allowed quantification of bound AAVP in the various experimental conditions. Values indicated are results from a representative example of four experiments. Error bars indicate standard deviation of the mean. The symbol * indicates a value of $p < 0.05$ and *** indicates a value of $p < 0.001$. 

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**Figure 9. Oct-AAVP-TNF internalizes into NCI-H727 cells.** Octreotide-targeted, but not insertless control, AAVP-TNF was found to internalize into NCI-H727 cells after both 2 and 24 hours incubation. A primary antibody against bacteriophage, capable of recognizing the phage-based portion of AAVP, followed by a Cy3-conjugated goat anti-rabbit secondary antibody, was used to identify AAVP within the cells (red fluorescence). Nuclear staining with DAPI (blue) indicated the presence of cells among the various experimental conditions.
Figure 10. **TNF expression after transduction with Oct-AAVP-TNF increases over time.** Octreotide-targeted or control AAVP-TNF was used to transduce NCI-H727 cells. Compared to insertless control AAVP, the levels of secreted TNF found in cell culture medium 8 and 10 days after transduction in the octreotide group was significantly increased. Values indicated are results of a representative example of two experiments. Error bars indicate standard deviation of the mean. The symbol * indicates a value of \( p < 0.05 \).

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**Oct-AAVP-TNF Activity in the MEN1 Transgenic Mouse Model**

As described previously, the mouse model of MEN1 syndrome used in this study, forms insulin-secreting tumors, or insulinomas, in the islets of the endocrine pancreas. The tumor microenvironment of the Men1 transgenic mouse model provides an opportunity to
evaluate the activity of the octreotide-targeted AAVP-TNF in a system that mimics the vulnerable vasculature found in MEN1 patients. Pancreatic NETs in the Men1 mouse model are highly vascularized. In addition to increased density compared to control islets, blood vessels in pancreatic NETs of the Men1 mice demonstrate structural abnormalities, including dilation and intense tortuosity [54]. Tumors in the Men1 mouse model also express SSTR2 for targeting with the octreotide peptide displayed in AAVP [1233]. Increased islet vascularity in Men1 transgenic mice correlates with both insulinoma development and disease progression [54]. These studies confirmed the suitability of the Men1 mouse model for targeted gene delivery using Oct-AAVP-TNF.

Insulinomas are functioning tumors with a measurable increase in serum insulin levels evident in these mice as the tumor progresses [54]. Men1 transgenic mice were evaluated prior to the initiation of the study to confirm the presence of an insulin-secreting tumor in the animals. Compared to control mice, the Men1 transgenic mice had significantly increased serum insulin levels, indicating the presence of functioning NETs (Figure 11). With the confirmation of tumor status, AAVP was administered via tail vein injection and allowed to circulate for 4 days. After this time point, the tissues were recovered and first

![Figure 11. Pancreatic NETs of the Men1 transgenic mice are functioning insulinomas.](image-url)

Compared to control mice of the same age, 12-month-old Men1 transgenic mice have increased levels of circulating insulin, indicating the presence of insulin-secreting tumors in the pancreas. Error bars indicate standard deviation of the mean. The symbol *** indicates a value of $p < 0.001$. 

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analyzed for AAVP homing. Octreotide-targeted AAVP-TNF was found to localize specifically in the insulinomas of the Men1 mice, but was not found in the islets of control mice (Figure 12). Evaluation of normal organs and tissues recovered from the Men1 transgenic mice revealed the presence of anti-phage fluorescence within the spleen, liver, and kidney (Figure 13), consistent with previous studies [30, 1144]. The amount of expression appears to be decreased compared to both earlier studies and previous laboratory experience, likely as a result of the extended time frame in our study compared to earlier reports with localization studies done at time points of 24 hours or less.

Transgene expression was also evaluated at both RNA and protein levels to evaluate the transduction capabilities of Oct-AAVP-TNF. First, an ELISA assay to evaluate TNF protein expression in the pancreas, including the tumors present, and control organs identified TNF only in the pancreases of the Men1 transgenic mice but not the pancreases of the control animals. In addition, no TNF was found in the control organs of from either

![Figure 12. Oct-AAVP-TNF localizes to insulinomas in the Men1 transgenic mice but not islets in the control mice.](image)

Using immunofluorescence, evidence of AAVP homing, expressed as green fluorescence associated with anti-phage antibody binding, was observed in the insulin-secreting tumors of Men1 transgenic mice. Anti-insulin staining, indicated in red, indicated the endocrine tissue.
The lack of TNF expression in the organs of the reticuloendothelial system (RES; or mononuclear phagocyte system (MPS)), including the spleen, liver, and kidneys was indicative of a lack of cellular transduction in these organs. Although the AAVP particles are commonly identified in these organs in phage homing studies, and our homing as well (Figure 13), a lack of transgene expression from these organs confirms previous reports that this accumulation is a transient artifact of particle processing, degradation, and excretion in vivo [30, 1144, 1191, 1245]. Of particular interest is the lack of TNF expression in the spleen, since this organ has been shown to be positive for SSTR2 expression [1246].

Using a quantitative real-time RT-PCR analysis, we were able to measure TNF RNA levels and confirm the results of the ELISA analysis, with TNF expression only evident in

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**Figure 13.** Oct-AAVP-TNF is found in the organs of the RES, but not the negative control tissues of the brain, heart, lung, and muscle. Evaluating the control organs of the Men1 transgenic mice revealed evidence of octreotide-targeted AAVP (green fluorescence) in the spleen, kidney, and liver, in agreement with previous studies. Non-specific binding to negative control organs (brain, heart, lung, or muscle) was not observed.
appreciable amounts in the pancreas, but not control organs, of the Men1 mice. Laser capture microdissection (LCM) enabled evaluation of endocrine tumors and exocrine tissue separately to determine the origin of the pancreatic TNF expression. Inclusion of these values in the graph revealed the pancreas expression originated solely in the insulin-secreting tumors of the endocrine pancreas, with no TNF expression found in the exocrine tissue (Figure 15). Microdissection of the parathyroid from the thyroid enabled evaluation of TNF in an endocrine organ expressing SSTR2\(^4\) as a control for tumor specificity and not

\(^4\) http://www.proteinatlas.org/ENSG00000180616/normal
With this confirmation of Oct-AAVP-TNF localization to insulinomas in the Men1 transgenic mice and measureable TNF expression in the tumors following our brief study period, we concluded that the octreotide-targeted AAVP-TNF is suitable for targeted gene delivery to pancreatic NETs, as designed.

Figure 15. TNF in the pancreas of Men1 mice originates solely in the insulinoma. Using quantitative real-time RT-PCR, increased TNF expression was found in the pancreas compared to control organs. However, measuring the pancreatic tumor and exocrine tissue independently, after isolation and extraction by LCM, indicated TNF expression is significantly increased in the tumor, with no appreciable TNF expression originating the exocrine tissue. Values were determined from the analysis of duplicate samples of each tissue from each of the three Men1 transgenic mice. Error bars indicate standard deviation of the mean. The symbol * indicates a value of $p < 0.05$. 

endocrine localization and transduction with the Oct-AAVP-TNF.
CONCLUSIONS

A thorough description of the generation of an AAVP hybrid vector expressing a peptide with known biological activity has been provided. This octreotide-targeted AAVP was engineered for the delivery of TNF specifically to the NET cells expressing SSTR2. Rather than the traditional phage library panning and selection process to identify tumor-specific ligand/receptor pairs for targeted applications, this project validated a known ligand/receptor pair in the phage system as suitable for ligand-directed gene delivery to tumor cells specifically. Phage library screenings provide a method to identify receptors expressed in the vasculature, typically of a specific tissue or tumor. Further validation to confirm receptor identification and expression allows for generation of a targeted imaging or therapeutic agent by conjugation of a clinical agent to the identified ligand. However, as our study demonstrates, identification and rational selection of a known ligand/receptor pair for targeted gene therapy circumvents the use of combinatorial phage libraries for ligand discovery and receptor mapping of a specific tumor. After preparation of a phage construct targeted by the amino acid sequence of the octreotide peptide, a somatostatin analog used clinically for symptomatic relief of hormone hypersecretion and imaging of tumors overexpressing SSTR2, we confirmed the selective and specific binding to SSTR2 and then generated an AAVP hybrid vector for gene therapy targeted by the octreotide amino acid sequence for delivery of TNF. Evaluations in vitro revealed Oct-AAVP-TNF activity in NET cells expressing SSTR2. Cell binding, AAVP internalization, and transgene expression were described, demonstrating the activity of a phage construct when targeted by a known peptide, which had not been previously shown, and encouraging the evaluation of our agent in vivo. Confirmation of both ligand-directed homing to pancreatic NETs in the Men1 transgenic mouse model and TNF expression originating solely from the tumor showed activity in a model of human disease.

Validation of AAVP homing and TNF expression in vitro and in vivo indicated the potential clinical utility of our octreotide-targeted AAVP and revealed a new avenue for design of gene therapy vectors targeted by known peptide ligands to receptors overexpressed in tumors specifically. We were able to demonstrate specific targeting of the tumor,
significant cellular transduction and gene expression, and no non-specific AAVP binding or TNF expression. These results indicate further investigation is warranted using our Oct-AAVP-TNF in preclinical studies. The clinical course of this targeted gene therapy could feasibly proceed rapidly as a candidate for patients with tumors identified with imaging studies utilizing somatostatin receptor scintigraphy. The SRS imaging study could be considered an imaging biomarker or a companion diagnostic test for a theranostic approach of targeted drug delivery. Clinicians evaluate the applicability of octreotide or other somatostatin analogs for treating pancreatic NETs with imaging studies that typically utilize radiolabeled octreotide peptide [997]. Therefore, an extension of this reality indicates tumor homing and localization of our AAVP targeted with the octreotide amino acid sequence should be possible to the same cells and tumor sites revealed in the SRS imaging studies.

There are, however, caveats in tumor susceptibility regarding SSTR2 expression down-regulation in response to octreotide treatment over time [728]. This has been observed in the treatment of acromegaly in pituitary tumors where resistance to octreotide is evident as a decrease in the effectiveness of octreotide against hormone secretion corresponding to a loss of SSTR2 expression in a subset of patients [1247]. This phenomenon is feasibly avoidable with the use of somatostatin analogs with a broader receptor binding profile, rather than SSTR2 specifically. However, it is possible that the use of octreotide for targeted drug delivery, as we propose, rather than long-term symptomatic relief, could diminish this resistance, especially with the use of a combinatorial approach including our octreotide-targeted AAVP. The addition of agents capable of producing a synergistic effect by enhancing either AAVP transduction efficiency [1183, 1248] or diminishing the effects of inhibitor of apoptosis proteins (IAPs) [1184], could further increase the efficacy of AAVP. Furthermore, specific targeting of the tumor cells, as opposed to the tumor vasculature as is the case with peptides identified in “mapping” studies done with phage library panning in vivo, provides an opportunity for dual or even synergistic combinations of targeted therapy agents. Inclusion of an anti-angiogenic agent, possibly an RGD (Arg-Gly-Asp) targeted agent known to disrupt the vasculature and provide an anti-tumor response could be one combinatorial approach to explore [36, 1144, 1182-1184, 1189, 1191, 1209, 1249]. Islet-specific vascular zip-codes, previously identified and confirmed for tumor expression in NETs of the pancreas in an alternative model of islet tumor formation [298, 1137], could
also provide a pancreas-specific anti-vascular therapeutic option capable of supplementing tumor cell-specific octreotide activity.

Initially, however, a long-term therapy study is currently underway to evaluate the utility of the octreotide-targeted AAVP-TNF as an anti-tumor agent in the Men1 transgenic mouse model. Weekly administration of Oct-AAVP-TNF for four months will provide a basis for understanding the efficacy of the targeted therapy in a pre-clinical model mimicking human disease. Over the course of the study, serum insulin levels will be evaluated routinely in addition to immediately following the first injection. Because the Men1 transgenic mice produce functioning insulinomas, analysis of insulin, in addition to serial imaging studies, provides an additional measurement for analyzing the tumor response to treatment over time. Any biochemical response to the treatment will be supported with an analysis of the resected tumors for any decrease in size as well as markers of apoptosis that will be evaluated after the four month study. This would provide a new avenue of investigation towards the goal of identifying novel agents with activity in pancreatic NETs with the aim of not only the promotion of disease stabilization and diminished growth, but tumor regression [1250]. Oct-AAVP-TNF could have broader indications than octreotide biotherapy in pancreatic NETs as well. While octreotide biotherapy is not indicated clinically for the treatment of non-functioning tumors, as these tumors have no secretory-related syndrome, non-functioning pancreatic NETs do express SSTR2 and can be localized by SRS, making them susceptible to ligand-directed gene delivery in our AAVP.

Additionally, numerous tumors of neuroendocrine origin beyond the pancreas express SSTR2, making them possible targets for evaluation with our octreotide-targeted AAVP [735, 1251]. These tumors originate in organs of the endocrine system or in tumors with a known hormonal component, and the majority of these tumors have been evaluated for SSTR2 expression and octreotide avidity using the SRS imaging technique (Table 5).

Finally, there are multiple known biologically active ligands with their corresponding receptors overexpressed in numerous tumor types that are being evaluated clinically for their ability to act as clinical agents for targeted drug delivery [1252]. Numerous peptide hormones are candidates for display in our AAVP for targeted delivery of TNF to tumors and overexpressing the receptors corresponding to the hormone ligands. Gonadotropin-releasing hormone (GnRH or luteinizing hormone-releasing hormone
Table 5. SSTR2 expression and SRS sensitivity in various tumor types

<table>
<thead>
<tr>
<th>Location (organ, gland, etc.)</th>
<th>Tumor type</th>
<th>SSTR2 expression (% samples positive)</th>
<th>SRS detection (% tumors visualized)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenal</td>
<td>Pheochromocytoma</td>
<td>60 - 100</td>
<td>40 - 86</td>
<td>[650, 668, 671, 672, 675]</td>
</tr>
<tr>
<td></td>
<td>Ganglioneuroma</td>
<td>100</td>
<td></td>
<td>[668]</td>
</tr>
<tr>
<td>Breast</td>
<td>Carcinoma</td>
<td>25 - 90</td>
<td>74 - 75</td>
<td>[672, 675, 728, 1253]</td>
</tr>
<tr>
<td>Central nervous system (CNS)</td>
<td>Astrocytoma</td>
<td>82</td>
<td>67 - 100</td>
<td>[672, 675]</td>
</tr>
<tr>
<td></td>
<td>Meningioma</td>
<td>70 - 100</td>
<td>100</td>
<td>[672, 675, 1254, 1255]</td>
</tr>
<tr>
<td></td>
<td>Paraganglioma</td>
<td>92</td>
<td>94 - 100</td>
<td>[672, 675, 1256]</td>
</tr>
<tr>
<td>Kidney</td>
<td>Renal cell carcinoma</td>
<td>72</td>
<td>100</td>
<td>[1257]</td>
</tr>
<tr>
<td>Lung</td>
<td>Small cell lung cancer</td>
<td>50 - 57</td>
<td>63 - 100</td>
<td>[668, 672, 675, 1258]</td>
</tr>
<tr>
<td>Peripheral nervous system (PNS)</td>
<td>Neuroblastoma</td>
<td>65</td>
<td>89 - 100</td>
<td>[672, 675]</td>
</tr>
<tr>
<td>Pituitary</td>
<td>Adenoma (undefined/non-functioning)</td>
<td>55 - 100</td>
<td>71 - 86</td>
<td>[672, 675, 1247]</td>
</tr>
<tr>
<td></td>
<td>GH-producing</td>
<td>98</td>
<td>70</td>
<td>[672]</td>
</tr>
<tr>
<td></td>
<td>TSH-producing</td>
<td>100</td>
<td></td>
<td>[672]</td>
</tr>
<tr>
<td>Thyroid</td>
<td>Medullary carcinoma</td>
<td>38 - 100</td>
<td>58 - 71</td>
<td>[668, 672, 675, 1259]</td>
</tr>
<tr>
<td></td>
<td>Carcinoid (bronchial)</td>
<td>68</td>
<td></td>
<td>[649]</td>
</tr>
<tr>
<td></td>
<td>Carcinoid (ileal)</td>
<td>100</td>
<td></td>
<td>[649]</td>
</tr>
<tr>
<td></td>
<td>Carcinoid (ovary)</td>
<td>100</td>
<td></td>
<td>[668]</td>
</tr>
<tr>
<td></td>
<td>Carcinoid (rectum)</td>
<td>91</td>
<td></td>
<td>[668]</td>
</tr>
<tr>
<td></td>
<td>Carcinoid (small intestine)</td>
<td>100</td>
<td></td>
<td>[668]</td>
</tr>
<tr>
<td></td>
<td>Carcinoid (testis)</td>
<td>100</td>
<td></td>
<td>[668]</td>
</tr>
<tr>
<td></td>
<td>Carcinoid (thymus)</td>
<td>50</td>
<td></td>
<td>[668]</td>
</tr>
<tr>
<td></td>
<td>Carcinoid (undefined)</td>
<td>80 - 88</td>
<td>77 - 96</td>
<td>[41, 671, 672, 675, 880, 1260]</td>
</tr>
</tbody>
</table>
(LHRH)) receptor expression in breast and gynecological cancers in women and prostate cancer in men [1261] and neurotensin or bombesin receptors found in lung, colon, pancreatic, breast, and prostate cancers [1262-1265], are examples. Exploiting these known receptors overexpressed in tumors for targeted imaging and therapeutic applications has been studied extensively [1266]. However, the utility of a gene therapy approach could extend the efficacy of this drug delivery design by easing toxicity concerns and, as is the case with octreotide, exploit current clinical imaging standards to identify a patient population for therapeutic drug delivery.
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