Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine 653

Oncolytic Adenovirus Therapy of Neuroendocrine Tumors

JUSTYNA LEJA
Dissertation presented at Uppsala University to be publicly examined in Rudbecksalen, Rudbecklaboratoriet, Dag Hammarskjöld's väg 20, Uppsala, Friday, April 15, 2011 at 09:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English.

Abstract

Neuroendocrine tumors (NETs), originally described as carcinoids, represent a rare and heterogeneous group of neoplasms associated with intensive secretion of hormones, bioactive peptides and amines. Most of the patients are diagnosed at a late stage of disease, often with liver metastases. Surgery remains the main treatment to control metastatic disease, but is not curative. Oncolytic virotherapy represents a promising approach to treat cancer and different strategies have been exploited to restrict viral replication to tumor cells. We developed an oncolytic adenovirus based on serotype 5, Ad5[CGA-E1A], where the chromogranin A (CgA) promoter controls expression of the E1A gene and thereby virus replication. We found that Ad5[CGA-E1A], selectively replicates in NET cells and it is able to suppress fast-growing human BON carcinoid tumors in nude mice. The activity of Ad5[CGA-E1A] was not completely blocked in liver cells. We further suppressed virus replication in hepatocytes by targeting E1A with miR122, an miRNA specifically expressed in the liver. miRNAs bind to mRNA and induce its cleavage or translational blockage. By insertion of tandem repeats of miR122 target sequences in 3’UTR of E1A gene, we observed reduced E1A protein expression and replication arrest in miR122 expressing liver cells. The oncolytic potency of the miR122-targeted virus was not affected in NET cells. Since some NET and neuroblastoma cells express high levels of somatostatin receptors (SSTRs), we introduced in the virus fiber knob cyclic peptides, which contain four amino acids (FWKT) and mimic the binding site of somatostatin for SSTRs. The FWKT-modified Ad5 transduces midgut carcinoid cells from liver metastases about 3-4 times better than non-modified Ad5. Moreover, FWKT-modified Ad5 overcomes neutralization in an ex vivo human blood loop model to a greater extent than Ad5, indicating that the fiber knob modification may prolong the systemic circulation time. NETs represent a huge therapeutic challenge and novel diagnostic markers are needed for early detection and effective treatment of NETs. We have profiled primary tumors and liver metastases of ileocecal NETs, using Affymetrix microarrays and advanced bioinformatics. We have identified six novel marker genes and show high similarity between primary lesions and liver metastases transcriptome by hierarchical clustering analysis.

Keywords: adenovirus, virotherapy, oncolytic virus, neuroendocrine tumors, chromogranin A, somatostatin receptors, microRNA, novel biomarkers

Justyna Leja, Department of Immunology, Genetics and Pathology, Rudbecklaboratoriet, Uppsala University, SE-751 85 Uppsala, Sweden.

© Justyna Leja 2011

ISSN 1651-6206
ISBN 978-91-554-8022-6
urn:nbn:se:uu:diva-146966 (http://urn.kb.se/resolve?urn=nbn:se:uu:diva-146966)
"People never learn anything by being told, they have to find out for themselves."
Paulo Coelho

To my parents
Dla moich kochanych rodziców
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


Reprints were made with permission from the respective publishers.

\[1\] Copyright © 2007 American Association for Cancer Research

\[2\] Copyright © Leja et al. Open access-article distributed under the terms of the Creative Commons Attribution License.

\[3\] Copyright © 2009 Nature Publishing Group
Leja J. 2011. Oncolytic Adenovirus Therapy of Neuroendocrine Tumors

**Opponent:** Prof Akseli Hemminki, MD, PhD  
*Cancer Gene Therapy Group, Molecular Cancer Biology Program, Transplantation Laboratory, HUSLAB, Haartman Institute, Finnish Institute for Molecular Medicine, University of Helsinki and Helsinki University Central Hospital*

**Committee members:**  
Assoc Prof Catharina Svensson, PhD  
*Department of Medical Biochemistry and Microbiology, Uppsala University*

Prof Tommy Linné, PhD  
*Faculty of Veterinary Medicine and Animal Sciences, Swedish University of Agricultural Sciences*

Assoc Prof Peter Stälberg, MD, PhD  
*Department of Surgical Sciences, Endocrine Surgery, Uppsala University*

**Chairman:** Assoc Prof Angelica Loskog, PhD  
*Department of Immunology, Genetics and Pathology, Uppsala University*

**Supervisor:** Prof Magnus Essand, PhD  
*Department of Immunology, Genetics and Pathology, Uppsala University*

**Co-supervisor:** Assoc Prof Valeria Giandomenico, PhD  
*Department of Medical Sciences, Endocrine Oncology, Uppsala University Hospital*
Contents

Introduction ................................................................................................... 11
1. Neuroendocrine tumors ........................................................................ 11
   1.1 Classification ................................................................................. 11
   1.2 Neuroendocrine cells and their markers ........................................ 12
   1.3 Gastroenteropancreatic neuroendocrine tumors (GEP-NETs) ...... 12
   1.4 Detection and treatment of GEP-NETs ......................................... 13
   1.5 Neuroblastoma ............................................................................... 14
   1.6 Oncolytic viruses as therapeutic agents for GEP-NETs and neuroblastoma? ............................................................... 15
2. Adenoviruses ........................................................................................ 15
   2.1 Adenovirus – classification and structure ...................................... 15
   2.2 Adenovirus life cycle ..................................................................... 16
   2.3 Adenoviruses as gene therapy vectors ........................................... 19
3. Adenoviruses as Oncolytic Agents ....................................................... 20
   3.1 Tumor-specific replication ............................................................. 21
   3.2 Transcriptional targeting ............................................................... 22
   3.3 Chromogranin A promoter ............................................................ 22
   3.4 Post-transcriptional targeting ......................................................... 23
   3.5 Liver-specific miRNAs .................................................................. 24
   3.6 Transductional targeting ............................................................... 26
   3.7 Clinical trials with oncolytic adenoviruses .................................... 26
4. Immune Responses against Adenoviruses ........................................... 28
   4.1 Innate immune responses ............................................................... 28
   4.2 Adaptive immune responses .......................................................... 29
   4.3 Interactions with blood components .............................................. 30
Aims of Study ............................................................................................... 32

Methods ........................................................................................................ 34
   Proximity Ligation ................................................................................... 34
   Laser Capture Microdisseccion ................................................................. 35

Summary of Papers ....................................................................................... 37
   Paper I ...................................................................................................... 37
   Paper II ..................................................................................................... 37
   Paper III .................................................................................................... 38
   Paper IV ................................................................................................... 39
Future Perspectives .......................................................................................... 40
Acknowledgements .......................................................................................... 44
References ......................................................................................................... 46
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad</td>
<td>Adenovirus</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenoviral death protein</td>
</tr>
<tr>
<td>APLP1</td>
<td>Amyloid precursor-like protein 1</td>
</tr>
<tr>
<td>CAR</td>
<td>Coxsackie adenovirus receptor</td>
</tr>
<tr>
<td>CD</td>
<td>Cytosine deaminase</td>
</tr>
<tr>
<td>CEA</td>
<td>Carcinoembryonic antigen</td>
</tr>
<tr>
<td>CgA</td>
<td>Chromogranin A</td>
</tr>
<tr>
<td>CpG</td>
<td>Cytosine-guanoside nucleotide</td>
</tr>
<tr>
<td>CR1</td>
<td>Complement receptor 1</td>
</tr>
<tr>
<td>CR2</td>
<td>Constant region 2</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>DBF</td>
<td>DRE binding protein</td>
</tr>
<tr>
<td>DBP</td>
<td>DNA-binding protein</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DRE</td>
<td>Distal regulatory element</td>
</tr>
<tr>
<td>DRR</td>
<td>Distal regulatory region</td>
</tr>
<tr>
<td>DSG2</td>
<td>Desmoglein 2</td>
</tr>
<tr>
<td>EC</td>
<td>Enterochromaffin cell</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>FGF2</td>
<td>Fibroblast growth factor 2</td>
</tr>
<tr>
<td>FX</td>
<td>Factor X</td>
</tr>
<tr>
<td>GEP-NET</td>
<td>Gastroenteropancreatic neuroendocrine tumors</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>GMCSF</td>
<td>Granulocyte macrophage colony stimulating factor</td>
</tr>
<tr>
<td>HCV</td>
<td>Hepatitis C virus</td>
</tr>
<tr>
<td>HSP</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>HSPGs</td>
<td>Heparin sulfate proteoglycans</td>
</tr>
<tr>
<td>HSV-TK</td>
<td>Herpes simplex virus-1 thymidine kinase</td>
</tr>
<tr>
<td>HVR</td>
<td>Hyper variable region</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IRF3</td>
<td>Interferon regulatory factor 3</td>
</tr>
<tr>
<td>IS</td>
<td>Intervening sequence</td>
</tr>
<tr>
<td>ITRs</td>
<td>Inverted terminal repeats</td>
</tr>
<tr>
<td>kb</td>
<td>Kilo base pairs</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>LDCV</td>
<td>Large dense core vesicles</td>
</tr>
<tr>
<td>MAGE-D2</td>
<td>Melanoma antigen family D2</td>
</tr>
<tr>
<td>miRNA</td>
<td>Micro RNA</td>
</tr>
<tr>
<td>MLP</td>
<td>Major late promoter</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiating factor</td>
</tr>
<tr>
<td>NAbs</td>
<td>Neutralizing antibodies</td>
</tr>
<tr>
<td>NAP1L1</td>
<td>Nucleosome assembly protein 1-like 1</td>
</tr>
<tr>
<td>NE</td>
<td>Neuroendocrine</td>
</tr>
<tr>
<td>NET</td>
<td>Neuroendocrine tumors</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor κB</td>
</tr>
<tr>
<td>NK cell</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>NSE</td>
<td>Neuron specific enolase</td>
</tr>
<tr>
<td>PDGFR</td>
<td>Platelet-derived growth factor receptor</td>
</tr>
<tr>
<td>PET</td>
<td>Pancreatic endocrine tumor</td>
</tr>
<tr>
<td>PFV-1</td>
<td>Primate foamy virus 1</td>
</tr>
<tr>
<td>PKR</td>
<td>Protein kinase R</td>
</tr>
<tr>
<td>PLA</td>
<td>Proximity ligation assay</td>
</tr>
<tr>
<td>P-LISA</td>
<td>Proximity ligation assay in situ</td>
</tr>
<tr>
<td>PNMA2</td>
<td>Paraneoplastic antigen Ma2</td>
</tr>
<tr>
<td>PRR</td>
<td>Proximal regulatory region</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate specific antigen</td>
</tr>
<tr>
<td>pTP</td>
<td>Terminal protein</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated upon Activation, Normal T-cell Expressed and Secreted</td>
</tr>
<tr>
<td>Rb</td>
<td>Retinoblastoma</td>
</tr>
<tr>
<td>RCA</td>
<td>Rolling circle amplification</td>
</tr>
<tr>
<td>RGD</td>
<td>Arginine-glycine-aspartic acid</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SLMV</td>
<td>Synaptic-like microvesicles</td>
</tr>
<tr>
<td>SST</td>
<td>Somatostatin</td>
</tr>
<tr>
<td>SSTR</td>
<td>Somatostatin receptor</td>
</tr>
<tr>
<td>Syn</td>
<td>Synaptophysin</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular endothelial growth factor receptor</td>
</tr>
<tr>
<td>VMAT1</td>
<td>Vesicular monoamine transporter 1</td>
</tr>
<tr>
<td>VMAT2</td>
<td>Vesicular monoamine transporter 2</td>
</tr>
</tbody>
</table>
Introduction

1. Neuroendocrine tumors

1.1 Classification

Neuroendocrine tumors (NETs) are a rare and highly heterogeneous group of neoplasms that arise from the dispersed neuroendocrine system. Recent insights indicate that almost any epithelial stem cell is able to transdifferentiate into a neuroendocrine cell. Therefore, NETs can arise from neural elements, endocrine glands, cell clusters (e.g., pancreatic islets) and single cells (e.g., thyroid C-cells).

NETs encompass a large group of neoplasms. The term NETs most commonly refers to two groups described as: carcinoids (NETs originating from gastrointestinal tract and the bronchopulmonary system) and pancreatic neuroendocrine tumors (PETs). Different NETs are often discussed separately and represent various neoplasms such as: phaeochromocytoma, neuroblastoma, medullary carcinoma of thyroid, multiple endocrine neoplasia type 1 and 2, Merkel cell cancer, paraganglioma. NETs of the gastrointestinal tract and pancreas, despite behavioral and genetic differences, are often described together as gastroenteropancreatic neuroendocrine tumors (GEP-NETs), because of similarities in cell features.

The term carcinoid was introduced by the pathologist Obendorfer in 1907 as a descriptive name for what he considered to be a benign neoplasm in the ileum. The traditional classification of carcinoids is based on their embryological origin and divided into: foregut (lung, thymus, stomach, upper part of duodenum), midgut (lower duodenum, jejunum, ileum, appendix and cecum) and hindgut (colon, rectum) carcinoids. The World Health Organization has presented a new classification of GEP-NETs based on their malignant potential as: well-differentiated neuroendocrine neoplasm (low and intermediate grade, benign behavior, uncertain malignant potential), poorly-differentiated neuroendocrine carcinomas (high-grade, malignant behavior) and mixed exocrine-endocrine tumors. The term carcinoid remains in use, both in the official WHO classification of NETs of the lung and as a synonym for NETs of other sites. In this thesis, the term carcinoids refers to gastrointestinal (GI) neuroendocrine tumors. Bronchopulmonary carcinoids are classified separately.
1.2 Neuroendocrine cells and their markers

Neuroendocrine (NE) cells are generally diffusely distributed as single cells, cell clusters or entire glands. NE cells are specialized in production, storage and regulated secretion of hormones, bioactive peptides and amines. The secretory products are stored in large-dense-core vesicles (LDCVs) or small synaptic-like vesicles (SSLVs), which correspond to the synaptic vesicles of neurons. The secretory functions of these vesicles are complex and precisely regulated. Peptide hormones are first stored and modified in the trans-Golgi, then packaged inside secretory granules and released in response to stimuli.

The NE cells in the GI tract constitute the largest hormone-producing group of cells in the body. They derive from GI stem cells and there are at least 15 types of gut NE cells, including enterochromaffin (EC) cells, which function as sensors and regulators of secretion and smooth muscle contraction in the GI tract. EC cells are located in the gut mucosa, in small numbers, deeply embedded in the mucosal crypts. These cells secrete various hormones and peptides, including serotonin, melatonin, substance P, guanylin and granins.

Gastrointestinal NE cells share a variety of antigens with neural cells, usually named as neuroendocrine markers, which are associated with different cellular compartments. Chromogranin A (CgA), which belongs to the chromogranins family, is an acidic hydrophilic glycoprotein and a major component of the LDCVs. CgA serves as a multivalent precursor of several biologically active peptides. Serum CgA is used as a diagnostic and prognostic marker for various NETs. Synaptophysin (Syn) is an integral membrane glycoprotein located in the SSLVs. Neuron-specific enolase (NSE) is the best known cytosolic marker, while vesicular monoamine transporter 1 (VMAT1) and 2 (VMAT2) are important markers for cellular type identification, which have distinct tissue distribution and pharmacologic properties. CgA, Syn, NSE, VMAT1 and VMAT2 are critical for histopathological identification and classification. In addition to CgA, NE cells secrete various bioactive substances such as: histamine, gastrin, somatostatin, neuropeptide K, chromogranin B, which are used for more specific diagnostics to identify phenotype or genotype of NETs.

Recent studies on gene expression profiling have indentified novel markers, such as NAP1L1, MAGE-D2, APLP1 and PNMA2, which potentially may be used in the diagnosis and prognosis of gastrointestinal NETs.

1.3 Gastroenteropancreatic neuroendocrine tumors (GEP-NETs)

GEP-NETs are rare malignancies with an incidence rate of 2.5-5 cases per 100,000 per year. Their prevalence has increased during the last 35 years.
mainly due to the introduction of more sensitive diagnostic tools as well as overall increased awareness among physicians\textsuperscript{1,3,34}.

Most GEP-NETs are sporadic, but some of them may occur as a part of inherited syndromes. The clinical behavior of GEP-NETs is extremely variable, since they may be functioning (when hormone secretion induces a clinical syndrome) or non-functioning, ranging from very slow-growing tumors, which are the majority, to highly aggressive and very malignant tumors. In general, malignancy, excessive secretion of hormones and anaplasia are observed simultaneously\textsuperscript{35}. The overall 5-year survival for carcinoid tumors is about 67%, whereas for PETs it varies from 97% to as low as 30%\textsuperscript{9}. The majority of PETs are functioning tumors, exhibiting diverse symptomatology. Carcinoid tumors grow more slowly than PETs, are often small in size and highly vascularized. Since GEP-NETs are mostly asymptomatic or induce nonspecific symptoms, the majority of patients are diagnosed with metastatic disease. The most common metastatic sites include liver, lymph nodes and lungs\textsuperscript{36,37}. Carcinoid patients with liver metastases have an excess of serotonin in the systemic circulation, therefore 5-HIAA, a metabolic product of serotonin, is used as a diagnostic marker. Secretion of active substances may occasionally cause the so called “carcinoid syndrome” with specific symptoms such as: diarrhea, cutaneous flushing, bronchospasm and heart-failure. The carcinoid syndrome is primarily associated with midgut carcinoids\textsuperscript{1,38-40}.

1.4 Detection and treatment of GEP-NETs

Assessment of the location and extent of disease by numerous imaging techniques is crucial for the management of GEP-NETs. Moreover, novel imaging modalities are under investigation with a major goal of increasing the sensitivity for detection of micro-metastases\textsuperscript{9,35}. Complete resection of primary tumor and local lymph nodes can cure some patients with local disease. Since most patients are diagnosed with metastatic disease, surgical treatment often reduces symptoms and improves survival, but is not curative. Another means of tumor reduction is hepatic artery embolization, which is also effective to control tumor-related symptoms. Conventional chemotherapy, radiotherapy as well as biotherapy with $\alpha$-interferon have limited antitumor responses. Cytotoxic therapies are considered more effective in progressive and poorly differentiated PETs, than in gastrointestinal carcinoids, which have very low response rates\textsuperscript{36-38,41}.

The majority of NETs express somatostatin receptors (SSTRs), which are G-protein-coupled plasma membrane receptors. There are five subtypes of the receptor (SSTR1-SSTR5) which regulate various functions. Activation of SSTRs triggers their internalization and has antisecretory and antiproliferatory effect on NET cells\textsuperscript{42,43}. Somatostatin (SST), the natural ligand for SSTRs has a short plasma half-life and it is not clinically used.
Therefore, stable synthetic analogs and their radiolabeled versions have been developed for clinical applications and have become important diagnostic and therapeutic tools for the management of hormonally active GEP-NETs. The administration of SST analogs is highly effective in symptomatic improvement and tumor stabilization. Recently published studies have associated the anti-proliferative effect of SST analogs therapy with prolonged survival of patients with metastatic NETs.

GEP-NETs are highly vascularized and express high levels of vascular endothelial growth factor (VEGF), together with its receptor (VEGFR). Specific therapies targeting VEGF signaling pathways have been evaluated in GEP-NETs. Bevacizumab is a monoclonal antibody directed against VEGF and was one of the first treatment evaluated in GEP-NETs and associated with some objective tumor responses and improvement in progression-free survival. Tyrosine kinase inhibitors have been used to target not only the VEGFR but also the platelet-derived growth factor receptor (PDGFR), RET and c-Kit. The new drugs triggered stable disease and partial responses in some patients. Furthermore, inhibitors of mammalian target of rapamycin (mTOR) represent a second class of agents that have shown preliminary evidence of activity in GEP-NETs. The mTOR functions downstream of a number of tyrosine kinase receptors, including the VEGFR, and serves a central role in regulating cell growth.

1.5 Neuroblastoma

Neuroblastoma is a tumor of the sympathetic nervous system and is the most common extracranial solid tumor of childhood. Most primary tumors (65%) occur within the abdomen, with at least half of them arising in the adrenal medulla. Other common sites of disease include the neck, chest, and pelvis. The median age of diagnosis is 17.3 months, with 40% diagnosed as infants and 98% by 10 years of age. The biological hallmarks of neuroblastoma relate to acquired genetic abnormalities and some of them have prognostic features. The main genetic markers are amplification of MYCN, ploidy changes (DNA content) and partial deletions of chromosome 1 and 11 and gain of chromosome 17. These markers are useful to classify stratification risk of patients and their selection for the treatments. Neuroblastoma shows striking differences in outcome. Some subsets of tumors undergo spontaneous regression, while some can relapse and progress. Most children with low and intermediate risk neuroblastoma achieve remission via a combination of surgery, radiation and chemotherapy. In high-risk patients, due to the recurrent disease, long term survival is lower than 40%. Relapsed malignancies are often resistant to apoptosis induced by chemo-and radiotherapies and inhibition of apoptosis occurs at multiple levels. The high-risk disease remains a clinical challenge, especially if taking
into consideration that high-dose chemotherapy of these patients has reached a level where further intensification of cytotoxic treatment is not likely to increase cure rates\textsuperscript{59,60}.

1.6 Oncolytic viruses as therapeutic agents for GEP-NETs and neuroblastoma?

Despite recent progress in the management of metastatic GEP-NET and high-risk neuroblastoma, there is an unmet need for new therapies. Viruses that selectively replicate in and kill tumor cells may represent such a therapy. Many viruses, particularly RNA viruses, have a natural selectivity for replication in tumor cells. Others can be genetically modified to selectively replicate in tumors cells. Genetic modification may be particularly appropriate for DNA viruses, which are genetically more stable than RNA viruses.

2. Adenoviruses

2.1 Adenovirus – classification and structure

Adenoviruses (Ads) are medium-sized (90-100 nm in diameter) non-enveloped viruses, with an icosahedral protein capsid and 12 extruding fibers (Figure 1)\textsuperscript{61,62}. The linear, double-stranded DNA genome is 26-45 kb in size (Figure 2). Adenoviruses were first discovered in 1953, isolated from cultures of human adenoid tissues\textsuperscript{61}. Since then, over 50 human adenovirus (Ad) serotypes have been characterized and classified into seven species (A – G), based on immunological distinctiveness and genomic sequence\textsuperscript{63,64}. Species B has been further divided into three groups (AdB/1, AdB/2 and AdB/3), based on their receptor specificity\textsuperscript{65}.

Adenoviruses are ubiquitous in the human population, causing mostly mild respiratory infections\textsuperscript{62,64}. However, depending on the infecting serotype, they may cause various other illnesses, such as acute respiratory disease (species E), gastroenteritis (species A, F and G), epidemic keratoconjunctivitis (species D) and urinary tract infections (species B)\textsuperscript{64}. Members of species C (Ad2 and Ad5) have been shown to be endemic in parts of the world where they have been studied, and infection is usually acquired during childhood. Adenoviral infections can cause severe complications especially in immunocompromised patients.

The adenoviral capsid consists of 240 homotrimeric hexons on the faces and edges of the capsid and 12 pentons, composed of penton bases and extended trimerized fibers (Figure 1)\textsuperscript{62,66}. Based on the location, four kinds of hexons, designated H1 – H4 exists. Sixty H1 are associated with pentons, while the remaining hexons form “group of nine” on the 20 faces of the
icosahedrons. Up to nine hypervariable regions (HVR) are presented by the amino acid loops on the surface of the hexon and account for diversity between serotypes. The penton base has pentameric structure, with irregular folds on the top part formed by loops of Arg-Gly-Asp (RGD). The fiber has a long trimeric structure with three distinct regions: tail, shaft and knob. The capsids of most serotypes contain one type of fiber, except species F and G. Minor proteins (IIIa, VI, VIII and IX proteins) are also located within the capsid and have mainly stabilizing functions. Five proteins (V, VII, Mu, IVa2 and terminal protein TP) are associated with the genomic DNA. The 23K virion protease is situated in the virus core and plays important role during early stage of infection, virus assembly and release.

**Figure 1. Structure of adenovirus**

### 2.2 Adenovirus life cycle

Adenoviruses can infect both dividing and non-dividing cells in a wide variety of cell types and tissues. Entry of most Ads involves high-affinity binding of the Ad fiber knob to the primary cellular receptor, coxsackie-adenovirus receptor (CAR), which is a transmembrane protein with two immunoglobulin-like domains and is present in specialized intercellular junctions (Figure 3). The secondary interaction between the RGD motif on the penton-base of the virus and cellular α,β-integrins, leads to endocytosis into clathrin-coated pits. The virus fibers are cleaved off and vertex proteins (penton base, IIIa, VI, VIII, IX) are released, mediating the escape of virus from the late endosomes. Transport of partially degraded virus to the nucleus, involves the participation of dynein and microtubules. The Ad genome is transferred through the nuclear pore complex into the
nucleus, where histones and other factors bind to the Ad DNA and transcription is initiated.

Some serotypes are able to bind additional receptors to CAR, including immunoglobulin superfamily members (species C) and sialoglycoproteins such as GD1a glycan (species D). Species B Ads do not utilize CAR as primary receptor\textsuperscript{78,79}. Serotypes from group AdB/1 (Ad16, Ad21, Ad35, Ad50) nearly exclusively use CD46 as a primary receptor\textsuperscript{80}. CD46 is a ubiquitously expressed inhibitory complement receptor\textsuperscript{81}. Group AdB/2 (Ad3, Ad7, Ad14) share a common entry molecule known as desmoglein-2 (DSG-2), which is a calcium-binding transmembrane glycoprotein belonging to the cadherin family\textsuperscript{80}. In epithelial cells, DSG-2 is a component of the cell-cell junction structure\textsuperscript{82,83}. Group AdB/3 (Ad11) preferentially uses CD46 for infection, but also interacts with DSG-2 if CD46 is blocked. Moreover, cell entry of Ads from species B is mediated by macropinocytosis, although a small portion of viruses may be internalized by endocytosis\textsuperscript{63}.

The Ad replication cycle can be divided into an early and a late phase, which occur before and after DNA replication, respectively (Figure 3). Transcription takes place on both DNA strands\textsuperscript{61}. Furthermore, most genes have multiple open reading frames and/or differentially spliced products\textsuperscript{61}. The genome organization is presented in Figure 2, while a detailed description of adenoviral gene products and their functions is summarized in Table 1. The first adenoviral gene, to be transcribed is E1A, which transactivates expression of Ad genes from other regions and generates a favorable environment for viral replication, by inducing into the S phase of the cell cycle\textsuperscript{84}. DNA replication begins from both DNA termini and requires sequences within inverted terminal repeats (ITRs) as origins of replication\textsuperscript{85}.

![Figure 2. Transcription map of the adenovirus genome. The adenovirus genome is organized in early (red), intermediate (blue) and late (green) transcriptional units.](image-url)
Table 1 Adenovirus gene products and their functions

<table>
<thead>
<tr>
<th>Phase</th>
<th>Gene</th>
<th>Products and their function</th>
</tr>
</thead>
<tbody>
<tr>
<td>early</td>
<td>E1A</td>
<td>Inactivates pRB to release E2F - cell cycle deregulation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Transactivates viral promoters</td>
</tr>
<tr>
<td></td>
<td>E1B</td>
<td>55kD targets p53 and participate in transport of late viral mRNA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>19kD is a Bcl-2 homologue - anti-apoptotic</td>
</tr>
<tr>
<td></td>
<td>E2A</td>
<td>Preterminal protein (pTP) and DNA polymerase (pol) - DNA replication</td>
</tr>
<tr>
<td></td>
<td>E2B</td>
<td>Single-strand DNA binding protein (DBP) - DNA replication</td>
</tr>
<tr>
<td></td>
<td>E3</td>
<td>gp19kD inhibits MHC I expression</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10.4kD/14.5kD (RID complex) inhibits tumor necrosis factor (TNF) apoptosis, internalizes TNF receptor and degrades Fas ligand</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14.7kD inhibits TNF apoptosis, stabilized NFκB</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11.6kD (ADP) induces cell lysis</td>
</tr>
<tr>
<td></td>
<td>E4</td>
<td>Products: orf1, orf2, orf3, orf4, orf6 and orf6/7 modulate viral mRNA metabolism, promote virus DNA replication and block host protein synthesis</td>
</tr>
<tr>
<td>intermediate</td>
<td>IVa2</td>
<td>Initiate the major late promoter (MLP), which regulates late genes</td>
</tr>
<tr>
<td></td>
<td>IX</td>
<td>Non-coding RNA that stimulates translation of viral genes and blocks double stranded RNA activated protein kinase R (PKR) during interferon response</td>
</tr>
<tr>
<td></td>
<td>VAI</td>
<td>Non-coding RNA that blocks PKR during interferon response</td>
</tr>
<tr>
<td></td>
<td>VAlI</td>
<td>Non-coding RNA that blocks PKR during interferon response</td>
</tr>
<tr>
<td>late</td>
<td>L1-L5</td>
<td>Structural proteins: L1 (IIIA); L2 (penton base, V, VII); L3 (hexon, VI, virus protease); L4 (VIII); L5 (fiber)</td>
</tr>
</tbody>
</table>

Proteins produced after translation of viral mRNA transcripts are transported from the cytoplasm to the nucleus for particle assembly. Adenoviral DNA incorporation to the pro-capsid is mediated by the packaging sequence. Infection with Ad results in lysis of host cells and release of progeny viruses (Figure 3). Species C Ads accumulate adenoviral death protein (ADP) at the last stages of infection, which seems to play role in cytolysis. The species B Ads do not encode ADP and the exact mechanism of virion release is not yet determined.
2.3 Adenoviruses as gene therapy vectors

In the past, a majority of gene therapy applications were focused on replacement of nonfunctional genes in monogenic disorders \(^{86,87}\). For these purposes, the first generation of Ad vectors had their transgenes incorporated into the E1 region, rendering them replication-deficient. Additional deletion of the E3 region allowed introduction of up to 7.5 kb of foreign DNA \(^{61,88}\). These Ad vectors have had limited application in corrective gene therapy due to induction of immune responses, which reduces transgene expression.
and efficacy of vector re-administration. Subsequent deletion of Ad genes led to the generation of helper–dependent Ad vectors (also called “gutless” vectors), which only contain the ITRs and the packaging signal 89. Even though they are less immunogenic and support longer transgene expression, gutless Ad vectors still induce innate immune responses and do not escape pre-existing immunity 90. The immunogenic nature of Ads and strong transgene expression have made them rather suitable as vectors for immunotherapy and vaccination strategies 91,92. For example, Ad vector encoding CD40 ligand has been used in local immunotherapy of bladder tumors with encouraging results 93. CD40 ligand is a potent inducer of T-cell immune responses. Therapy with this vector was safe and successful in activating immune responses within the tumors.

In cancer gene therapy, replication-deficient Ads showed no significant therapeutic effect, mainly due to low transduction rates and no spread of Ad vectors within the tumor. Therefore, recent developments have been redirected from replication-deficient vectors to replicating Ads with oncolytic potential.

3. Adenoviruses as Oncolytic Agents

Virotherapy, i.e., the use of genetically modified viruses that replicate in and destroy tumors, is an emerging therapeutic approach for cancer. Adenoviruses are one of the most commonly used viruses as oncolytic agents, due to their favorable features and safety profile. Ads infect a wide variety of both quiescent and proliferating cells and have a naturally lytic replication cycle without integration of their genome into the host cell chromosomes. Manufacturing and handling of Ads benefit from the ease of virus production at high titers and the high stability of adenoviral particles 94-97.

Adenoviruses are able to replicate in tumor cells and spread within the tumor, which may result in an amplification of the input dose and more effective elimination of cancer cells. Adenoviral replication is not dependent on proliferative status of the tumor cells. This is important for targeting of cancer stem cells, which often present often low proliferation ratios 98-100. Moreover, Ads circumvent traditional therapy resistance mechanisms, since their killing properties are not dependent on the apoptotic property of the target cell 58. In addition to direct lysis at the end of the replicative cycle, oncolytic effect of Ads can be further enhanced through expression of toxic proteins and inflammatory cytokines 64,101.

Several strategies have been proposed to improve the outcome of oncolytic therapy. A combination of viruses with cytotoxic drugs, radiation therapy and targeted small molecules undergoes constant progress in order to achieve additive or synergistic effects. A strategy to use different viruses
together may also be an attractive option. Furthermore, recent studies have shown that oncolytic virotherapy may benefit from redirection and increase of immunological antitumor responses. Viruses are highly immunogenic and can often alter immunosuppressed tumor microenvironment and trigger infiltration of immune cells to the tumors. Immune responses directed against infected tumor cells may subsequently lead to adaptive immune responses targeting tumor-associated antigens as a form of in situ vaccination. Due to limited systemic delivery of viruses, especially in immunized host, new approaches focus on means to increase virus delivery to the tumor. One of them is to use tumor-trafficking carrier cells to hide viruses from immune system. This approach can be complementary and combined in the next generation therapies.

3.1 Tumor-specific replication

Application of viruses in oncolytic cancer therapy requires efficient transduction and replication of viruses in cancer cells to promote tumor cell killing. Since it is equally important to minimize damage and toxicity to healthy tissues, researchers have modified Ads to restrict Ad replication to cancerous cells. The first strategy aims at achieving tumor specific replication of Ads and it is based on mutations or deletions of certain viral genes, which can be complemented in tumor cells, but not in normal cells. The earliest and most known example is the dl1520 virus, also called ONXY-015. This Ad lacks the E1B-55kD gene, which inactivates the pro-apoptotic p53 protein and was intended to replicate specifically in p53-deficient tumors. However, different studies revealed that it is the late functions of E1B-55kD protein, namely the nuclear export of viral mRNAs and shutoff of host protein translation, that determine the tumor selectivity. Another example is the Δ24 adenovirus, in which a 24bp deletion in the CR2 region of the E1A gene results in a protein unable to bind to the retinoblastoma (Rb) tumor suppressor, a pivotal cell-cycle regulator protein. This modification precludes viral replication in cells with an intact G1-S phase checkpoint.

Restriction of viral infection and replication as well as improvement of the safety profile of oncolytic viruses can be further achieved by different mechanisms. Transcriptional and post-transcriptional strategies utilize phenotypic differences between tumor and normal cells to control viral replication. Transductional targeting involves genetic or chemical modification of capsid proteins in order to enhance specific infection of tumor cells.
3.2 Transcriptional targeting

Transcriptional control of adenoviral replication can involve insertion of cellular or artificial promoters or enhancers, which are selectively active in tumor cells. These elements are used mainly to replace the viral promoter, which controls expression of the E1A gene. The promoter for the gene of choice has to be regulated at the transcription level and its regulatory sequence should be characterized and functional outside the chromatin context. Moreover, promoter sequences need to retain their selectivity also within the Ad genome, which contains several elements for transcriptional control that may interfere with a foreign promoter. Regulatory eukaryotic DNA sequences known as insulators are commonly used in order to shield promoters from unspecific activation of viral enhancers. The choice of promoter is not restricted to genes that are specific or overexpressed in tumor cells. In the case of tumors arising from specialized tissues or cell groups that are dispensable, tissue-restricted expression will be sufficient to control virus replication.

The earliest example of transcriptional targeting was the use of the prostate-specific antigen (PSA) promoter to regulate prostate specific replication of the adenovirus CV706 [1]. Since then, various tissue-specific promoters have been investigated, for example: tyrosinase for melanoma therapy, osteocalcin for bone metastasis of prostate cancer or COX-2 for gastric cancer [12-14]. In addition, promoters of genes exclusively active in certain dividing or embryonic cells have been utilized, such as: telomerase reverse transcriptase, survivin, carcinoembryonic antigen (CEA) and α-fetoprotein [15-17].

3.3 Chromogranin A promoter

The human chromogranin A (CgA) gene is localized on chromosome 14 and the CgA transcript contains eight exons. It is selectively expressed in neuroendocrine cells. The chromogranin A promoter contains both positive and negative control domains that ensures cell type specific pattern of transcription [18,19]. Two 5’ regions of the transcription start site have been identified: a distal regulatory region (DRR) located between -726 and -455 upstream to the start site and a proximal regulatory region (PRR) between -60 and -26. The DRR domain plays a role in enhancing CgA gene transcription and the enhancing effect is specific for the neuroendocrine cells. Within the DRR domain, the region responsible for the transcriptional effect was found between -576 and -550. It contains an AP-1 motif and has been described as the distal regulatory element (DRE). Moreover, it has been demonstrated that the DRE by itself was substantially more effective at enhancing transcription than the entire 271 bp DRR, which suggests the presence of negative elements within the DRR fragment [19]. It has also been
shown that mutation in the AP-1 motif abolished the enhancing effects of the DRE on transcription. Studies indicate that transcriptional enhancement of CgA by the DRE is dependent on a unique neuroendocrine-specific DRE-binding factor (DBF), which specifically and directionally binds the DRE to assemble and synergize a functional transcriptional complex. Within the PRR sequence a cAMP response element (CRE) was found, separated from the TATA box by a conserved intervening sequence (IS). The human IS is similar to the Sp1-binding site consensus sequence and due to its location it may possess an ability to affect the transcriptional activity of the CgA gene. The CRE is necessary, but not sufficient for CgA transcription in neuroendocrine cells. It is likely that the CRE facilitates the formation of a functional transcriptional complex at the TATA box region, whereas DRR is required to activate the transcriptional complex and to enhance the transcription in neuroendocrine cells.

3.4 Post-transcriptional targeting

A new strategy to regulate viral replication includes modulation of mRNA stability and translation of mRNA into protein. The mechanism is based on gene silencing, where the mRNA expression level is altered by small RNA molecules (20-24 bp) called microRNA (miRNA). They bind to mRNA in a sequence-specific manner, resulting in either mRNA cleavage or translational blockage. MicroRNA molecules are produced from larger transcripts that form hairpin precursors (Figure 4). These precursors are known as primary miRNA (pri-miRNA) and are generally transcribed by RNA polymerase II. Nuclear RNase III, called Drosha performs cleavage of the pri-miRNA and generates 60-70 bp stem loop miRNA precursor (pre-miRNA). Pre-miRNA is transported to the cytoplasm by the export receptor Exportin-5. The nuclear cut by Drosha defines one end of mature miRNA molecule and the cytoplasmic cut by Dicer, also RNase III endonuclease, defines the opposite one. Dicer recognizes the pre-miRNA and cuts both strands at the site of a stem loop to generate a miRNA duplex. One strand of the miRNA duplex is associated with the RNA-inducing silencing complex (RISC) and it binds to target mRNA sequences, usually present in the 3’untranslated region (3’UTR) of the mRNA molecule. Partial complementarity in base-pairing between miRNA and target mRNA can act to suppress mRNA translation, but upon high-sequence homology, miRNA causes catalytic degradation of target mRNA.

The first miRNA was discovered in C.elegans and since then over 500 human miRNAs have been predicted or described. They play a key role in regulation of gene expression in many organisms. RNA silencing takes part in development, cell differentiation, cell proliferation, cell death and chromosome structure. Many miRNAs are also involved in antiviral response by targeting viral gene expression. Replication of the
primate foamy virus 1 (PFV-1) is inhibited in human cells expressing miR32, which has a binding site in the PFV-1 genome. It has been proposed that viruses are under selective pressure to avoid sequence homology to cellular miRNA in the tissues where they replicate. However, in the case of hepatitis C virus (HCV), the presence of miR122 in the target cell enhances viral replication, by specific binding to a 5’UTR of HCV. This phenomenon can be a result of viral adaptation and exploitation of the cellular defense system for their own benefit. Moreover, herpesviruses, adenoviruses, polyomaviruses and retroviruses, encode viral miRNAs to modulate both viral and cellular mRNA expression. By regulation of both viral and host genes, viral miRNAs play an important role in enhancing replication or minimizing the exposure of viral proteins to the host immune system.

3.5 Liver-specific miRNAs

Many miRNAs show highly differential tissue expression. Therefore they have been used as tools to mediate tissue-specific targeting of gene therapy vectors. Introduction of miRNA target sequences into a viral gene region essential for viral replication creates a virus that is regulated by specific miRNA molecules. This strategy has the potential to further increase a potent defense against replication in normal cells. Furthermore, miRNA sequences are short and easy to add to the compact viral genome. They do not attenuate viral replication or their lytic capacity and the strategy is applicable both for RNA and DNA viruses. Naldini et al. were the first to use miRNA target sequences to suppress transgene expression from a lentiviral vector by including target sequence of tissue-specific miRNAs present in hematopoietic cells or hepatocytes. Kelly et al. were able to restrict replication of an oncolytic coxsackievirus A21 (CVA21) virus by incorporation of miRNA target sequences recognized by muscle-specific miRNAs, using a similar approach. Virus replication was reduced in normal muscle tissue leading to reduction of muscle toxicity without compromising tumor-killing ability.

The safety of adenoviral-based gene therapy has in recent years focused on how to avoid liver toxicity, which together with antiviral immune responses, is considered the main limitation of this strategy. Several miRNAs are known to be specifically expressed in the adult mouse liver, such as: miR122, miR22, miR125b, miR24, miR92a, miR194. However, miR122 is the most abundant among them and it consists of up to 70% of the total miRNA pool isolated from the mouse liver, with a range of 50,000 – 82,000 copies per average cell.
Figure 4. The biogenesis and functions of micro RNAs (miRNAs) and other small interfering RNAs (siRNAs). miRNAs are genomically encoded, while siRNAs originate from extracellular precursors (dsRNA, plasmids encoding siRNA sequences).

Primary human hepatocytes express even higher levels of miR122, approximately 135,000 copies per cell. Profile expression of miRNAs is partially different in the fetal liver, indicating developmental regulation of miRNAs expression. Investigation of miR122 target genes has given proofs and indications of a variety of pathways regulated by this molecule, such as: cellular stress response, hepatocarcinogenesis and lipid metabolism.
3.6 Transductional targeting

Transductional targeting affects adenoviral cell entry and may add tropism to new targets or/and detarget virus from binding to their natural receptors. The expression of CAR is downregulated in some tumors, which reduces transduction of Ad5-based viruses. To circumvent the low expression of CAR, several strategies have been applied. For example, insertion of a RGD-motif in the HI loop of the virus fiber knob redirects primary interactions of the virus towards $\alpha_v\beta$-integrins, which are abundantly expressed on many tumor cells \(^{135-137}\). Replacing part of the Ad5 capsid with components from other Ad serotypes is also commonly utilized \(^{138,139}\). Fiber proteins from serotype 3 and 35 are most commonly used to overcome CAR-deficiency and to increase the infection rate in various tumors. Chimeric Ad5/35 viruses present enhanced oncolysis in the treatment of gliomas and B-cell tumors, while Ad5/3 viruses were shown to infect malignant glioma and ovarian cancer cells more efficiently \(^{140-143}\). The use of fiber proteins from non-human Ads such as bovine, porcine and mouse was also investigated to modify tropism \(^{144,145}\). In addition to direct genetic modification, transductional targeting can be achieved by bridging the Ad and a cell surface receptor with adapter-molecules such as bi-specific antibodies, cell-selective ligands and chemical conjugates \(^{78}\).

3.7 Clinical trials with oncolytic adenoviruses

Adenoviruses have been widely used in clinical trials for various tumors. Most of these trials are phase I dose escalation safety studies, but some Ads have entered phase II and III trials. The general experience with Ad as therapeutic agent is positive, since adverse events are mild. The oncolytic virus with the most published clinical data is ONYX-015, which has been evaluated in head and neck cancer, malignant gliomas, hepatocellular, pancreatic, ovarian and metastatic colorectal carcinomas, using intratumoral, intravenous, intrarterial and intraperitoneal routes \(^{146}\). In preclinical models, hepatic toxicity was the primary dose-related toxicity observed. Treatment with ONYX-015 was well-tolerated and more importantly no significant liver toxicity was reported to date after Ad infusions including numerous injections directly into the hepatic artery \(^{147}\). ONYX-015 showed localized efficacy when injected directly into head and neck cancer \(^{148}\). However, for other malignancies ONYX-015 was safe but not efficient as a single treatment \(^{149}\). To improve antitumor efficacy, ONYX-015 was combined with chemotherapy. Combination of ONYX-015, cisplatin and 5-fluorouracil demonstrated encouraging responses with 19 out of 30 injected tumors decreasing 50% in size \(^{150}\). Trials with ONYX-015 were continued with various tumor types presenting bad prognosis, such as: metastatic colorectal cancer, pancreatic carcinoma, glioblastoma and ovarian carcinoma \(^{146}\).
Treatment with ONYX-015 in combination with 5-fluorouracil and leucovorin was beneficial for metastatic colorectal cancer patients \(^{151}\). Treatment resulted in tumor regression in 46% of patients and their median survival increased to 19 months, as opposed to the expected 6-8 months in patients receiving only chemotherapy \(^{152}\). A adenovirus similar to ONYX-015, named H101 has been approved in China for treatment of neck and head cancer in combination with chemotherapy \(^{153}\). H101 is the first oncolytic virus approved for human use.

Combination of radiation therapy with an E1B-deleted adenovirus, carrying a fusion gene of two prodrug activating enzymes, cytosine deaminase (CD) and herpes simplex thymidine kinase (HSV-TK) was applied for the first time in therapy for prostate cancer \(^{154}\). Combination of oncolytic Ads, prodrugs and radiation provided remarkable clinical benefits for prostate cancer patients together with lack of toxicity. None of the intermediate risk prostate cancer patients showed evidence of disease after treatment based on their prostate biopsies, PSA levels and rectal examination.

Oncolytic Ads controlled by tumor- or tissue- specific promoters have also been well tolerated in patients. Intratumoral treatment with CV706, designed to treat prostate cancer, proved to be safe at virus doses up to \(10^{13}\) virus particles and produced encouraging responses \(^{155}\). All patients achieved reduced PSA levels in a dose-response manner. A similar prostate-specific virus, named CG7870 was administered intravenously to patients with hormone–refractory metastatic prostate cancer \(^{156}\). The treatment was well tolerated up to a dose of \(6 \times 10^{12}\) virus particles with reduction of PSA levels observed in 5 out of 23 patients.

Accumulated evidences indicate that besides effective virus replication within the tumor, treatment efficacy also depends on the extent of induction of host immune responses. Oncolysis is an immunogenic event, which may trigger activation of the immune system against virus-infected tumor cells. Therefore, Ads have been armed with immunostimulatory molecules in order to enhance this effect. Oncolytic Ad expressing granulocyte macrophage colony-stimulating factor (GM-CSF) has been used in patients with advanced solid tumors \(^{157}\). Signs of efficacy were seen in 8 out of 15 radiologically evaluable patients, including two patients with complete remissions. The treatment resulted in induction of both tumor-specific and virus-specific immunity. Comparable antitumor activities were observed in a similar study performed with a chimeric Ad5/3 coding for GM-CSF \(^{158}\). Another immunostimulatory strategy includes administration of a serotype 2 adenovirus (Ad2), called H103 coding for a heat shock protein 70 (HSP70). A dose of \(3 \times 10^{12}\) viral particles given intratumorally was well tolerated \(^{159}\). Most of the 27 patients were classified with stable disease and 3 with partial responses.
A new class of oncolytic Ads with multiple genetic modifications has entered clinical testing, demonstrating promising results. These viruses are designed to maximize antitumor effect by enhanced infection, replication as well as expression of various therapeutic genes. Safety and preliminary efficacy were observed in patients with advanced solid tumors following treatments with a transcriptionally targeted chimeric Ad5/3 virus carrying the gene for the immunostimulatory cytokine GM-CSF. In this study objective evidence of anti-tumor responses were observed in 11 out of 18 patients. Moreover, the results suggest that anti-tumor efficacy can be obtained despite induction of neutralizing antibodies (NAbs) in patients. ICOVIR-7 represents another multi-targeted oncolytic Ad, where an RGD-motif in the HI loop of the fiber knob enhances tumor infectivity, while replication selectivity is ensured by a 24bp deletion in E1A and a tumor-specific E2F promoter. A single round of injection of 1x10^12 viral particles demonstrated good safety and antitumor responses in more than half of the patients with advanced solid tumors.

Clinical trials with oncolytic Ads are mainly performed in patients with advanced diseases excessively difficult to treat. Moreover, phase I and phase II trials are primarily designed to address safety. Therefore, the treatment protocols may not be ideal to assess antitumor effects.

4. Immune Responses against Adenoviruses

4.1 Innate immune responses

Adenoviruses are highly immunogenic and elicit potent innate and adaptive immune responses. Since adenoviral infections are common, the majority of adults have acquired immunity, often against multiple Ad serotypes.

The innate response is comprised of various cells, including macrophages, dendritic cells (DCs), neutrophils and natural killer (NK) cells as well as serum proteins such as chemokines, cytokines and complement. Structural components of Ad, both DNA and capsid proteins, play roles in triggering innate responses and the outcomes can vary depending on Ad species and infected cell type. Many of these pathways lead to activation of NF-κB and interferon regulatory factor 3 (IRF3), followed by production of inflammatory mediators and interferons (IFNs). Type I IFN represents one of the most important antiviral defense mechanisms.

Adenoviral components are detected by pattern recognition receptors such as toll-like receptors (TLRs). In mammals, there are at least 13 TLRs with unique specificities. Both TLR2, TLR9 have been implicated in Ad recognition. TLR2 is a cell surface receptor, that is known to bind peptidoglycan and zymosan moieties present in bacteria. Studies have shown that TLR2 is a key mediator in responses to some dsDNA viruses as well,
however the exact ligand has not been characterized\textsuperscript{170-172}. TLR9 is an endosomal receptor recognizing DNA with unmethylated CpGs motifs. Sensing of Ads by TLR9 and the adaptor molecule MyD88 is a main mode of recognition in plasmocytoid DCs\textsuperscript{168}. In addition, Ad genome may be detected by cytoplasmic DNA sensors as it has been described in macrophages and conventional DCs\textsuperscript{168,173}. The action of these sensors and downstream adaptors are under investigation. It is proposed that cytosolic pathways play a main role in Ad-elicited IFN responses\textsuperscript{174}. Upregulation of cytokines and chemokines such as: IL-1, IL-6, IL-8, IL-12, RANTES, macrophage inhibitory protein-1/2 (MIP-1/2) and TNF-α has been observed in response to Ad infection\textsuperscript{174,175}. Macrophages throughout the body, including tissue residential macrophages such as Kupffer cells and DCs are the principal source of these cytokines and chemokines\textsuperscript{163,176}.

The complement system is an important defense mechanism, which induces rapid destruction and phagocytosis of pathogens\textsuperscript{177,178}. Complement activation can occur by antigen-antibody complexes (classical pathway), direct binding of C3b to antigen surfaces (alternative pathway) or by interaction of mannose binding protein to antigen surfaces (lectin pathway). Adenoviruses can trigger the classical and the alternative pathway, in the presence or absence of anti-Ad antibodies\textsuperscript{179}.

4.2 Adaptive immune responses

Cellular immune responses to Ads have been observed both for CD8+ cytotoxic T cells (CTLs) and for CD4+ T helper cells\textsuperscript{180}. Multiple class I and class II-restricted epitopes have been mapped within the conserved region of the hexon\textsuperscript{180,181}. Only minimal cellular responses to other capsid proteins, fiber or penton base, were detected. CTLs kill infected cells by multiple mechanisms including perforin, Fas-L and TNFα and thereby the Ad life cycle is disrupted before progeny viruses are released\textsuperscript{181}. Specific CD4+ T cells play a critical role in driving B-cell activation and differentiation. Activated B-cells undergo formation into plasma cells, which produce antibodies directed against adenoviral epitopes located on the major capsid proteins, \textit{i.e.}, hexon, penton and fiber\textsuperscript{182}. Binding of antibodies induces effective clearance of viruses from the circulation and enhances the interaction of Ads with leukocytes through the Fcγ- and complement receptors\textsuperscript{177,178}. Adenoviral infection generates serotype-specific neutralizing antibodies (NAbs). Anti-hexon NAbs were initially proposed as the most important in the neutralization process\textsuperscript{183-185}. However, recent data indicate that NAbs against fiber and penton proteins may also have great relevance\textsuperscript{160,186-188}. It seems like antibodies directed against various Ad capsid components synergize in the neutralization process\textsuperscript{189}.
4.3 Interactions with blood components

In patients with localized tumors, direct intratumoral administration of oncolytic Ad is the most efficient mode of delivery. While for patients with inaccessible tumors or metastases, systematic administration represents a more beneficial option. However, as several different clearance mechanisms are present, including the action of NAbs and the complement system, intravenous delivery of Ad has not been effective.

Interaction of Ads with blood may dramatically affect virus biodistribution and toxicity. Studies with serotype 5 adenovirus (Ad5) demonstrated that over 90% of applied Ad associate with blood cells after incubation with human blood. It is mainly a result of interaction of Ad5 with human erythrocytes, which express CAR on their surface and therefore can bind to Ads using CAR as their primary receptor. Erythrocytes as well as leukocytes express complement receptor 1 (CR1), which binds Ad5 in the presence of antibodies and complement proteins. Murine erythrocytes lack both CAR and CR1, therefore Ad5 association with murine blood cells is less than 0.1%. Intravenously administrated Ad5 rapidly binds to and activates circulating platelets. Direct Ad-platelet binding causes release of cytokines and sequestration to the reticulo-endothelial system in the liver. Induction of thrombocytopenia after intravenous injection of Ad5 was observed both in mice and humans. However, the precise mechanism of action remains unknown. Other blood cells that play an important role in Ad recognition and clearance are neutrophils. The interactions with neutrophils occur in the presence of complement and antibodies and are mediated by CR1 and Fcγ receptors. Formation of complexes between Ads with Nabs can facilitate infection of various immune cells such as monocytes or antigen presenting cells, which express Fcγ receptors.

In mice, after intravenous injection of Ad5, 99% of delivered virus is sequestered by the liver. Liver residential macrophages, also known as Kupffer cells, accumulate large amounts of intravenously delivered Ads and they may serve as the first clearance mechanism of Ads in the liver. Kupffer cells express CR1 and Fcγ receptors, which are involved in engulfing Ad-immune complexes. However, different studies suggest that Ad binding to Kupffer cells may take place via multiple mechanisms. Elimination of Kupffer cells leads to a great increase of hepatocyte transduction. In contrast to infection of Kupffer cells, infection of hepatocytes by Ad5 does not depend on virus interactions with CAR or integrins, but it is mediated by blood factors. Studies demonstrated that coagulation factor X (FX) binds with high affinity to the Ad5 hexon protein through interaction with the hypervariable regions. Subsequently, FX mediates Ad5 uptake via binding to heparin sulphate proteoglycans (HSPGs)
Interaction with coagulation factors is not specific for all Ads, since several Ad serotypes were found unable to bind FX \(^\text{205}\).

The complexity of Ad interactions with blood cells and proteins may strongly influence the development of Ad-based therapies in humans. The current existing animal models may likely provide only partial information regarding biodistribution and toxicity of Ads in humans. For example, intravenous injection of Ads rapidly induces liver toxicity in mice, however in human no profound liver-related toxicity has been reported. Moreover, various Ad serotypes may engage different molecular mechanisms resulting in divergent clearance modes and cell/organ targeting.
Aims of Study

Both diagnosis and treatment of NETs represent a challenge and there is a slow progress in the development of more sensitive biomarkers and effective therapeutics. The overall aim of this thesis was to make substantial and meaningful advances in the field of NET therapy. Papers I, II and III describe the development of neuroendocrine-selective oncolytic adenoviruses as novel agents for the treatment of neuroendocrine tumors and their liver metastasis. This is the first cancer virotherapy designed to specifically target neuroendocrine malignancies. Paper IV presents a study of gene expression profiling of NETs, which has successfully contributed to the discovery of a novel and more sensitive biomarker for NETs.

Specific aims presented by the individual papers are:

I. To construct an oncolytic adenovirus for the therapy of neuroendocrine tumors, where the chromogranin A promoter controls the expression of the E1A gene, Ad(CgA-E1A). To evaluate the specificity and potency of the Ad(CgA-E1A) in vitro and in vivo.

II. To improve safety and reduce the replicative capacity of the neuroendocrine-selective adenovirus Ad(CgA-E1A) in the liver cells by introducing miR122 target sequences in 3’ UTR of E1A. To evaluate the specificity and potency of the miR122-targeted oncolytic adenovirus in vitro and in vivo.

III. To alter adenovirus tropism towards neuroendocrine tumor cells expressing somatostatin receptors, by modification of the Ad fiber knob with four amino acids (FWKT) to mimic the binding site of somatostatin to somatostatin receptors. To evaluate the FWKT-modified Ad on various neuroendocrine cell types. To evaluate interactions of the FWKT-modified Ad with human blood.
IV. To indentify genes and proteins, which are specifically expressed in gastrointestinal neuroendocrine carcinoma and enterochromaffin cells, the cell type giving rise to the tumor, with the aim to discover novel markers for neuroendocrine tumors.
Methods

Material and methods used in the papers are described in the “Material and Methods” sections of each paper. Methods of a particular interest are described in more detail in the present chapter.

Proximity Ligation

The proximity ligation (PLA) technology was developed in Ulf Landegren’s group at the Department of Immunology, Genetic and Pathology, Uppsala University. This method offers sensitive and specific detection of proteins by utilizing in vitro DNA amplification. It relies on a simultaneous and proximate recognition of target molecules by pairs of affinity probes, giving rise to an amplifiable detection signal. The PLA technique has undergone continuous adaptation for numerous purposes. For example, studies of the presence and activity of low-abundance proteins in serum or body fluids give PLA a great potential in future diagnostics. Another application involves multiplex analysis to test multiple interactions in cells or many different proteins in one sample.

In paper III, proximity ligation in situ assay (P-LISA) was applied, where detection of protein interactions can be monitored directly in individual cells or tissues by highly specific and localized DNA amplification. This method allows for studying binding of capsid-modified adenoviruses with various cellular receptors and to my knowledge; this is the first successful use of the PLA method to study virus interactions with cellular surface molecules.

In our P-LISA, cells were incubated with adenoviruses and fixed to a slide prior to addition of primary and secondary proximity probes. The primary probes are pair of antibodies, where one is against the adenoviral hexon protein and the other binds the extracellular domain of the surface molecule that serve as primary receptor for viral entry (Figure 5A). The secondary probes are antibodies that recognizeFc domains of the primary probes and to which oligonucleotides have been conjugated (Figure 5B). If the oligonucleotides are brought in proximity, two additional connector oligonucleotides can hybridize to them, creating a template of ligation (Figure 5C). Therefore, hybridization and ligation are possible only when both primary probes have bound proximal epitopes and subsequently have
been recognized by secondary probes. Successful ligation results in formation of circular DNA molecule, which can serve as an “endless” temple for rolling circle amplification (RCA) \(^{212}\). The amplification is primed from the oligonucleotide attached to one of the secondary probe. RCA results in production of single-stranded DNA composed of concatemeric copies of circular DNA template (Figure 5D). The RCA product can be visualized by hybridization of fluorescently labeled oligonucleotides complementary to the sequence encoded in the original DNA circle. Each detected interaction produces one RCA product, therefore they can be enumerated. Distinct, bright and sub-micrometer sized RCA products are easily distinguishable from potential background fluorescence in an epifluorescence microscope.

Figure 5. Scheme of proximity ligation in situ assay. A) Primary probes (primary antibodies) bind to target proteins. B) Secondary probes (secondary antibodies) with conjugated oligonucleotides recognize the primary probes. C) Additional oligonucleotides are hybridized and ligated to form circular DNA molecule. D) The newly formed DNA molecule can be amplified by rolling circle amplification. A concatemeric amplification product can be detectable by hybridization of fluorescently labeled oligonucleotides.

Laser Capture Microdissection

The laser-based microdissection system is a powerful technology that provides rapid, accurate isolation of specific cell types for a variety of molecular analyses \(^{213,214}\). Briefly, a laser is coupled to a microscope and focuses on a tissue attached to a slide. By movement of the laser by optics or the stage the focus follows a trajectory which defines the area of interest. The area is then cut out and separated from the adjacent tissue. Sample extraction can be done in several ways. The laser microdissection process does not alter or damage the morphology and chemistry of the excised cells,
therefore it is a useful technique for DNA, RNA and protein collection for further analyses.

In paper IV, microdissection was performed with help of the PALM® Robot Microbeam laser capture microdissection system, where collection of the excised cells is done by the laser pressure catapult method. This system was used to isolate groups of ileocecal neuroendocrine tumor cells from primary tumors and metastases as well as normal epithelial cells of ileal mucosa (Figure 6). Snap-frozen specimens of tumors or normal ileum were cut into 10 µm thick cryosections and mounted on polyethylene-naphtalen (PEN) membrane slides, followed by hematoxilin staining and dehydration. A narrow beam ultraviolet laser was used to cut the selected group of cells from surrounding tissues. The microdissected cells were then deposited by defocused laser pulse into the collection tube containing proper RNA extraction buffer. Approximately 5000 cells were collected from each section.

Figure 6. Laser capture microdissection of tissue sections. Laser microdissection of ileal crypts in normal ileum (A and B) and neuroendocrine tumor cells in mesentery metastasis of midgut carcinoid (C and D). Pictures were taken before (A and C) and after (B and D) collection of the excised cells by the laser pressure catapult system.
Summary of Papers

Paper I

Neuroendocrine tumors (NETs) are a rare and heterogeneous group of neoplasms, which originate from the dispersed neuroendocrine system. NETs, often referred to as carcinoids, occur mostly in the gastrointestinal tract and pancreas, with secondary location in the bronchopulmonary system. They are often associated with intensive secretion of hormones, bioactive peptides and amines. Most of the patients are diagnosed at a late stage of disease with metastases, located mainly in the mesentery and in the liver.

In this paper we developed an oncolytic adenovirus for neuroendocrine tumor therapy where we used the chromogranin A (CgA) promoter to control adenoviral replication selectively in neuroendocrine cells. CgA is a glycoprotein abundantly expressed by cells with neuroendocrine features and it is used as a prognostic and diagnostic marker of neuroendocrine tumors. We constructed a serotype 5-based E1B-deleted adenovirus (Ad) with the E1A gene controlled by the human CgA promoter. The mouse H19 insulator with enhancer blocking activity was placed upstream the CgA promoter to shield it from unspecific activation from viral elements. The oncolytic potency of the virus, Ad[CgA-E1A], was evaluated both in vitro and in vivo. It efficiently replicates in and kills neuroendocrine cells, such as: BON, a cell line derived from a metastatic human carcinoid of the pancreas as well as neuroblastoma cell lines: SH-SY-5Y and SK-N-DZ. Moreover, it dramatically inhibited the growth of BON xenografts in nude mice. The replication ability of Ad[CgA-E1A] was attenuated in cell lines without neuroendocrine features as well as in freshly isolated normal hepatocytes. However, despite the fact that CgA is not expressed in human hepatocytes, the activity of Ad[CgA-E1A] was not completely blocked in liver cells leaving the risk of unwanted toxicity in a clinical setting.

Paper II

Since neuroendocrine tumors often metastasize to the liver and intrahepatic injection is the most likely route of delivery for treatment, it is crucial that normal liver cells do not support virus replication. In order to improve the safety of the neuroendocrine-selective adenovirus Ad[CgA-E1A] from paper
I, we set out to deregulate E1A gene expression in normal hepatocytes by a liver-specific miRNA, miR122. MicroRNA-mediated regulation is a new strategy based on gene silencing, where small noncoding RNA molecules bind to mRNA and trigger translational blockage or cleavage of mRNA. We constructed two oncolytic adenoviruses, where either six or twelve target sequences for miR122 were inserted in the 3’UTR of the E1A gene, Ad[CgA-E1A-miR122] and Ad[CgA-E1A-miR122x2]. The double control mechanism of E1A expression by the CgA promoter and miR122 molecules combine specific targeting of virus replication to neuroendocrine tumor cells and detargeting of virus replication from normal liver cells. The miR122-targeted adenoviruses demonstrated reduced E1A protein expression as well as replication arrest in normal hepatocytes. Moreover, incorporation of miR122 target sequences into the E1A 3’UTR did not interfere with E1A expression and the potent viral replication observed in neuroendocrine cells, which are negative for miR122 expression. The strategy exploiting transcriptional and post-transcriptional regulation offers the possibility to use higher doses of adenoviruses for more efficient and specific tumor treatment without inducing liver toxicity.

**Paper III**

The previously described, neuroendocrine-selective adenovirus Ad[CgA-E1A-miR122] is based on human adenovirus serotype 5 (Ad5) and infects target cells by binding to the coxsackie-adenovirus receptor (CAR) and integrins on the cell surface. Some neuroendocrine tumor (NET) and neuroblastoma cells express low levels of CAR and are therefore poorly transduced by Ad5. However, they often express high levels of somatostatin receptors (SSTRs). We introduced cyclic peptides containing four amino acids (FWKT), which mimic the binding site of somatostatin for SSTRs in the virus fiber knob. We show that FWKT-modified Ad5, Ad5fkFWKT binds to SSTR2 on NET cells, by using the proximity ligation method. We also show that Ad5fkFWKT transduces midgut carcinoid cells from liver metastases about 3-4 times better than non-modified Ad5. Moreover, FWKT-modified Ad5 overcomes neutralization in an ex vivo human blood loop model to a greater extent than Ad5, indicating that the fiber knob modification may prolong the systemic circulation time.

Our studies indicate that the FWKT-modified neuroendocrine-selective adenovirus Ad5fkFWKT[CgA-E1A-miR122] may be a promising therapeutic agent for carcinoid tumor treatment that may translate into an experimental therapeutic approach for neuroendocrine tumors.
Paper IV

Neuroendocrine tumors represent a huge therapeutic challenge and new criteria are needed to identify novel diagnostic, prognostic and predictive markers for early detection and effective treatment of tumors. We have profiled primary tumors and liver metastases of ileocecal NETs, using Affymetrix microarrays and advanced bioinformatics. We selected genes of interest, which were further verified to be expressed on mRNA level, by quantitative real-time PCR analysis on laser captured cells and on protein level by immunohistochemistry. We identified six novel marker genes previously not associated with ileocecal NETs: Glutamate receptor ionotropic AMPA2 (GRIA2), SERPINA10, paraneoplastic antigen Ma2 (PNMA2), testican-1 precursor (SPOCK1), G protein-coupled receptor 112 (GPR112) and olfactory receptor family 51 subfamily E member 1 (OR51E1), of which GRIA2 is specifically expressed by NET cells while the others are also expressed by normal enterochromaffin cells, supplied by R. V. Lloyd. Further studies were needed to investigate whether the GRIA2, SERPINA10, PNMA2, SPOCK1, GPR112 and OR51E1 genes encode novel diagnostic biomarkers for ileocecal NET. Furthermore, we analyzed similarity between primary lesions and liver metastases transcriptome by hierarchical clustering analysis. Two genes, chemokine (C-X-C motif) ligand 14 (CXCL14) and NK2 transcription factor related, locus 3, (NKX2-3) were expressed to a lower level in liver metastases than in primary tumors and normal EC cells, which may imply a role in neuroendocrine carcinoma differentiation. The study provides new information about the gene expression profile of neuroendocrine tumors.

Findings presented in Paper IV have contributed to the discovery that autoantibodies against paraneoplastic antigen Ma2 are novel and specific biomarkers for more sensitive diagnosis and progression of NETs. Quantification of Ma2 autoantibodies is more accurate in prediction of tumor recurrence than measurement of CgA levels, which currently is considered the most important indicator of tumor progression.
Future Perspectives

In paper I, we have described a novel neuroendocrine-specific chromogranin A-driven (CgA) oncolytic Ad5 virus, which demonstrated efficient killing of neuroendocrine tumor cells both in vitro and in vivo. The CgA promoter works well in gastrointestinal NETs but we observe varying activity in neuroblastoma cell lines. Therefore, we have recently focused on other members of the chromogranin family, i.e., secretogranin (Sg)III and neuroendocrine secretory protein 55 (NESP55). The proximal promoters of SgIII and NESP55 have not been characterized. We identified that the 0.5 kb genomic 5’ flanking sequences of both SgIII and NESP55 can drive transgene expression in neuroblastoma cell lines. Therefore, these sequences were subcloned in front of E1A and oncolytic adenoviruses were produced. Specific viral replication and virus-induced cell lysis will be evaluated in neuroblastoma cell lines.

In paper II, side-by-side comparison of various oncolytic Ads showed that E1B-deleted Ad5 had decreased lytic ability in relation to unmodified Ad5. Therefore, oncolytic property of our neuroendocrine-specific Ad could be improved by restoration of the E1B-55kd gene. In order to introduce new material into Ad genome, the insulator or the CgA promoter need to be partially deleted or replaced with shorter variants.

Systematic administration of viruses faces a major obstacle in the form of the host immune system. The human blood stream is a challenging environment for Ads due to effective clearance mechanisms performed by innate and adaptive immune responses. In paper III, we compared the activities of fiber-modified Ad5 viruses with unmodified Ad5 in the ex vivo blood loop model. Interestingly, the FWKT-modified Ad, which differs from unmodified Ad5 in only a few amino acids in the HI loop of the fiber knob, overcame neutralization in the whole blood to a greater extent than unmodified Ad5. On the other hand, Ad5f35 virus, where the whole Ad5 fiber was replaced by the fiber from serotype 35, did not escape the neutralization process. These preliminary results indicate that even small modifications may have a relevant effect on Ad activity after systemic administration, thus outcome of anti-tumor therapy. Replacement of both hexon and fiber proteins have been reported helpful for systemic administration \^160,183,216. Several studies have demonstrated the ability to produce Ad5 viruses with Ad5 hexon replaced with hexons derived from
various Ads, such as: Ad1, Ad2, Ad3, Ad6, Ad12, Ad48 \(^{91,183}\). Each of these chimeric vectors has shown an ability to escape from NAbs.

An alternative method, which is also evaluated in our group, is the development of oncolytic Ads based entirely upon rare alternative Ad serotypes or non-human-derived Ads \(^{144,217-219}\). We have selected different human Ad serotypes, including: Ad4, Ad11, Ad12, Ad17, Ad37 in order to investigate their biological characteristics. High titer production of various Ad serotypes has proved to be difficult. Due to their different natural tropism, cell lines derived from corneal cells, such as keratocytes (for species D) or gastric cells (for species A) may be found more suitable for Ad production. Therefore, to improve production of Ad serotypes, we are testing various producer cells and focus on spinning system of cell culture. The Ads with the most attractive features such as strong oncolytic activity, prolonged circulation in the blood and low seropr evalence have a potential therapeutic value. We have preliminary data indicating that Ad11 from species B and Ad17 from species D have as high oncolytic activity as Ad5 in different cancer cell lines. Moreover, Ad11 and Ad17 effectively evaded neutralization in plasma samples from healthy volunteers as well as human immunoglobulins for intravenous use (IvIg), which represents immunoglobulins from a pool of 10,000 individuals. Further studies will be conducted to evaluate activity of Ads in human blood from various species in the \textit{ex vivo} human blood loop model in order to select the serotypes which may be the most suitable for systemic administration.

One popular approach has been to coat the virus with polyethylene glycol (PEG) to shield epitopes recognized by antibodies \(^{220}\). However, despite promising results in mouse model, PEG coating (PEGylation) of Ads gives only partial improvements in the \textit{ex vivo} human blood loop model \(^{221}\). Another chemical approach include coating the virus with hydrophilic polymers based on N-[2-hydroxypropyl] methacrylamide (HPMA) \(^{222}\). HPMA polymers have been shown to effectively cover adenoviral capsids and decrease NAbs binding and interaction with human blood cells \(^{223,224}\). Despite the fact that polymer-coated Ads lose activity \textit{in vitro}, viruses become infectious when they accumulate within tumors as a result of the enhanced permeability and retention effect \(^{225,226}\). Although the mechanism is unknown, the restoration of virus infectivity seems to be tumor-specific event. Preclinical studies of virus-blood interactions in mice likely give misleading results. Therefore, the use of the \textit{ex vivo} blood loop model may be the best approach to assess activity of HPMA-coated virus in human blood.

Another approach to improve delivery of viruses into the tumor sites and at the same time shield the virus from immune system is to use carrier cells that naturally migrate to tumors. Mesenchymal stem cells (MSCs) pre-loaded with oncolytic adenovirus successfully delivered virus into advanced breast and lung tumors in mice \(^{227}\). Furthermore, four children with
metastatic neuroblastoma, refractory to conventional therapies, received several doses of autologous MSCs loaded with oncolytic adenovirus. A complete clinical response was documented in one case and the child is in complete remission after this therapy. Other cell types also investigated as carriers of oncolytic viruses are: T-cells and cytokine-induced killer cells with specificity for a tumor-associated antigen, endothelial progenitor cells and macrophages that are attracted to tumor areas by released cytokines, chemokines and hypoxia. Macrophages were used as a carriers to systematically deliver a prostate-specific oncolytic adenovirus to human prostate tumors in a nude mouse model. The same study demonstrated effective hypoxia-induced replication and release of adenovirus by macrophages infiltrating hypoxic areas in tumors. The combination of cell therapy with carriage of oncolytic viruses offers excellent opportunities to improve both modalities. Cells play a role in packaging, protection and delivery of viruses, while virus immunogenicity may break the immunosuppressive tumor microenvironment and enhance anti-tumor immune responses. Further studies are required to test which virus and cell type can be matched for the most beneficial synergistic effect. Identification of which cellular markers and phenotypes are associated with effective tumor homing, could improve cell engineering to generate high numbers of carrier cells for clinical use.

In paper IV, we have identified six potential markers for neuroendocrine tumor cells by using Affymetrix microarrays analysis. The consecutive studies are performed in the research group of assoc. prof. V. Giandomenico, PhD, at department of Medical Sciences, Uppsala University Hospital, Uppsala and I am also taking part in them. The initial follow-up research focused on PNMA2 and has led to the discovery that autoantibodies against paraneoplastic antigen Ma2 are novel and specific biomarkers for a more sensitive detection of recurrence in small intestine-NETs. The GRIA2 gene expression is present in primary and metastatic NETs, but not in normal EC cells. There is no evidence of GRIA2 protein expression in neuroendocrine tumor cells either by Western blot or immunohistochemistry analyses. Therefore, GRIA2 is investigated as a potential diagnostic mRNA marker. Evaluation of GRIA2 mRNA expression by QRT-PCR have already started in clinical samples of lung NETs. The results from the Q-RT-PCR analysis will be followed up by in situ hybridization analysis on laser capture microdissection of tumor cells. The GPR112 and OR51E1 genes, which encode intra-membrane plasma proteins, were not detected in NET cells with commercially available antibodies either by Western blot or immunohistochemistry analyses. The GPR112 and OR51E1 transcripts will be isolated, cloned and sequenced to evaluate any modification in the wild-type sequences that could be responsible for alteration in the translation phase. Both, GPR112 and OR51E1 represent interesting targets for immuno-
or radioimmunotherapy if the protein expression in the plasma membrane
can be visualized with *i.e.*, novel specific antibodies.
Acknowledgements

This thesis was performed at the Department of Immunology, Genetics and Pathology at Rudbeck Laboratory, Uppsala University, Uppsala. The work was supported by funding from the Swedish Cancer Society (Grant 4419-B05-06XBC and CAN2007/885), the Swedish Research Council (K2005-31X-15270-01A and K2008-68X-15270-04-3), the Verto Institute, the Swedish Children Cancer Foundation (PROJ08/006), Gunnar Nilsson’s cancer Foundation (E50/08), The European Community on behalf of GIANT (Grant LSHB-CT-2004-512087) and Tore Nilsson’s Foundation.

I would like to express my sincere gratitude to those people that contributed to the work presented in this thesis. I would especially like to thank:

My supervisor, Professor Magnus Essand, for all your support and advices, patience and proofreading that led to the completion of this thesis.

My co-supervisor, Associate Professor Valeria Giandomenico, for your guidance, introduction to various laboratory techniques as well as constructive criticism that shaped me as a scientist.

Associate Professor Angelica Loskog, for your kind help with all administrative problems, your great energy regardless the situation and for giving me my own place in the Satellite lab.

Berith Nilsson, for your kindness, tolerance and invaluable help with many cloning experiments.

Jan Grawe, for your invaluable help with many laboratory equipments.

I would like to thank current and former members of the GIG group for creating a creative workplace with a great atmosphere: Dr. Björn Carlsson, Dr. Wing Cheng, Dr. Sofia Vikman, Dr. Christina Ninalga, Dr Arian Sadeghi, Viktoria Rashkova, Jing Xu, Dr. Angelica Danielsson – thank you for helping me with CBA experiments, Dr Fredrik Eriksson – thank you for interesting speculations, fruitful discussions and careful revision of my thesis, Chuan Jin – thank you for instant help with many experiments. Special thanks goes to Di Yu, a hard-working PhD student and great colleague, you have helped me enormously both with lab work and the traitorous PCs. Sincere thanks goes to Linda Sandin, a talented PhD student with an energetic and strong personality, that became my dear friend and supported me when I needed it the most.
I would like to acknowledge my good friend Andrew Friberg, thank you for all the incredible stories, candies and chat sessions in Rudbeck’s corridors.

I would like to express my extensive thanks to all fantastic girls at the Satellite lab that accepted me into their group with open arms and gave me a lot of support, energy and warmth: Dr. Sara Mangsbo, Dr. Moa Fransson, Dr. Camilla Lindqvist, Lisa Christiansson, Hannah Karlsson, Lina Liljenfeldt and Gabriella Paul Wetterberg. Jag värdesätter er vänskap och jag är tacksam för den härliga tid vi haft tillsammans, för alla intensiva lunchdiskussioner och all den choklad vi delat. Sara, jag beundrar din passion för vetenskap, tack för alla kommentarer om immunsystemet, som inte gav mig någon panik över huvud taget, eller? Tack Moa, för din positiva attityd, du sprider optimism och glädje när det behövs som mest. Tack Camilla, för alla diskussioner om livet, såväl glada som allvarliga. Hannah, jag tycker väldigt mycket om din stora personlighet och ditt ännu större skratt, tack också för all hjälp med envisa maskiner. Lina, jag avundas dig ditt tålamod och norrländska lugn, du lyckas förmedla dina poänger med få ord. Ett särskilt stort tack till Lisa, den bästa dansaren, för att du har varit min översättare och för ditt tålamod med mina första steg med svenskan.

I would like to extend my gratitude to all the wonderful friends I met in Uppsala that shared with me memorable times and supported me in various ways: Dr. Hans-Henrik Fuxelius, Dr. Roberta Sommaggio, Maren Ziegler, Kim Nevelsteen, Ammar Zaghlool, Ariel Martinez, Monica Ricão Canelhas, Petar Kovachev, Kasia Zaremba, Grzegorz Niedźwiedzki, Kasia Planeta, Peter Björnskär, Kasia Kulma, Kasia Rogóż, Agata Zięba, Ania Płaczek, Kasia Ciosek. Special thanks to Dr. Dieter Fuchs, like Yoda, you always encouraged me with good counsel and exquisitely sarcastic comments.

Chciałabym wyrazić swoją wdzięczność dla wszystkich moich polskich przyjaciół w Uppsali, za nieustanne wsparcie, zrozumienie i cudowną przyjaźń.

Chciałabym podziękować moim kochanym rodzicom, Kazimierzowi i Barbarze Leja, za miłość, wsparcie i wiarę we mnie na każdym etapie mojego życia.


Min älskling och min glädje, Daniel Jarblad, du är källan till den styrka jag behöver. Tack för att du gör mig lycklig varje dag, jag älskar dig.
References


Acta Universitatis Upsaliensis

Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine 653

Editor: The Dean of the Faculty of Medicine

A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)