Oncolytic Newcastle Disease Virus as Treatment for Pancreatic Cancer

Pascal Buijs

Printing of this thesis was financially supported by Erasmus MC, Afdeling Heelkunde Erasmuc MC, ABN Amro, Applied Medical, Care10, ChipSoft, Covidien, Drost Loosdrecht, ERBE Nederland and Ipsen Farmaceutica.

The research for this thesis was performed within the framework of the Erasmus Postgraduate School Molecular Medicine.

Printing of this thesis was financially supported by Erasmus MC, Afdeling Heelkunde, ABN Amro, Care10, ERBE Nederland, Applied Medical, Covidien, ChipSoft, Ipsen Farmaceutica, Drost Loosdrecht

ISBN: 978-90-824231-0-5
Cover design: Michiel Voûte
Layout & Printing: Tromp Drukkerij BV

Oncolytic Newcastle Disease Virus as Treatment for Pancreatic Cancer

Oncolytisch Newcastle disease virus
als behandeling voor alvleesklierkanker

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de rector magnificus

Prof.dr. H.A.P. Pols

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
woensdag 30 september 2015 om 09:30 uur

Pascal Rolin Antoine Buijs
geboren te Landgraaf

ERASMUS UNIVERSITEIT ROTTERDAM

Promotiecommissie

Promotoren:	Prof.dr. C.H.J. van Eijck Prof.dr. R.A.M. Fouchier
Overige leden:	Prof.dr. R.C. Hoeben Prof.dr. C.M.F. Dirven Prof.dr. L.J. Hofland
Copromotor:	Dr. B.G. van den Hoogen

Table of Contents

Chapter 1 General Introduction 7

Chapter 2 Different Responses of Human Pancreatic Adenocarcinoma Cell Lines to Oncolytic Newcastle Disease Virus Infection
Cancer Gene Ther. 2014 Jan;21(1):24-30 17

Chapter 3 Recombinant Immunomodulating Lentogenic or Mesogenic Oncolytic Newcastle Disease Virus for Treatment of Pancreatic Adenocarcinoma
Viruses. 2015 Jun;7(6):2980-2998 33

Chapter 4 Intravenously Injected Newcastle Disease Virus in Non-Human Primates is Safe to Use for Oncolytic Virotherapy
Cancer Gene Ther. 2014 Nov;21(11):463-71 51

Chapter 5 Pigeon Paramyxovirus Type 1 from a Fatal Human Case Induces Pneumonia in Cynomolgus Macaques (*Macaca Fascicularis*)
In preparation 69

Chapter 6 Summarizing Discussion
Adapted from: Oncolytic Viruses: from Bench to Bedside with a Focus on Safety
Hum Vaccin Immunother. 2015 Jul 3;11(7):1573-84 83

Chapter 7 Nederlandse Samenvatting 101

Chapter 8 Reference List 107

Chapter 9 Appendices

- Dankwoord 135
- About the Author 139
- List of Publications 141
- Erasmus MC PhD Portfolio 143

chapter 1

General Introduction

Pancreatic ductal adenocarcinoma

Pathology

Several malignant and benign neoplasms can arise from the pancreas, stemming from ductal, acinar or islet cells. Since 85 percent of all pancreatic neoplasms are represented by pancreatic ductal adenocarcinoma (PDAC), the commonly used term 'pancreatic cancer' usually refers to this entity. The broader term 'exocrine pancreatic neoplasms' includes all tumors arising from ductal and acinar cells and their stem cells, making up 95 percent of all malignant neoplasms of the pancreas. Less than 5 percent of pancreatic neoplasms are endocrine, arising from the islet cells [1]. Most PDACs (60-70%) are found in the head of the pancreas mainly in the upper half, which cause obstructive jaundice as the presenting symptom. The minority of PDACs is located in the uncinated process and body or tail of the pancreas.

Exocrine pancreatic neoplasms can be classified according to the WHO nomenclature, describing benign, premalignant and malignant lesions [2]. Premalignant lesions can be pancreatic intraepithelial neoplasias (PanIN), mucinous cystic neoplasms (MCN) or intraductal papillary mucinous neoplasms (IPMN) with varying grades of dysplasia, and are considered at risk for progression to invasive malignancy.

Malignant PDACs are mostly hard tumors with dense stromal fibrosis and moderate to poor differentiation. Local extension of tumors typically includes adjacent structures like duodenum, portal vein or superior mesenteric vessels, and a striking perineural invasion pattern is often observed [3].

Molecular etiology

Most commonly found genetic mutations in pancreatic adenocarcinoma can be divided into three broad categories: early activation of oncogenes such as K-ras [4-6]; subsequent inactivation of tumor suppressor genes such as TP53 [5,7], p16/CDKN2A [8-10], and SMAD4 [5,11]; and inactivation of genome maintenance / DNA damage repair genes such as hMLH1 and hMSH2 [5,12,13]. Careful genetic analysis of pancreatic tumors and its precursors have helped to develop a genetic progression model for PDAC [14]. PanIN lesions harbor many of the same mutations as PDAC, starting with K-ras gene mutation in PanIN-1 [15], p16/CDKN2A gene mutations in PanIN-2, and SMAD4 and TP53 inactivation in PanIN-3 [14,16].

Incidence

An estimated 338.000 new cases of PDAC arise worldwide yearly, and 330.000 patients die of their disease, making PDAC the eight leading cause of death from cancer in men and women [17,18]. Incidence rates vary between 7-9 per 100.000 in men and 5-6.5 per 100.000 in women, resulting in a male-to-female ratio of 1.3:1. Around 80 percent of cases become clinically apparent in patients 60-80 years (median age at diagnosis is 71 years); cases below the age of 40 years are rare.

Risk factors

The major risk factors for pancreatic cancer include hereditary factors like positive family history [19,20], hereditary pancreatitis [21,22], ataxia-telangiectasia [23], and germline mutations in oncogenes like BRCA1 [24,25], BRCA2 [25-27], STK11/PRSS1 (Peutz-Jeghers syndrome) [28] or CDKN2A (familial atypical

multiple-mole melanoma syndrome) [29,30]. Non-hereditary influenceable factors include cigarette smoking [31-33], high body mass index and lack of physical activity [34], diabetes mellitus [35], and non-hereditary chronic pancreatitis [36-39].

Symptoms

Early clinical signs of pancreatic cancer include abdominal pain, unexplained weight loss, jaundice and pruritus, but symptoms can vary with the location of the tumor [40,41]. 70 percent of patients suffer from diabetes mellitus, usually with an onset of less than 2 years [42]. Later occurring symptoms of progressive disease are related to liver metastasis and/or invasion of adjacent organs like stomach, colon and/or peritoneal cavity.

Diagnosis

Unfortunately, there is no screening tool to detect asymptomatic premalignant or early malignant pancreatic tumors. Although there is consensus that patients with a (inherited) predisposition for pancreatic cancer should be screened, there is no consensus on the most effective method or the optimal interval of screenings [43].

In general, the diagnostic evaluation of a patient with suspected pancreatic cancer includes serologic evaluation and abdominal imaging. Computed tomography (CT) of the abdomen with a specific pancreas protocol is preferred [44-46] since abdominal ultrasound is often an incomplete examination due to overlying bowel gas and low sensitivity for small (<3 cm) pancreatic lesions [47-49]. Additional testing (including biopsy) may be unnecessary before surgical intervention if the appearance of the pancreatic tumor on CT is typical, the CT scan provides enough information to assess resectability, and the patient is fit to undergo a major surgical resection. However, additional procedures may be indicated in cases where the diagnosis is doubtful with symptoms pointing to chronic or autoimmune pancreatitis, when resectability is uncertain, or when a therapeutic intervention is needed (like biliary tree decompression). Endoscopic retrograde cholangiopancreatography (ERCP) or endoscopic ultrasound (EUS) can either confirm or dismiss the diagnosis of PDAC, with the opportunity to conduct brush cytology or preferably fine needle aspiration biopsies of the tumor [50-53].

The tumor-node-metastasis (TNM) system is used as oncologic staging system for PDAC. The goal of the staging work-up is to define the extent of disease to identify patients who are eligible for curative resection. Pancreatic tumors are categorized on a continuum from resectable, borderline resectable to unresectable according to either the involvement of adjacent structures and/or the presence of distant metastases. Locally advanced, unresectable tumors usually invade the celiac artery, superior mesenteric artery and vein, portal vein, and/or hepatic artery. Tumors that are not definite resectable or unresectable are called 'borderline resectable'. After careful assessment, only 15-20 percent of patients who present with a pancreatic tumor are considered candidates for resection.

Treatment

Radical (R0) surgery by means of pancreaticoduodenectomy (Whipple procedure) for tumors in the head or neck of the pancreas, or (laparoscopic) distal pancreatectomy for tumors in the body or tail of the pancreas is the only potential curative treatment option to date for stage I/II PDAC. Variations in technique or more extensive surgery have not lead to improvement in outcomes [54], and irradical

resections (R1) are still common [55]. Adjuvant chemotherapy (gemcitabine or fluorouracil) has been shown to improve overall survival slightly [56-58], while the efficacy of adjuvant radiation therapy is still under debate [59-61].
Patients with locally advanced and thus unresectable stage III or metastatic stage IV PDAC have shown to receive little survival benefit from palliative chemotherapy [62-65]. Also, radiation therapy for stage III PDAC was shown not to improve survival [66,67].

Prognosis
The prognosis after treatment is dependent mostly on TNM stage and histologic grade of the primary tumor. However, even for the 10–15 percent of patients with resectable disease, the prognosis is poor: median survival after resection of pancreatic tumors and adjuvant chemotherapy has been around 24 months and 5-year survival 20 percent [57], although numbers for true PDAC (excluding the 15% other types of pancreatic malignancies) are probably worse [68]. For patients with locally advanced or metastatic pancreatic cancer, the outlook is even grimmer: with palliative chemotherapy, median survival has been only 6 months [62]. Patients with an irradical resection have shown a slightly longer survival rate than patients with stage III PDAC not undergoing resection [55].

Oncolytic viruses

Oncolytic viruses (OVs) are viruses that selectively infect and damage malignant tumors without damaging normal tissues, and are employed in what is called oncolytic virotherapy [69]. Most malignant tumors have evolved to escape immune surveillance, and resist apoptosis and growth inhibition, which are not only hallmarks of cancer, but also play an important role in antiviral responses [70-73]. As such, cancer cells are more susceptible to (oncolytic) virus infection and virus induced killing. OVs use infected cancer cells' cellular resources to produce progeny virus, while infection results in a variety of cell death types, ranging from apoptosis, necrosis and autophagy, to immunogenic cell death and necroptosis. In addition, virus induced local and systemic responses to OVs including destruction of tumor vascularity, triggering anticancer immune responses, and therapeutic activity of OV expressed transgene-encoded proteins can enhance the oncolytic efficacy of the therapy.
Cancer selectivity of OVs can be either naturally occurring as with Newcastle disease virus, reovirus, mumps virus and Moloney leukemia virus. Other viruses, such as measles virus, adenovirus, vesicular stomatitis virus, vaccinia virus and herpes simplex virus have been genetically engineered to make them cancer specific. With the advent of reverse engineering techniques, modifications attributing to efficacy and safety of OVs have marked the introduction of new generations of recombinant OVs. Most recent developments have focused on conditional replication in tumor cells, expression of (therapeutic) transgenes as well as targeting and/or enhanced delivery of OVs [74-77].
Over 60 years ago, cancer patients were treated with (impure) naturally occurring OV preparations in clinical trials that would not pass the critical evaluations of today's standards [78-80]. Some successes were reported, but patients also frequently became ill or died when infection spread to normal tissues. Today, the newer generations of oncolytic viruses have been evaluated extensively for their efficacy in preclinical trials, and have shown to be more effective than first generation vectors on

many occasions [81-85]. Talimogene laherparepvec (an oncolytic herpes simplex virus) has recently undergone evaluation in a phase III clinical trial in patients with advanced or metastatic melanoma. This will probably be the first oncolytic virus to obtain FDA approval, while a marketing authorization application for the European Union has also been submitted to the EMA [86]. For most of the used OVs, safety in terms of toxicity has been studied in laboratory animals and humans [82]. However, studies focusing on environmental shedding and possible recombination of OVs with wildtype viruses are relatively scarce [87].

Newcastle disease virus (NDV)

NDV as an oncolytic virus

NDV has been described as a naturally occurring oncolytic virus as early as 1952 [88-90]. Since then, numerous clinical trials have employed wildtype NDV strains (MTH-68, 73-T, PV701, HUJ) either as a direct oncolytic agent, or as an oncolysate vaccine for treatment of patients with various types of advanced stage cancer [91-101]. Results of these early trials have been relatively disappointing, illustrated by the lack of further development of these treatment strategies.

NDV selectively replicates in and destroys tumor cells while sparing normal cells, presumably because of defective interferon (IFN) signaling pathways in tumor cells [102-106], although discrepancies for this theory have also been reported [107-109]. The tumor-specific replication and subsequent induced cytolysis, and the inability of the virus to spread among healthy cells, make NDV a safe cancer therapeutic agent. The IFN produced by normal cells surrounding the tumor cells upon NDV infection, besides having antiviral effects, might exert an antiproliferative effect against cancer cells, like human pancreatic adenocarcinoma cell lines (HPACs) [110,111].

In more recent years, the interest in oncolytic NDV has revived with the advent of recombinant viruses [112,113]. Several approaches have been used to improve oncolytic efficacy of recombinant NDV (rNDV), including increasing virulence by increasing the cleavability of the F protein [114-119], transfer of therapeutic or immunomodulating transgenes [114,115,120-122], targeting of tumor cells with modified attachment proteins [123,124], and combination therapy targeting immune checkpoints [125]. Increasing virulence of rNDV improved the direct oncolytic efficacy most, and most preclinical studies have employed mesogenic virulent rNDVs for expressing transgenes [113]. NDV has been shown to be very safe in tumor models using mice or rats, even when used in high dose and injected intravenously [116,118,119,126].

Safety of oncolytic NDV

Surprisingly, detailed information on (pre)clinical safety testing of oncolytic NDVs is scarce, and a comparison of different (non-)virulent strains in non-human primate models has never been described. Preclinical studies employing NDV as a vaccine vector using subcutaneous, intranasal and/or intratracheal administration has shown that NDV as vaccine vector is highly attenuated in non-human primates [127-132]. However, virulent NDV strains pose an environmental risk, as birds (specifically poultry) are very susceptible to infection with mesogenic or velogenic strains. Relevant shedding of live virus was observed after infection in early clinical trials [93,99]. Although NDV is an RNA virus with

an inherent high viral polymerase error rate due to the lack of proofreading, (recombinant) viruses with or without transgene(s) have shown to be genetically very stable upon passaging [120,122,133].

Clinical virology of NDV

NDV belongs to the genus *Avulavirus* within the family of *Paramyxoviridae* and has a natural avian host range comprising over 240 species of bird [134]. Newcastle disease, and consequently NDV, has been named after an described outbreak in Newcastle-upon-Tyne, although the disease entity has been reported earlier in the Dutch East Indies [135,136]. NDV was previously also known as avian paramyxovirus type 1 (APMV-1). NDV infections cause high mortality and economic losses in the poultry industry. NDV strains vary widely in pathogenicity and have been classified accordingly into three pathotypes: lentogenic (low virulent, no mortality in susceptible hosts), mesogenic (intermediate virulent, < 10 percent mortality) or velogenic (highly virulent viscerotropic or neurotropic, 10-100 percent mortality) [112,137]. NDV spreads primarily through direct contact between infected and healthy birds and via oral, nasal and ocular secretions and droppings. Infection occurs by virus inhalation, ingestion or conjunctival contact. Symptoms of NDV infection in poultry include respiratory (sneezing, coughing, gasping and nasal discharge), gastrointestinal (green waterish diarrhea), neurological (paralysis, twisting of head and neck, ataxia and clonic spasms) and general symptoms (loss of appetite, drop of egg production and peri-ocular tissue swelling). Prophylactic vaccination of (commercial) poultry for NDV is currently applied on a large scale and (suspected) outbreaks have been rigorously acted upon to prevent further spread [138], although outbreaks still occur regularly and the virus is considered endemic in some countries lacking resources for adequate disease prevention and control [139].

Zoonotic NDV

Although NDV can cause severe and lethal disease in poultry, infection is relatively asymptomatic in humans. Human infections with NDV have been observed after exposure to virus while handling infected birds or cadavers and lyophilized or aerosolized NDV vaccines. The associated symptoms have been described as mild: acute conjunctivitis and laryngitis, occasionally accompanied by low-grade fever and chills, with a rapid and spontaneous resolution [140-146]. Human-to-human transmission has never been reported.

Pigeon paramyxovirustype 1 (PPMV-1)

In the early 1970s, cases of NDV infection in racing pigeons were reported, and the antigenically distinct NDV variant that could be isolated is now best known as PPMV-1 [147,148]. Based on their difference in reactivity in hemagglutination assays, and their host specificity for columbiform species, all PPMV-1 strains cluster in lineage VIb/I of genotype VI chicken NDV strains [149-152]. Since the first outbreaks, the disease has been spread to all parts of the world as a result of contact between birds at races and exhibitions. PPMV-1 is now endemic in both domestic and wild pigeons and other columbiform species in Europe and North America, from where it regularly spreads to poultry, causing substantial outbreaks [149,153-160]. Although all PPMV-1 strains analyzed to date contain a multibasic amino acid motif at the F protein cleavage site, not all PPMV-1 strains are virulent in chickens (see also the next paragraph on molecular virology of NDV). However, serial passaging can result in virulent variants [161-166]. PPMV-1 disease signs in pigeons generally include a series of nervous disorders: bilateral or

unilateral locomotor disturbances of wings or legs, torticollis, and watery green diarrhea. Respiratory signs are not often seen [167]. Two cases of human infection with PPMV-1 associated with fatal disease have been reported: one in the USA [168] and one in the Netherlands (unpublished data). Both cases involved severe pneumonia and an immunosuppressive condition as an underlying comorbidity.

Molecular virology of NDV

NDV is an enveloped, non-segmented negative-sense RNA virus harboring a 15.186, 15.192 or 15.198 nucleotides long genome which contains six open reading frames (ORFs) [169-171]. These ORFs encode the nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN) and the RNA dependent RNA polymerase (L). Conserved sequences called 'gene-start' and 'gene-end' flank all six genes and serve as a transcriptional promoter and terminator, respectively. In addition, intergenic non-coding nucleotide stretches lie between the genes varying in length from 1 to 47 nucleotides [172]. The 3' and 5' ends of the genome make up the leader and trailer regions of 55 and 114 nucleotides long, respectively [172]. RNA editing during P gene transcription results in two additional structural proteins, V and W [173].

An NDV virion consists of genomic viral RNA encapsidated within the ribonucleoprotein (RNP) complex, which on itself consists of the NP, P and L proteins. The M protein surrounds this complex and a lipid bilayer completes the envelope, from which glycoproteins HN and F protrude. NDV's HN glycoprotein can bind to sialic acids on the host cell membrane, triggering F protein-mediated fusion of the viral envelope with the cellular membrane. After entry, the RNP complex dissociates from the M protein and is released into the cytoplasm, where NDV undergoes its complete replication cycle. The viral polymerase complex first transcribes viral genomic RNA into mRNAs that code for viral proteins until sufficient amounts have accumulated. It then switches to synthesize full-length plus-strand anti-genomic RNA, which serves as template for the production of minus-strand genomic RNA. Under direction of the M protein new RNP complexes are transported back to the cell membrane, where they are assembled into new virions, which are then released from the host cell by budding. HN activity is necessary for virus detachment and also prevents self-aggregation of viral particles.

Virulence of NDV can be attributed mostly to the (multi)basic cleavage site of the fusion protein [174,175]. Cleavage of the precursor protein F0 into active F1 and F2 by host cell proteases is needed for progeny viruses to become infective [176]. Lentogenic NDV strains possess a monobasic amino acid motif at the F protein cleavage site, which makes them only infective when cleaved by trypsin-like proteases found only extracellular in the respiratory and intestinal tract. In contrast, mesogenic and velogenic NDV strains possess a multibasic amino acid motif at the F protein cleavage site, which can be cleaved intracellular by ubiquitous furin-like proteases. As a result, these strains can cause systemic disease, which may be fatal. In cell culture conditions, these virulent strains also cause F protein mediated cell-to-cell fusion, leading to the formation of so-called syncytia: giant multi-nucleated cells that serve as virus production factories. Besides the F protein cleavage site, other viral factors also contribute to NDV virulence, including HN, which also determines viral tropism, and the viral polymerase complex [175]. The V protein is also important for virulence, as it is a potent IFN antagonist in susceptible hosts [177-180].

Innate immunity in oncolytic virotherapy

During optimizing of the effect of oncolytic viruses for use in cancer therapy, most efforts have focused on increasing virulence, expressing transgenes and targeting tumor cells. Less is known about the potential role of the innate immune system in oncolytic virotherapy. The innate immune system is capable of sensing intracellular pathogen-associated molecular patterns occurring when a normal cell becomes infected with a (viral) pathogen like NDV [71]. This will lead to the production of type I interferons (IFNs), which act as an autocrine and paracrine signal to upregulate IFN-stimulated genes (ISGs). Besides having antiviral effects, type I IFNs, in particular IFNβ, also exert anti-proliferative, pro-apoptotic and pro-inflammatory effects in tumor cells, which could attribute to oncolytic virus efficacy [110,111,125,181,182]. On the other hand, viruses use different strategies to counteract the innate immune system to increase infectivity and replication efficiency, which could potentiate oncolytic efficacy [120], but might also have an impact on perceived safety of oncolytic NDV. The non-structural NS1 protein of influenza A virus is one of the most potent antagonists of innate immunity characterized to date [183,184]. NDV's V protein is also a potent IFN antagonist in susceptible hosts, but not in human cells [177-180].

Outline of this thesis

Pancreatic ductal adenocarcinoma (PDAC) still remains a difficult to treat disease. The work presented in this thesis was undertaken to develop a new treatment modality for PDAC, virotherapy using oncolytic Newcastle disease virus (NDV).

To obtain 'proof of principle' for oncolytic efficacy of NDV for PDAC we investigated the responses of several human pancreatic adenocarcinoma cell lines (HPACs) to inoculation with NDV in **chapter 2**. We focused on the oncolytic potential of a wildtype strain NDV, and assessed the innate immune response of the different cell lines to evaluate the basis of oncoselectivity of NDV in PDAC.

Subsequent improvement of the oncolytic efficacy of NDV was evaluated in **chapter 3**, describing experiments undertaken using recombinant viruses with immunomodulating capacity, as well as more virulent recombinant NDVs both *in vitro* and in a nude mouse model for PDAC. We focused on the innate immune responses and oncolytic efficacy in HPACs, in addition to efficacy of intratumoral injection of different rNDVs in a nude mouse model for PDAC.

Chapter 4 focuses on the safety of oncolytic NDVs. Although much is known on the oncolytic potential of NDV, studies on safety are relatively scarce. We therefore evaluated the toxicity and shedding of lentogenic and mesogenic (recombinant) NDV strains when injected intravenously in high doses in non-human primates.

In **chapter 5**, we present a study with a PPMV-1 strain isolated from a fatal human case. The virus was investigated for the zoonotic potential, e.g. causing disease in a non-human primate model, when inoculated via the natural route.

In **chapter 6**, the findings presented in this thesis, and the progress in the field of OVs is discussed.

Chapter 2

Different Responses of Human Pancreatic Adenocarcinoma Cell Lines to Oncolytic Newcastle Disease Virus Infection

Pascal R.A. Buijs
Casper H.J. van Eijck
Leo J. Hofland
Ron A.M. Fouchier
Bernadette G. van den Hoogen

Cancer Gene Therapy 2014;21(1):24-30

Abstract

Newcastle disease virus (NDV) is a naturally occurring oncolytic virus with clinically proven efficacy against several human tumor types. Selective replication in and killing of tumor cells by NDV is thought to occur because of differences in innate immune responses between normal and tumor cells. In our effort to develop oncolytic virotherapy with NDV for patients with pancreatic cancer, we evaluated the responses to NDV infection and interferon (IFN) treatment of 11 different established human pancreatic adenocarcinoma cell lines (HPACs). Here we show that all HPACs were susceptible to NDV. However, this NDV infection resulted in different replication kinetics and cytotoxic effects. Better replication resulted in more cytotoxicity. No correlation was observed between defects in the IFN pathways and NDV replication or NDV induced cytotoxicity. IFN production by HPACs after NDV infection differed substantially. Pretreatment of HPACs with IFN resulted in diminished NDV replication and decreased the cytotoxic effects in most HPACs. These findings suggest that not all HPACs have functional defects in the innate immune pathways, possibly resulting in resistance to oncolytic virus treatment. These data support the rationale for designing recombinant oncolytic NDVs with optimized virulence, which should likely contain an antagonist of the IFN pathways.

Introduction

An estimated 277.000 new cases of pancreatic adenocarcinoma arise worldwide yearly, and 266.000 patients die of this disease [185]. Radical surgery is the only potential curative treatment option to date. However, even for the 10-15% of patients with resectable disease, the prognosis is poor: median survival after resection is 24 months and 5-year survival 20% [57]. For patients with locally advanced or metastatic pancreatic cancer, the outlook is even grimmer: with palliative chemotherapy median survival is just 6 months [62]. Development of novel treatment options for pancreatic cancer is of crucial importance.

The local or systemic administration of oncolytic viruses such as Newcastle disease virus (NDV) to cancer patients is a promising treatment strategy with encouraging results in a variety of tumor types [83,96,113,186]. NDV is a replication competent oncolytic virus belonging to the family *Paramyxoviridae* with a natural avian host range. The virus has a replication cycle confined to the cell cytoplasm without integration or recombination. NDV strains are categorized in three different groups based on the severity of disease they cause in birds: lentogenic (avirulent), mesogenic (intermediate virulent) and velogenic (virulent). While NDV can cause severe and lethal disease in poultry, infection is relatively asymptomatic in humans [101].

NDV selectively replicates in and destroys tumor cells while sparing normal cells, presumably due to defective interferon (IFN) signaling pathways in tumor cells [102-106]. Infection of normal cells leads to IFN production which inhibits viral replication. The tumor-specific replication and subsequent induced cytolysis, and the inability of the virus to spread among healthy cells, make NDV a safe cancer therapeutic agent. The IFN produced by normal cells surrounding the tumor cells upon NDV infection, besides having anti-viral effects, might exert an anti-proliferative effect against human pancreatic adenocarcinoma cell lines (HPACs) [110].

Several naturally occurring NDV strains have shown anti-tumor activity without major side effects in phase I-II clinical trials [92,95,96,101]. However, the success of these therapies was only marginal, likely due to the IFN sensitivity or over-attenuation of the virus. In addition, some tumor cells are still capable of mounting effective antiviral responses, which may at least partially explain the observed resistance of some tumors to oncolytic NDV therapy [114,120]. The generation of recombinant viruses has provided opportunities to improve the efficacy of oncolytic NDV virotherapy [114,115,120,121,187-189].

Pancreatic tumors are heterogeneous in cell composition, and information is lacking on defects in IFN pathways in these cells. Our ultimate goal is to generate an optimized recombinant NDV (rNDV) with destroying capacity of all different cell types of pancreatic tumors. For a rational design, we evaluated the susceptibility of 11 HPACs for NDV, response to this infection with regard to IFN production and cytotoxicity, and response to IFN treatment.

Material and Methods

Cell lines and culture conditions

The human pancreatic adenocarcinoma cell lines were obtained from the American Type Culture Collection (Wesel, Germany) and authentication was performed using Short Tandem Repeat profiling [190]. Cells were used not more than 25 passages after thawing. PANC-1, MIA PaCa-2, BxPC-3, Hs 700T, Hs 766T, CFPAC, SU.86.86, AsPC-1, Capan-1, Capan-2 and HPAF-II were cultured in Roswell Park Memorial Institute 1640 medium (RPMI-1640) supplemented with 100 U ml^{-1} penicillin, 100 U ml^{-1} streptomycin, 2 mM L-glutamine (PSG) and 5% or 10% HyClone Characterized Fetal Bovine Serum (FBS HC; Thermo Fischer Scientific, Breda, the Netherlands). The non-neoplastic human lung fibroblast cell line MRC-5 was cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with PSG and 10% FBS HC. 293T cells were cultured in DMEM supplemented with PSG, 0.5 mg ml^{-1} geneticin, 1 mM sodium pyruvate, 1% non-essential amino acids and 10% FBS HC. Vero clone 118 cells [191] were cultured in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with PSG and 10% FBS HC. All cells were kept at 37 °C with 5% CO_2 in a humidified incubator. Periodically, cells were tested and confirmed to be *mycoplasma*-free. All media and supplements were purchased from GIBCO (Life Technologies, Bleiswijk, the Netherlands).

Newcastle disease virus

All infection experiments were performed with lentogenic wild-type NDV isolated from a cloacal swab of a wild female mallard taken on 17th of October 2002 in Lekkerkerk, the Netherlands. The virus was cultured in embryonated chicken eggs using standard techniques. The F cleavage site was sequenced and the deduced amino acid sequence was found to be ^{112}GRQGRL^{117}, confirming this to be a lentogenic NDV. All infection experiments were performed in the presence of reduced concentration FBS HC (3%), without the addition of trypsin. Virus stocks were titrated by end point dilution assay in Vero clone 118 cells. To read out infection, cells were stained with chicken polyclonal anti-NDV antibody and rabbit-FITC labeled anti-chicken IgG antibody (1:2000 & 1:1000 dilution respectively; both from Abcam, Cambridge, UK) 72 hours after inoculation. Viral titers were calculated using the method of Reed & Muench [192]. In infection experiments, the term m.o.i. refers to multiplicity of infection. An m.o.i. of 1 equals one 50% tissue infective dose ($TCID_{50}$) per cell. Low, medium and high m.o.i. are defined in this manuscript as an m.o.i. of 0.01, 0.1 and 1 respectively.

Replication curves

1.5 x 10^6 cells in T25 flasks (Corning, Amsterdam, the Netherlands) were inoculated with NDV at low m.o.i. After 1 hour incubation, cells were washed three times with PBS, and fresh infection medium was added. At time points 0, 6, 12, 24, 48 and 72 hours after washing, duplicates of 100 µl supernatant were collected, mixed with 100 µl 50% (w/v) sucrose, and frozen at -80 °C. Samples were titrated in quadruplicate as described before.

Cytotoxicity assay

Quadruplicates of 2 x 10^4 cells per well in 96-well plates (Corning) were either mock inoculated or inoculated with NDV at different m.o.i. (low – medium - high). After 48 hours, 100 µl fresh medium was added. At time points 0, 24, 48, 72, 96 and 120 hours post inoculation, cells were washed once with PBS and lysed by incubation with 100 µl 0.9% Triton X for 45 minutes at 37 °C. Lactate dehydrogenase (LDH)

in 50 µl of lysate sample was assayed using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Leiden, the Netherlands) following the manufacturer's instructions. Cell viability is presented as percentage absorption (450 nm, Tecan Infinite F200, Giessen, the Netherlands) of inoculated versus mock inoculated cells, which were considered to be 100% viable. Although measurements were performed at multiple time points, we only show data from time point 5 days post inoculation, since this represented differences between observed cytotoxicity best.

IFN measurement with luciferase bioassay

To determine the presence of type I IFN in the supernatants of infected cells, a bioassay using a plasmid with the interferon stimulated response element (ISRE) fused to the firefly luciferase gene (pISRE-Luc; Agilent Technologies, Amstelveen, the Netherlands) as reporter was used. 24 hours post inoculation, supernatants of (mock-)infected cells were collected and stored at -20 °C. This time point was chosen to be able to measure IFN production without possible interference from decreasing cell viability and/or secondary IFN signaling loops. 293T cells were transfected using the calcium phosphate method [193] with pISRE-Luc as reporter plasmid and Simian virus 40-*Renilla*-luciferase (pRL-SV40; Promega) as internal control. The next day, the collected supernatants were treated with UV to inactivate virus present in the supernatant and 300 µl was placed on the transfected 293T cells, together with 200 µl fresh 293T medium. After 24 hours, luminescent signals were generated using the Dual-Glo Luciferase Assay System (Promega) according to the manufacturer's instructions and detected with a Tecan Infinite F200 microplate reader. *Renilla* luciferase signals were used to normalize firefly luciferase signals to correct for 293T cell number and transfection efficiency. IFN produced by NDV-infected cells is presented as fold change as compared to mock infected cells. Recombinant human IFN-β-1a (Merck Calbiochem, Darmstadt, Germany) was used as a positive control.

NDV staining and Fluorescence-activated cell sorting (FACS) analysis

Cells were harvested by trypsinization and subsequently fixed and permeabilized using Cytofix/Cytoperm following the manufacturer's instructions (BD, Breda, the Netherlands). Next, cells were incubated on ice for 1 hour with chicken polyclonal anti-NDV antibody (1:800 dilution), washed and subsequently incubated on ice for another hour with rabbit-FITC labeled anti-chicken IgG antibody (1:400 dilution). Cells were washed and fixed in 2% paraformaldehyde. FITC signals were detected using a BD FACS Calibur cytometer and FACS data was analyzed using BD CellQuest Pro software.

RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

RNA was isolated using the High Pure RNA Isolation kit (Roche, Woerden, the Netherlands) following the manufacturer's instructions. RNA was reverse transcribed using a two-step protocol. First, 22 µl (of a total of 50 µl) of eluted RNA was mixed with 2 µl random primers (500 µg ml^{-1}; Promega), 2 µl dNTP's (10 mM) and 1 µl RNAse inhibitor (40 U μl^{-1}; Promega). This mix was heated for 5 minutes at 65 °C and immediately put on ice. Next, 8 µl FS buffer (5x), 2 µl DTT (0.1 M), 2 µl Superscript III RT (200 U μl^{-1}; Life Technologies) and 1 µl RNAse inhibitor (40 U μl^{-1}) was added. This mix was incubated for 5 minutes at 25 °C and for 1 hour at 60 °C to synthesize cDNA. qRT-PCR was performed using 4 µl of cDNA in an ABI PRISM 7000 Sequence Detection System (Life Technologies), using TaqMan gene expression assays for ISG56, OAS1 and Mx1 (Hs00356631, Hs00973637 and Hs00895608; all from Life Technologies). β-actin was used as household gene (forward primer 3'-ggcatccacgaaactacctt-5', reverse primer 3'-agcactgtgttggcgtacag-5', probe

3'-atcatgaagtgtgacgtggacatccg-5'). Results are presented as fold change of treated samples versus control samples (triplicates), calculated using the $2^{-\Delta\Delta C}{}_{T}$ method [194].

Statistical analysis

Continuous data were compared between the groups using the Mann-Whitney U test. Correlation between continuous data was calculated using Spearman's rank correlation coefficient (rho). P values of <0.05 were considered statistically significant.

Results

For a rational design of recombinant NDVs with improved oncolytic efficiency acting against all different sorts of pancreatic tumor cells, we evaluated the responses to NDV for 11 different HPACs with regards to cytotoxic effects and innate immune responses: PANC-1, MIA PaCa-2, BxPC-3, Hs 700T, Hs 766T, CFPAC, SU.86.86, AsPC-1, Capan-1, Capan-2 and HPAF-II.

HPACs differ in susceptibility to NDV replication and subsequent cytotoxic effects

To assess the susceptibility of the 11 HPACs to NDV replication and NDV induced cytolysis, multicycle replication kinetic studies with wild-type lentogenic NDV were conducted. Cells were inoculated at low m.o.i. and virus titers in the culture supernatant were determined at different time points post inoculation. MRC-5, a normal human fibroblast cell line known to be susceptible to NDV infection, was taken as control.

Based on NDV replication capacity in the different HPACs, the cells were categorized into 3 groups (table 1; figure 1): supporting high replication (peak titer > $1{,}0 \times 10^5$: Capan^{-1}, HPAF-II and SU.86.86), supporting low to medium replication (peak titer $1{,}0 \times 10^4$ to $1{,}0 \times 10^5$: Capan-2, BxPC-3, AsPC-1, PANC-1 and CFPAC) and supporting minimal replication (peak titer < $1{,}0 \times 10^4$: Hs 766T, Hs 700T and MIA PaCa-2).

Table 1: NDV replication. Virus yield obtained after inoculation of MRC-5 (normal fibroblast) and 11 different human pancreatic adenocarcinoma cell lines (HPACs) with NDV.

Cell line	Peak titer ($TCID_{50}$/ml)
MRC-5	$1{,}0 \times 10^4$
Capan-1	$3{,}4 \times 10^6$
HPAF-II	$1{,}6 \times 10^6$
SU.86.86	$1{,}7 \times 10^5$
Capan-2	$6{,}6 \times 10^4$
BxPC-3	$5{,}5 \times 10^4$
AsPC-1	$3{,}1 \times 10^4$
PANC-1	$1{,}3 \times 10^4$
CFPAC	$1{,}2 \times 10^4$
Hs 766T	$4{,}6 \times 10^3$
Hs 700T	$2{,}8 \times 10^3$
MIA PaCa-2	$2{,}7 \times 10^3$

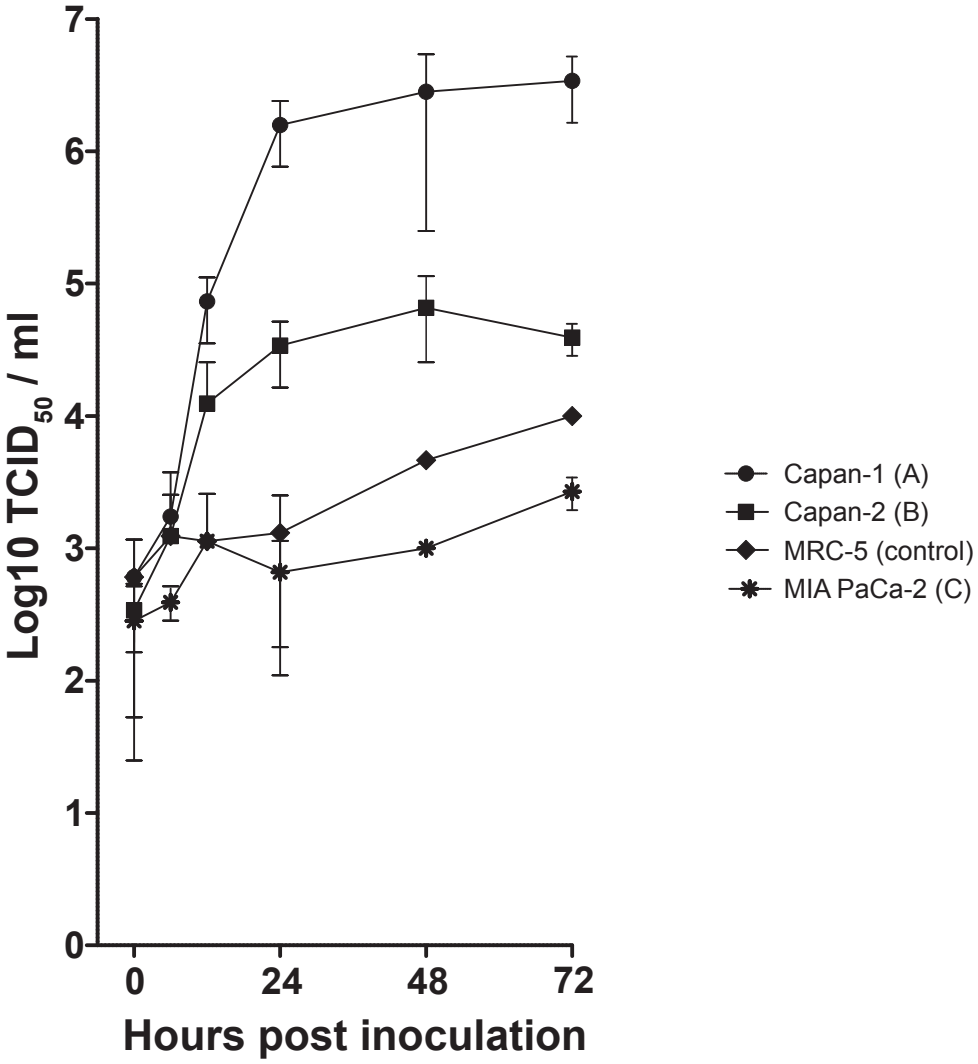


Figure 1: Replication kinetics of NDV in HPACs and MRC-5 fibroblasts. Representative HPACs for each category are shown (A = high replication: peak titer > 1,0 x 10^5; B = medium replication: peak titer 1,0 x 10^4 to 1,0 x 10^5; C = no replication: peak titer < 1,0 x 10^4). Samples were taken at indicated time points and titrated by end-point dilution assay in Vero clone 118 cells. Means and standard deviations of duplicate titrations are plotted.

The differences observed in NDV replication were reflected in the induced cytotoxicity (figure 2). In general, cells with high virus replication showed cytotoxicity already at low m.o.i. (SU.86.86, Capan-1, HPAF-II) while cells displaying low to medium virus replication required inoculation at medium m.o.i. to kill most cells. Inverse correlation was observed between peak viral titers and percentage cell survival at low and medium m.o.i. (Spearman's rho -0,77 and -0,74 respectively). MIA PaCa-2 and Hs 700T did not support virus replication and displayed cytotoxic effects only at high m.o.i. These findings indicate that NDV replication is an important factor to cause cytotoxicity in HPACs.

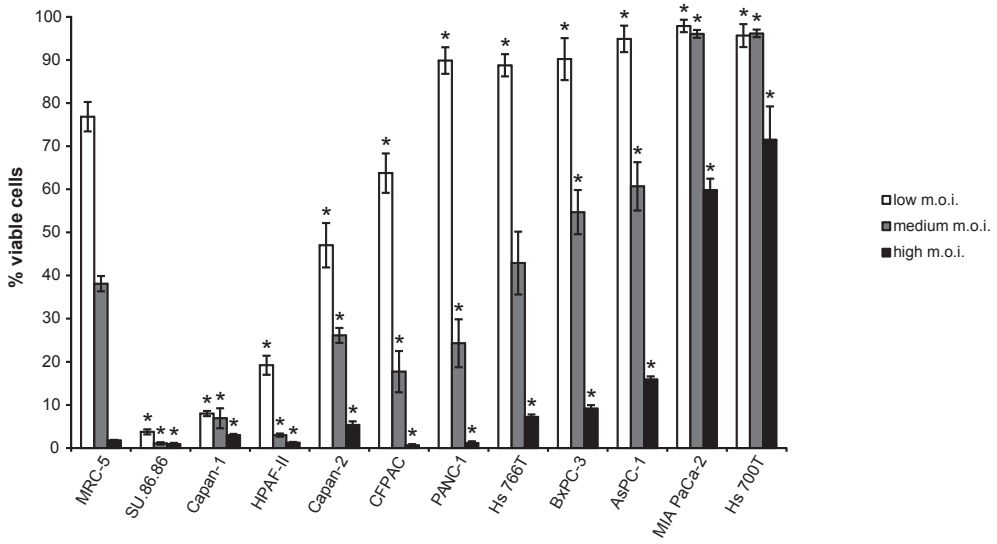


Figure 2: Cytotoxicity induced by NDV infection on all HPACs and MRC-5. Cells were either mock inoculated (not shown) or inoculated with NDV at low (white bars), medium (grey bars) or high m.o.i. (black bars). Cytotoxicity was measured daily for 5 days by LDH assay, with day 5 post inoculation being depicted. Results are presented as percentage of surviving cells as compared to mock inoculated cells, which were considered to be 100% viable. Means and standard deviations of quadruplicate experiments are plotted. * = $p < 0,05$ as compared to MRC-5 cells.

HPACs differ in IFN production and signaling pathways

For most HPACs, variation of NDV replication corresponded with differences in cytotoxic effects induced by the NDV infection. The observed variation in replication might result from differences in innate immune responses between the HPACs, as was described in earlier studies in other cell lines [103-106].

To investigate differences in the IFN production pathway, cells were inoculated with NDV at high m.o.i. to obtain high percentages of infected cells and 24 hours later IFN content in the supernatant was determined (figure 3A). Six out of 11 HPACS (SU.86.86, Capan-1, Capan-2, BxPC-3, AsPC-1, MIA PaCa-2) failed to induce substantial IFN production upon NDV infection. This lack of IFN production was not due to low percentage of infected cells, as all HPACs displayed more than 68% infected cells by FACS analysis upon inoculation with this high m.o.i. The insensitivity of MIA PaCa-2 cells to NDV infection and induced cytotoxic effects could not be explained by IFN production as these cells did not produce IFN upon NDV infection. Interestingly, five HPACs (HPAF-II, CFPAC, PANC-1, Hs 766T and Hs 700T) did induce

IFN production upon NDV infection, as did control MRC-5 fibroblasts. This is not an unusual finding as IFN production by tumor cells has been demonstrated before [104,106,187]. No correlation was found between virus replication or cytotoxic effects of NDV and IFN production in these cells (Spearman's rho -0,27; -0,08; -0,13 and -0,32 for replication; cytotoxicity at low, medium and high m.o.i. respectively). This is best demonstrated by the HPAF-II cell line, which produces relatively large amounts of IFN, yet is highly susceptible to NDV.

The tumor selectivity of NDV has also been shown to be due to defects in the IFN signaling pathway [104]. To test for defects in the JAK-STAT signaling pathway, cells were either mock treated or treated with 1000 IU ml^{-1} of recombinant human interferon beta (IFN-β). After 24 hours, expression of mRNA of three important interferon-stimulated genes (ISGs: interferon-stimulated gene 56 (ISG56), 2'-5'-oligoadenylate synthetase 1 (OAS1) and myxovirus resistance 1 (Mx1) was determined (figure 3B). As expected, all three tested ISGs in normal human lung fibroblast MRC-5 cells were upregulated upon IFN treatment, while these cells also produced IFN upon NDV infection. Of the 5 IFN producing HPACs (HPAF-II, CFPAC, PANC-1, Hs 766T and Hs 700T), 4 displayed a diminished upregulation as compared to MRC-5 fibroblasts for at least one of the mRNAs. Although lower than MRC-5 control, they still displayed at least a tenfold upregulation of at least one of the assayed ISG-mRNAs, which might still be biologically significant. For Hs 700T cells, which demonstrated production of IFN upon NDV infection, 2 out of 3 tested ISGs were highly upregulated upon IFN treatment. Thus, Hs 700T cells produce IFN upon NDV infection, and a mostly intact IFN signaling pathway in these cells results in expression of antiviral genes. Failure to upregulate OAS1 mRNA suggests that Hs 700T cells have a mutation in or deletion of this antiviral gene, but nevertheless they were still protected from infection. In summary, most of the tested HPACs showed a retained ability to respond to type I IFN, and the significance of the lower magnitude of response in some HPACs is unclear. Furthermore, no statistical correlation (tested with Spearman's rank) was found between virus replication or cytotoxic effects of NDV and ISG-mRNA upregulation.

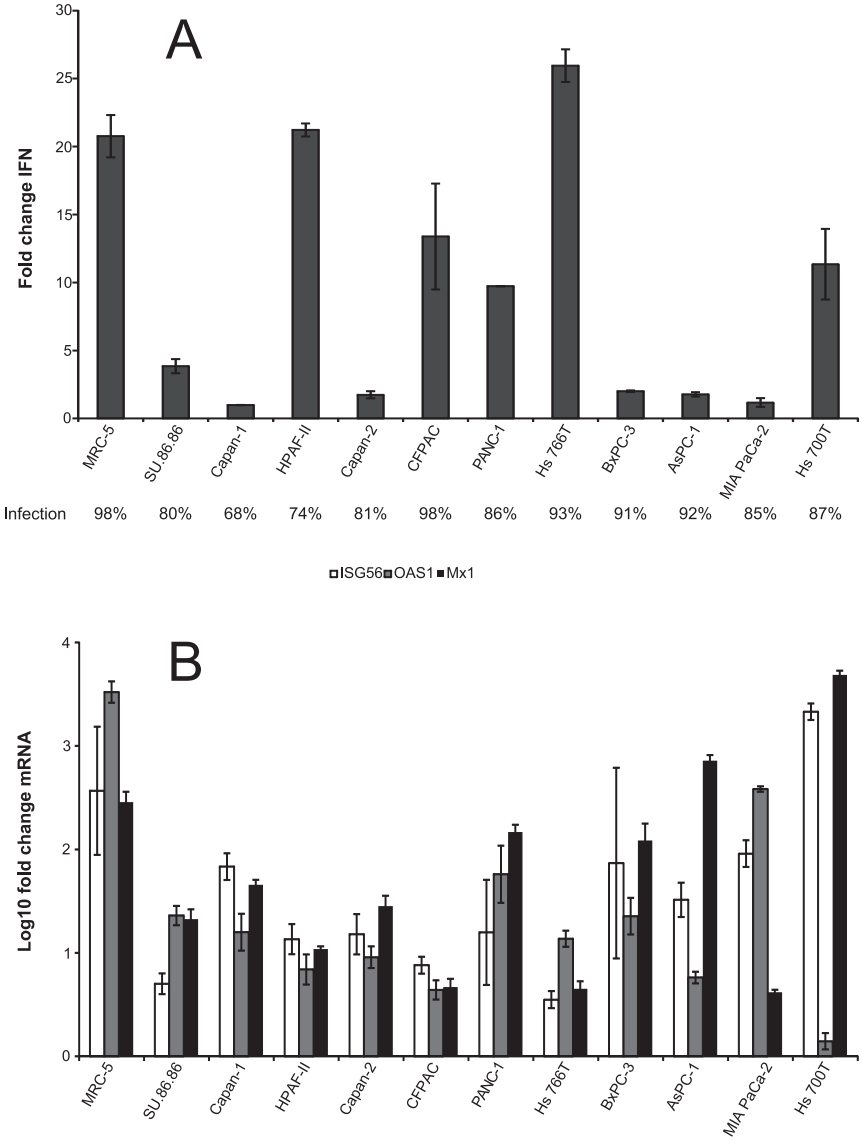


Figure 3

(A) IFN production 24 hours after NDV infection. Cells were either mock inoculated or inoculated with NDV at high m.o.i. After 24 hours, supernatants were tested for IFN content. Results are presented as fold change in IFN content in supernatants of NDV inoculated versus mock inoculated cells. Means and standard deviations of duplicate measurements are plotted. Indicated underneath the cell line names are infection percentages determined by FACS analysis.

(B) Expression levels of anti-viral ISGs upon IFN-β treatment. Cells were either mock treated or treated with 1000 IU of recombinant human IFN-β. After 24 hours, RNA was isolated and real time RT-PCR was performed for ISG56-mRNA (white bars), OAS1-mRNA (grey bars) and Mx1-mRNA (black bars). Results are presented as fold change gene induction of treated versus mock treated cells calculated using the $2^{-\Delta\Delta C}{}_{T}$ method. Means and ranges of triplicate experiments are plotted.

IFN-β pre-treatment can hamper NDV replication and protect from NDV induced cytotoxicity in most HPACs

To study whether the observed differences in IFN signaling also reflected a functional effect on replication of NDV in these cells, multicycle replication curves for NDV were generated in HPACs pre-treated with IFN-β. As shown in figure 4A, pre-treatment of the HPACs with IFN-β decreased virus replication in most cells to levels comparable to resistant HPACs, and replication was delayed in Capan-1 and SU.86.86 cells by 24 hours (figure 4B).

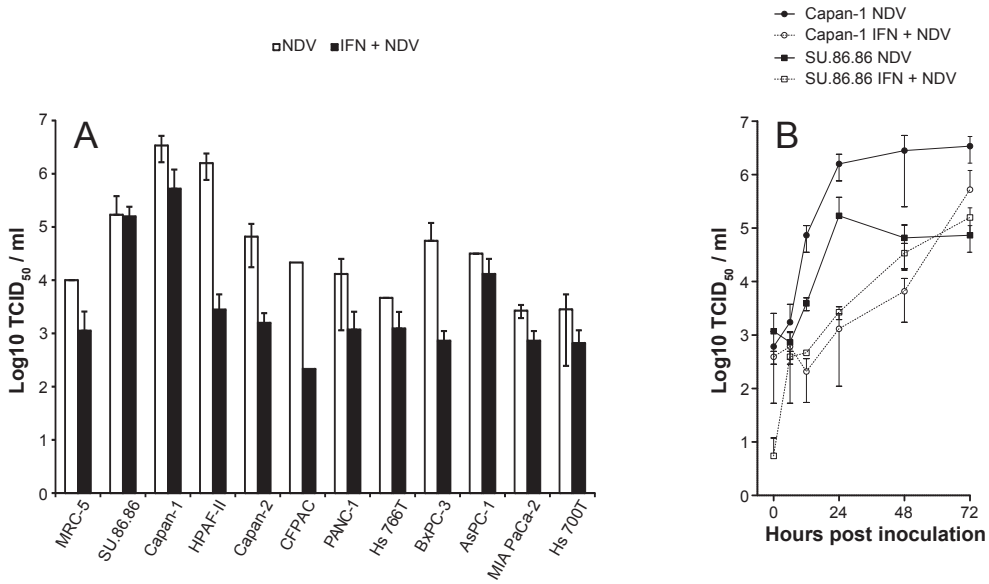


Figure 4

(A) Peak virus titers obtained after infection at low m.o.i. of cells either mock treated (white bars) or IFN-β treated (1000 IU ml^{-1}; black bars) for 24 hours. Samples were taken at different time points and titrated by end-point dilution assay in Vero clone 118 cells. Peak titers are depicted with corresponding standard deviation of two independent titrations.

(B) Growth kinetics of NDV infection in selected HPACs after IFN-β pre-treatment of cells. Cells were mock-treated (straight lines, filled symbols) or treated with 1000 IU ml^{-1} IFN-β (dashed lines, open symbols) for 24 hours. After pre-treatment, multicycle replication kinetics were performed, starting with low m.o.i. infection.

IFN-β pre-treatment also decreased NDV induced cytotoxic effects in most cells (figure 5), while IFN-β did not induce measurable cytotoxicity by itself after 24 hours (data not shown). HPACs like HPAF-II, Capan-2, CFPAC, PANC-1 and Hs 766T showed almost complete survival of cells after IFN-β pre-treatment and subsequent NDV infection. Interestingly, HPAF-II was one of the HPACs that showed complete protection for NDV induced cytotoxicity after exogenous IFN pretreatment, while these cells are capable of producing IFN upon NDV infection without being protected. It is important to note that cells were pretreated with a relatively high amount of exogenous IFN-β (1000 IU) for 24 hours. This in contrast to lower amounts of endogenous IFN without a pretreatment period in cells infected at lower m.o.i. This could explain the seemingly contradictory results in infection experiments with and

without IFN pretreatment. Capan-1 cells, in which IFN-β pre-treatment did not completely block viral replication, still displayed NDV induced cytotoxicity, but at lower levels as compared with cells not pre-treated with IFN-β, while IFN-β pre-treatment displayed no relevant protective effect on SU.86.86 cells. This again indicated that virus replication was important for the cytotoxic effects.

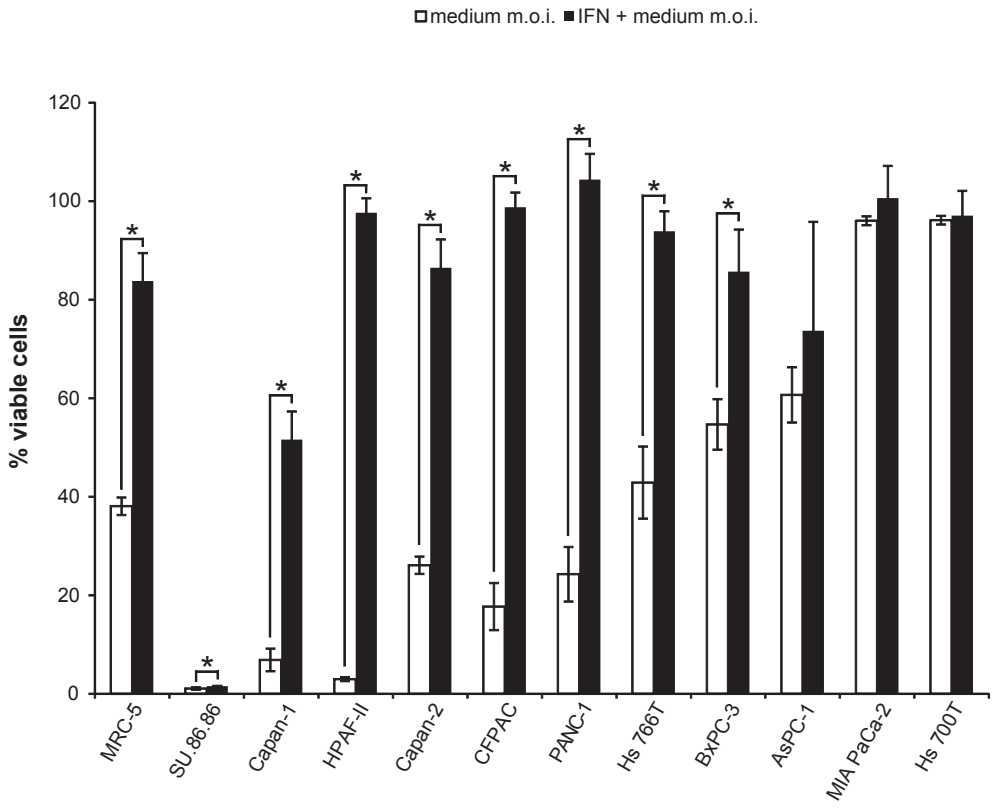


Figure 5: Induced cytotoxicity upon NDV inoculation after IFN-β pre-treatment of HPACs and MRC-5 cells. Cells were either mock inoculated (not shown) or inoculated with NDV at medium m.o.i. after mock treatment (white bars) or IFN treatment for 24 hours (black bars). Cytotoxicity induced by NDV infection was measured daily for 5 days by LDH assay (only day 5 is depicted). Results are presented as percentage of surviving cells as compared to mock inoculated cells, which were considered to be 100% viable. Means and standard deviations of quadruplicate experiments are plotted. * = $p < 0,05$.

Discussion

In our search for new therapies for pancreatic adenocarcinoma, we have focused our attention on the use of NDV as an oncolytic agent. Several wild-type NDV strains (MTH-68, 73-T, Ulster, PV701, HUJ) have been described as being effective oncolytic viruses, killing tumor cells originating from various cancer types *in vitro* as well as *in vivo* [96,113]. However, the success of these viruses in treatment of cancer patients was only marginal [93,101]. The development of reverse genetic techniques has revived the use of oncolytic viruses, and the oncolytic efficiency of NDV has been improved *in vitro* and *in vivo* by either generating viruses with higher virulence [126,195] or generating viruses that express immune modulatory genes [114,115,120,121,188,189].

For a rational design of a recombinant oncolytic NDV to treat patients with pancreatic tumors, we here evaluated the effect of wild-type NDV infection on human pancreatic adenocarcinoma cell lines (HPACs) *in vitro*. A broad panel of 11 different HPACs was chosen in order to study a heterogeneous population of pancreatic tumor cells, which resembles the heterogeneity of these tumors in patients.

Our results demonstrate that variations in NDV replication in the different HPACs generally coincided with differences in induced cytotoxic effects, with cell lines displaying more cytotoxic effects upon better NDV replication. This suggests that virus replication itself is a critical factor for NDV induced cytotoxicity, as was described in earlier studies in other cells [102-107,187,196-198]. This would argue for increasing the fusogenicity of an optimized recombinant NDV for treatment of pancreatic tumors. We observed multicycle replication of a lentogenic strain of NDV in some HPACs without addition of exogenous trypsin. Pancreatic tumor cells have been shown to produce trypsinogen [199,200], and this endogenous present trypsin could explain this observation and possibly differences between HPACs in supporting multicycle NDV replication.

It is thought that replication of oncolytic NDV is tumor specific because tumor cells have defects in the innate immune responses; however, discrepancies for this theory have also been reported [107-109]. Numerous studies have focused on defective IFN production as a reason for tumor-specific NDV susceptibility, however only limited sets of tumor cell lines were used, which could have resulted in non-representative findings [105,106]. By studying a more elaborate panel of HPACs, we convincingly demonstrate that IFN production by the HPACs does not correlate with NDV susceptibility. This is in accordance with findings reported by some other groups [107-109]. Interestingly, a recent study focusing on oncolytic Vesicular stomatitis virus (VSV) infection of a similar panel of HPACs reported mostly the same HPACs to be capable of producing IFN upon viral infection [201]. However, where we could not detect a correlation between IFN production and resistance against NDV infection, this was detected for VSV infection of HPACs.

Besides lack of IFN production, defective IFN signaling through the JAK-STAT pathway has been indicated to be responsible for tumor specific replication of NDV [103-105]. Although our results with 11 different HPACs show a highly variable and generally low anti-viral gene expression pattern after IFN stimulation, we also show that most HPACs can still use IFN to signal to an antiviral state. IFN pretreatment protected the cell lines from NDV replication and induced cytotoxicity. These results indicate that IFN signaling pathways are still functional in most HPACs, and it is possible that while we measured 3 important and well-known ISG-mRNA expressions, we missed others that play a role in anti-viral defense. A gene expression profiling approach could provide more detailed information on the regulation of different ISGs in these HPACs.

IFN induced by other tumor cells or bystander cells could be able to protect tumor cells from viral replication and NDV induced cytotoxic effects. Indeed, earlier reports also showed that IFN pretreatment could hamper NDV replication, although with a slightly lower effectiveness when compared to normal cells [104,105]. We argue that IFN production within a tumor environment could prevent oncolytic NDV from replicating in most tumor cells, thus preventing eradication of heterogeneous tumors.

Few other groups have demonstrated that IFN production and signaling are not the only factors to consider. Cells over expressing anti-apoptotic proteins like Rac1 [108], Livin [109], and Bcl-xL [107] also showed markedly increased susceptibility to NDV replication and virus mediated killing, irrespective of IFN production and signaling. It is possible that differences in apoptotic, necrotic or autophagy pathways account for the differences observed between HPACs. Further research is needed to elaborate on this theory.

IFN not only hampers viral replication, it also has direct anti-proliferative effects on pancreatic cancer cells [110,181,202,203] and it initiates signaling towards a potentially very effective antiviral and antitumor adaptive immune response [182]. It is under debate whether an improved oncolytic NDV should stimulate the IFN pathway [114,115] or suppress the immune system [120]; both approaches have shown to be feasible and effective. As a consequence, tuning of the balance between virulence and interaction with the IFN pathways of oncolytic viruses may be needed depending on the ultimate application.

This study was conducted to obtain more information for a rational design of an improved recombinant oncolytic NDV. On the one hand we studied the heterogeneity of HPACs in response to NDV infection. This allowed us to categorize the cells in three groups, based on susceptibility to NDV infection and induced cytotoxicity. These differences provide a rationale for improving the virulence of NDV, and subsequently test various recombinant NDVs against prototype HPACs of these three groups. In addition, this study, as shown by others, demonstrated that NDV infection and replication is hampered by IFN treatment of tumor cells. This underlines the need to also improve the IFN antagonistic properties of oncolytic NDV. The best recombinant oncolytic NDV *in vitro* could be one that encodes an IFN antagonistic protein and has higher fusogenicity due to inclusion of a different fusion protein. In conclusion, NDV is a promising candidate oncolytic virus for further preclinical testing on pancreatic cancer models, and reverse genetic techniques will enable us to generate NDV with improved oncolytic effect.

chapter 3

Recombinant Immunomodulating Lentogenic or Mesogenic Oncolytic Newcastle Disease Virus for Treatment of Pancreatic Adenocarcinoma

Pascal R.A. Buijs
Stefan van Nieuwkoop
Vincent Vaes
Ron A.M. Fouchier
Casper H.J. van Eijck
Bernadette G. van den Hoogen

Viruses 2015 Jun;7(6):2980-2998

Abstract

Oncolytic Newcastle disease virus (NDV) might be a promising new therapeutic agent for the treatment of pancreatic cancer. We evaluated recombinant NDVs (rNDVs) expressing interferon (rNDV-hIFNβ-F_0) or an IFN antagonistic protein (rNDV-NS1-F_0), as well as an rNDV with increased virulence (rNDV-F_{3aa}) for oncolytic efficacy in human pancreatic adenocarcinoma cells. Expression of additional proteins did not hamper virus replication or cytotoxic effects on itself. However, expression of interferon, but not NS1, resulted in loss of multicycle replication. Conversely, increasing the virulence (rNDV-F_{3aa}) resulted in enhanced replication of the virus. Type I interferon was produced in high amounts by all tumor cells inoculated with rNDV-hIFNβ-F_0, while inoculation with rNDV-NS1-F_0 resulted in a complete block of interferon production in most cells. Inoculation of human pancreatic adenocarcinoma cells with rNDV-F_{3aa} caused markedly more cytotoxicity compared to rNDV-F_0, while inoculation with rNDV-hIFNβ-F_0 and rNDV-NS1-F_0 induced cytotoxic effects comparable to those induced by the parental rNDV-F_0. Evaluation *in vivo* using mice bearing subcutaneous pancreatic cancer xenografts revealed that only intratumoral injection with rNDV-F_{3aa} resulted in regression of tumors. We conclude that although lentogenic rNDVs harboring proteins that modulate the type I interferon pathway do have an oncolytic effect, a more virulent mesogenic rNDV might be needed to improve oncolytic efficacy.

Introduction

Patients with pancreatic adenocarcinoma still have very poor survival rates, and current therapies are of limited effect [57,62]. Oncolytic Newcastle disease virus (NDV) could be a promising new therapeutic agent for the treatment of pancreatic cancer. NDV has been described as a naturally occurring oncolytic virus as early as 1952 [88-90]. Since then, numerous clinical trials have employed wild type NDV strains either as a direct oncolytic agent, or as an oncolysate vaccine for treatment of patients with various types of advanced stage cancer [91-96]. Results of these early trials have been relatively disappointing, illustrated by the lack of further development of these treatment strategies. With the advent of recombinant DNA techniques it has become possible to genetically engineer NDV [112], and interest in the use of recombinant NDV (rNDV) as an oncolytic virus has revived over the last decade [113].

NDV selectively replicates in and destroys tumor cells while sparing normal cells, presumably because of defective interferon (IFN) signaling pathways of the innate immune system in tumor cells. Previously, we reported that a lentogenic wild type NDV strain replicated in and was cytotoxic for 11 human pancreatic adenocarcinoma cell lines (HPACs) with high variability. These differences in the response of HPACs were not due to defects in innate immunity pathways as a number of these cell lines produced type I IFN upon NDV infection [204]. We, and others, have also shown that IFN produced by tumor cells and the normal cells surrounding the tumor cells exerts anti-proliferative, pro-apoptotic and pro-inflammatory effects in tumor cells, possibly attributing to oncolytic efficacy [110,111,125,181,182]. Therefore, rNDVs armed with the IFN gene might result in a virus with higher oncolytic efficacy. However, the antiviral activity of IFN hampers NDV replication in tumor cells [204]. Viruses use different strategies to counteract the IFN pathway to increase infectivity and replication efficiency. The non-structural NS1 protein of influenza A virus is one of the most potent antagonists of the IFN response of the innate immunity characterized to date [183,184]. Oncolysis of tumor cells depends on efficient virus infection and replication, therefore, incorporation of an IFN antagonist, such as the NS1 protein of Influenza A, in the genome of rNDV could be a way to improve the oncolytic efficacy of rNDV.

NDV strains are categorized in three different groups based on the severity of the disease they cause in birds: lentogenic, mesogenic, and velogenic and this classification correlates with their oncolytic properties in cancer cells. Increasing the virulence of rNDV has been shown to improve direct oncolytic efficacy most, resulting in preclinical studies using mesogenic virulent rNDVs expressing transgenes, such as IFN and the NS1 protein of influenza virus [113]. However, virulent NDV strains pose an environmental risk, as birds (specifically poultry) are very susceptible to infection with mesogenic or velogenic strains.

We hypothesize that arming lentogenic rNDV with IFN-modulating genes, either accelerating or blocking the IFN response, would improve the oncolytic effect of NDV sufficiently to circumvent using virulent rNDV with the associated potential biosafety risks. In the present study we compared lentogenic rNDVs expressing either hIFNβ or the IFN antagonistic protein (NS1) of the Influenza virus with those of a virulent rNDV (rNDV-F_{3aa}) for direct oncolytic efficacy.

Material and Methods

pNDV cloning

A full length cDNA clone of lentogenic NDV strain La Sota (pNDV-F_0) and expression plasmids pCIneo-NP, pCIneo-P and pCIneo-L, as well as cloning plasmid pGEM-T-PM-cassette were kindly provided by Ben Peeters from the Central Veterinary Institute of Wageningen UR, the Netherlands [112,205]. To create a full length rNDV-F_0 expressing either GFP, hIFN-β or NS1 protein of influenza strain A/PuertoRico/8/1934, a DNA fragment containing the open reading frame (ORF) encoding these proteins was inserted into the intergenic region between the P and M genes of pNDV-F_0 flanked by appropriate NDV-specific transcriptional gene-start and gene-end signals. Cloning strategies resulted in full length NDV genomes that complied with the rule-of-six [206]. To create a full length NDV cDNA clone with a multibasic cleavage site in the fusion protein, the amino acid sequence of the protease cleavage site was changed from ^{112}GRQGR↓L^{117} (lentogenic) to ^{112}RRQRR↓F^{117} (mesogenic; pNDV-F_{3aa}) by means of site-directed mutagenesis as described earlier [114]. Full length NDV plasmids were sequenced using a 3130*xL* Genetic Analyzer (Life Technologies, Bleiswijk, the Netherlands) to exclude incidental mutations that could arise during the cloning process.

Recombinant virus rescue

Recombinant NDVs were rescued using a method adapted from the original method described previously [112]. Briefly, BSR-T7 cells were transfected with 5 µg full length pNDV, 2.5 µg pCIneo-NP, 1.25 µg pCIneo-P and 1.25 µg pCIneo-L using 10 µl lipofectamine (Life Technologies). Three days later, 200 µl BSR-T7 supernatant was injected into the allantoic cavity of 10 day old specified pathogen free (SPF) embryonated chicken eggs. After incubation in a humidified egg incubator at 37°C for two or three days (mesogenic or lentogenic rNDVs, respectively), allantoic fluid was harvested and presence of virus demonstrated by hemagglutination assay [207]. Samples displaying hemagglutination were passaged once more in eggs to increase virus titer, and allantoic fluid was harvested after 2 or 3 days. Pooled fresh allantoic fluid was purified and concentrated by ultracentrifugation at 27.000 rpm for two hours at 4°C using a 30%/60% sucrose gradient. rNDV stocks were stored at -80°C.

Recombinant influenza virus A/PuertoRico/8/34 was rescued and titrated as described before [208].

Titration of rNDV

Virus stocks were titrated by end point dilution assay in Vero-118 cells, as described before [204]. All infection experiments were performed in the presence of reduced concentration FBS HyClone (3%; Thermo Fischer Scientific, Breda, The Netherlands), without the addition of exogenous trypsin.

Characterization of rNDV

RNA from virus stocks was isolated using the High Pure RNA Isolation kit (Roche, Woerden, the Netherlands) following the manufacturer's instructions and reverse transcribed into cDNA using a two-step protocol, as described before [204]. Amplicons generated with RT-PCR using primers flanking the P-M intergenic or F protein cleavage site region were sequenced to confirm the sequence of the inserted gene between the P and M genes and the protease cleavage site of the fusion protein (F_0/F_{3aa}). Expression of NS1 protein was assayed in Vero-118 cells (mock-)inoculated at m.o.i. 3 with either rNDV-F_0,

rNDV-NS1-F_0 or positive control influenza A/PuertoRico/8/34. Whole cell-lysates taken 18 hours post-inoculation were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a Hybond-C Extra nitrocellulose membrane (GE Healthcare Life Sciences, Diegem, Belgium). Membranes were stained for NS1 using primary monoclonal mouse anti-influenza A NS1 antibody (AB_2011757 / sc-130568; 1:1000 dilution; Santa Cruz Biotechnology, Heidelberg, Germany) and secondary peroxidase-conjugated polyclonal goat anti-mouse antibody (P0447; 1:2000 dilution; Dako, Heverlee, Belgium) or for β-tubulin using peroxidase-conjugated monoclonal rabbit anti-β-tubulin antibody (9099; 1:1000 dilution; Cell Signaling Technology, Leiden, the Netherlands). Chemiluminescent signals were generated using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences) following manufacturer's instructions and detected using a ChemiDoc MP system (Bio-Rad, Veenendaal, the Netherlands).

Cell lines and culture conditions

The HPACs SU.86.86, HPAF-II, BxPC-3, PANC-1, MIA PaCa-2, Hs 700T, CFPAC, Hs 766T, AsPC-1, and Capan-2 were obtained from the American Type Culture Collection and authenticated using Short Tandem Repeat profiling [190]. Cells were used not more than 25 passages after thawing. HPACs, non-neoplastic human lung fibroblasts MRC-5, Vero-118, BSR-T7 and 293T cells were cultured as described before [204,209].

Replication curves

For MIA PaCa-2 cells 3.0×10^6 and for SU.86.86, HPAF-II, BxPC-3, PANC-1 and Hs 700T 1.5×10^6 cells in T25 flasks (Corning, Amsterdam, The Netherlands) were inoculated at m.o.i. 0.1, in triplicate. After a 1 h incubation, cells were washed three times with PBS and fresh medium was added. At time points 0, 2 12, 24, 48, and 96 h after washing, duplicates of 100 µL supernatant were collected, mixed with 100 µL 50% (w/v) sucrose, and frozen at −80 °C. Samples were titrated by end point dilution assay in quadruplicate as described before [204].

RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR) for hIFNβ mRNA

Twenty-four hours after inoculation with rNDV at m.o.i. 3, cells were lysed with 300 µL lysis buffer of the Total Nucleic Acid Isolation kit (Roche) and RNA was isolated using a MagNA Pure LC machine (Roche) following the manufacturer's instructions. qRT-PCR (30 cycles) was performed with 20 µL RNA in an ABI PRISM 7000 Sequence Detection System (Life Technologies), using TaqMan gene expression assay for human IFNβ1 (Hs00277188_s1, Life Technologies). The primers in this assay map to the extreme 3' end of the hIFNβ gene (Hs00277188_s1, www.lifetechnologies.com), with the reverse primer annealing downstream of the stop codon of the hIFNβ coding sequence. This region is not present in the rNDV-hIFNβ-F_0 virus. Therefore, the qRT-PCR assay is not able to detect IFN-mRNA transcribed from the virus, and the assay will only detect endogenous transcribed IFN-mRNA. To detect both endogenous and exogenous expressed IFN, primers and probes mapping in the IFN coding region were used with an in-house developed assay and β-actin was used as household gene. The sequences of the primers and probes for the IFN-coding region and β-actin have been described before [210]. Results are presented as fold change of inoculated samples versus mock-inoculated samples (duplicates), calculated using the $2^{-\Delta\Delta C}{}_T$ method [194].

IFN measurement with luciferase bioassay

Cells were inoculated in triplicate at m.o.i. 3 and 24 hours post inoculation, supernatants were collected and assayed for IFN contents using a bioassay as described before [204]. IFN produced by NDV-inoculated cells is presented as fold change in luminescence compared to mock inoculated cells.

Cytotoxicity assay

Quadruplicates of 2 x 10^4 cells per well in 96-well plates (Corning) were either mock inoculated or inoculated with rNDV at different m.o.i. (range 0.0001 - 100). After 48 hours, 100 µl fresh medium was added. At time point 120 hours post inoculation, cell viability was determined using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Leiden, the Netherlands) as described before [204]. Prism for Windows version 5.03 (GraphPad software, La Jolla, CA) was used to analyze data using the *log(inhibitor) vs. normalized response (variable slope)* function to obtain LD_{50} values. The extra sum-of-squares F test was used to compare LD_{50} values. P-values of <0.05 were considered statistically significant.

Ethics statement

All experiments involving animals were conducted strictly according to European guidelines (EU directive on animal testing 86/609/EEC) and Dutch legislation (Experiments on Animals Act, 1997). The experimental protocol was reviewed and approved by an independent animal experimentation ethical review committee, not affiliated with Erasmus MC (DEC consult number EMC2921).

Animals and experimental design

Groups of 30 athymic nude mice (strain NMRI-Foxn1^{nu}; Charles River, Sulzfeld, Germany) were injected subcutaneously in their flank with 3 x 10^6 SU.86.86, BxPC-3 or MIA PaCa-2 cells. Tumor width (w) and length (l) were measured using a digital caliper (VWR International, Amsterdam, the Netherlands) and tumor volume was calculated using the modified ellipsoid formula w^2 x l / 2 [211,212]. Tumors were allowed to grow until the average tumor volume per group reached 50 mm^3 (3-5 weeks) and animals were appointed randomly stratified for tumor size to one of five treatment groups: PBS, rNDV-F_0, rNDV-hIFNβ-F_0, rNDV-NS1-F_0 or rNDV-F_{3aa}. Animals were injected intratumorally every other day for a total of 4 injections with 5 x 10^7 $TCID_{50}$ rNDV in a total volume of 50 µl or an equivalent volume PBS. After injection, tumor sizes were recorded two times weekly. Animals were euthanized if tumor volume exceeded 2000 mm^3, non-healing tumor ulcerations or excessive weight loss occurred, and ultimately 40 days after first injection with rNDV or PBS. Last observed tumor volumes were carried forward to calculate median volumes per group. Continuous data were compared between the groups using the Mann–Whitney U-test. One-sided P-values of <0.05 were considered statistically significant.

Results

Cloning, rescue and characterization of recombinant NDVs

Five different rNDVs were generated which were used throughout this study: rNDV-F_0, rNDV-GFP-F_0, rNDV-hIFNβ-F_0, rNDV-NS1-F_0 and rNDV-F_{3aa} (Figure 1a). Amplicons of the P-M intergenic (Figure 1b) and F protein cleavage site were sequenced to confirm identity of the rNDV stocks. Upon titration of virus stocks, only infection with rNDV-F_{3aa} lead to syncytia formation in cell cultures, characteristic for infection with mesogenic NDV (data not shown). In addition, western blot assay confirmed the expression of NS1 by rNDV-NS1-F_0 (Figure 1c).

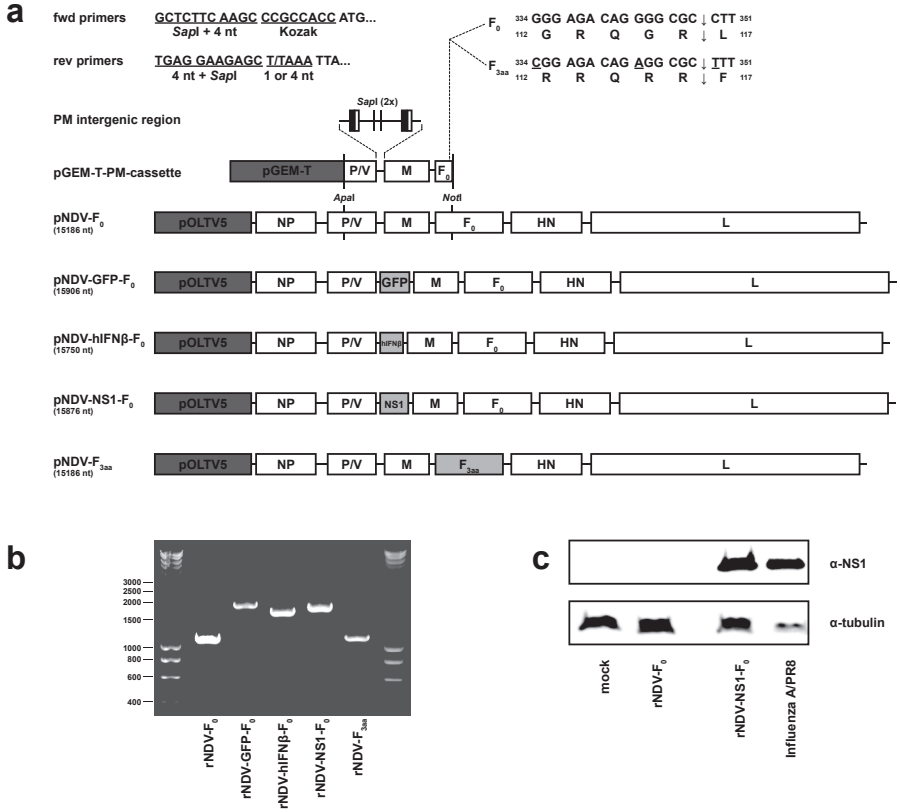


Figure 1. Generation and characterization of rNDVs. (a) Cloning strategy to obtain full length NDV plasmids. The nucleotide length of the NDV genome is noted below the plasmid names and all full length plasmids and pGEM-T-PM-cassette are drawn to scale. The 3'-terminal leader, intergenic regions and 5'-terminal trailer are shown as horizontal lines. Gene-end and gene-start sequences in the PM intergenic region are depicted as vertical black and white rectangles, respectively. fwd: forward; rev: reverse; nt: nucleotides; pGEM-T: plasmid backbone of pGEM-T-PM-cassette; NP: nucleoprotein gene; P: phosphoprotein gene; V: accessory V gene; M: matrix gene; F: fusion gene; HN: hemagglutinin-neuraminidase gene; L: large protein gene. **(b)** PCR product of PM intergenic region. RNA of indicated rNDV stocks was reverse transcribed into cDNA and the PM intergenic region was amplified with RT-PCR using flanking primers. **(c)** Western blot for expression of NS1 protein. Whole cell lysates of Vero-118 cells (mock-) inoculated with indicated viruses were assayed for expression of the NS1 protein or tubulin protein.

Replication kinetics of rNDVs

To test whether the expression of additional transgenes or a change in cleavability of the F protein had an effect on replication kinetics of rNDVs, replication curves were generated for the different viruses on six different HPACs. HPACs were selected based on replication efficiency of the wild type virus as reported in our previous study [204].

Upon inoculation, no significant differences were observed for replication of the lentogenic viruses rNDV-F_0, rNDV-GFP-F_0, and rNDV-NS1-F_0 on all cells. SU.86.86 and HPAF-II cells supported replication of these viruses to high titers, while replication was less efficient in the other four cell lines (Figure 2).

Inoculation with rNDV-hIFNβ-F_0 resulted in attenuated replication in all cells, with significant lower titers in SU.86.86, HPAF-II and BxPC-3 cells compared to inoculation with rNDV-F_0.

In contrast, inoculation with the mesogenic virus rNDV-F_{3aa} resulted in efficient multicycle replication in most cell lines. Inoculation of SU.86.86 with rNDV-F_{3aa} resulted in significant higher titers at t = 24 compared to those of rNDV-F_0 and after this time point titers of rNDV-F_{3aa} declined, due to the loss of cells as an effect of the efficient replication. Inoculation with rNDV-F_{3aa} of BxPC-3, PANC-1, MIA PaCa-2, and Hs 700T cells resulted in significant higher titers at t = 24, 48 and 96 compared to those of rNDV-F_0. Although the titers for rNDV-F_{3aa} in HPAF-II cells were higher than those of the other viruses in HPAF-II cells these differences were not significant (Figure 2).

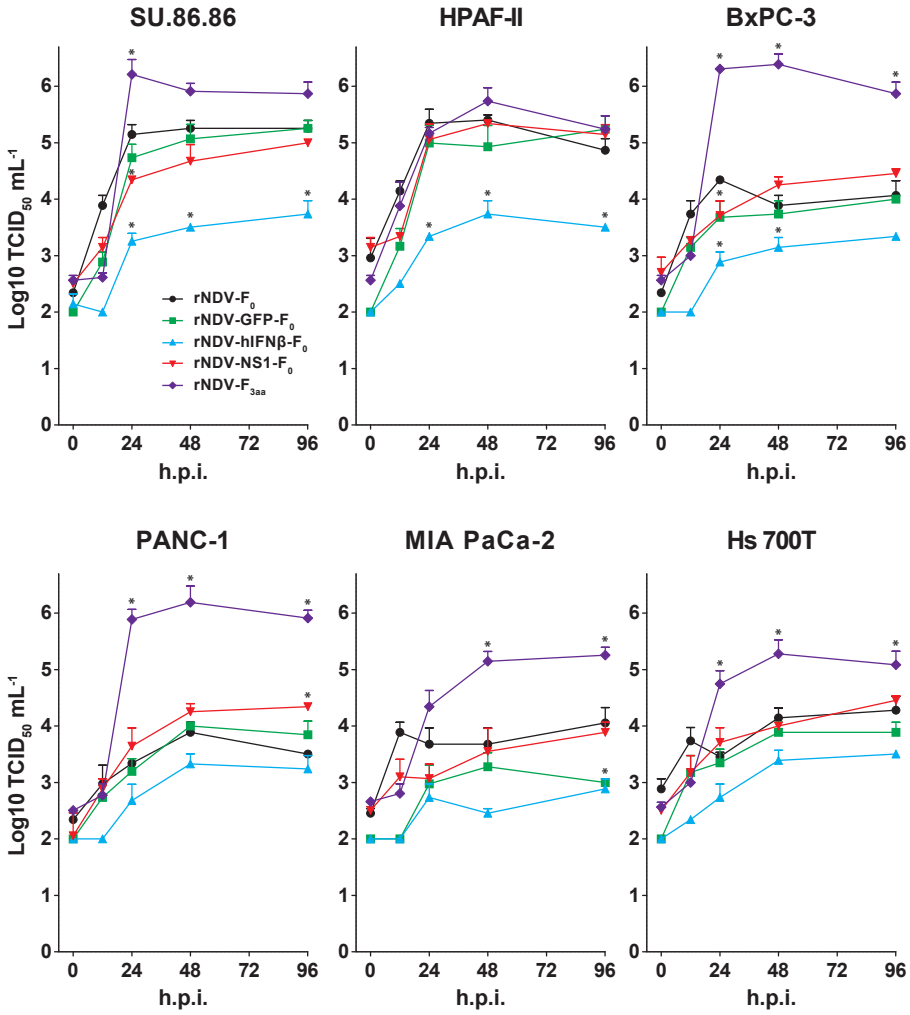


Figure 2. Replication kinetics of rNDVs in 6 different human pancreatic adenocarcinoma cell lines (HPACs). Cells were inoculated in triplo with m.o.i. 0.1 and samples were taken at indicated time points and titrated by end-point dilution assay in Vero-118 cells. Means and standard deviations of triplicate titrations are plotted. H.p.i.: hours post inoculation. * = $p < 0.05$ vs. rNDV-F_0 (one-way ANOVA + Bonferroni post-test), tested for time points 24, 48 and 96 h.p.i.

Modulation of IFN response by rNDVs

As IFN has a direct effect on oncolysis, the capacity of the viruses to induce, enhance or block IFN production was evaluated. Ten HPACs, as well as Vero cells (lacking the endogenous IFN genes) and MRC-5 cells (fully IFN competent), were inoculated with the five different rNDVs and at 24 hours after inoculation both hIFNβ protein production as well as gene expression levels were measured. The gene expression assay for endogenously expressed IFN maps to the extreme 3" end of the hIFNβ gene, with the reverse primer annealing downstream of the stop codon of the hIFNβ coding sequence. This region is not present in the rNDV-hIFNβ-F_0 virus, thus only the endogenous transcribed IFN-mRNA is

detected and not IFN-mRNA transcribed from the rNDV-hIFNβ-F_0 virus.

Inoculation with rNDV-F_0 or rNDV-GFP-F_0 resulted in upregulation of endogenous hIFNβ gene expression in MRC-5 cells and in eight of the HPACs. No upregulation was observed in SU.86.86, MIA PaCa-2 and, as expected, Vero cells. These findings were mostly in agreement with our findings using wild type NDV, except for BxPC-3 cells [204]. Inoculation with rNDV-hIFNβ-F_0 did not change this pattern of endogenous hIFNβ gene expression. An in-house assay detecting expression of both endogenous and exogenous IFN genes revealed similar expression levels as detected with the validated endogenous assay for cells inoculated with rNDV-F_0 or rNDV-GFP-F_0, but, as expected, higher expression levels for rNDV-hIFNβ-F_0 inoculated cells (data not shown). With this assay, expression of exogenous expressed hIFNβ mRNA was also detected in rNDV-GFP-F_0 inoculated SU.86.86, MIA PaCa-2 and Vero cells, in which endogenous IFN genes are absent or not upregulated (Figure 3a). Inoculation with rNDV-NS1-F_0 resulted in a marked decrease in endogenous hIFNβ gene expression levels in HPAF-II, BxPC-3, CFPAC, AsPC-1, Capan-2 cells, and MRC-5 fibroblasts, while no differences were observed in PANC-1 and Hs 766T cells, compared to expression levels in these cells inoculated with rNDV-F_0.

To test whether the differences in expression of the hIFNβ genes also resulted in differences in protein production, functional IFN protein content was determined in the supernatants of inoculated cells. Due to biosafety issues, we could not measure IFN in supernatants of cells inoculated with rNDV-F_{3aa}.

Upon inoculation with rNDV-F_0, cells demonstrated variation in the extent of IFN production. Six of the 10 HPACs produced significant less IFN compared to IFN-competent MRC-5 cells, while HPAF-II, CFPAC, and Hs 766T had a similar range of IFN production. Incorporation of GFP in the genome of rNDV-F_0 did not change this pattern in most HPACs. Only HPAF-II and Hs 766T cells produced significant less IFN upon inoculation with rNDV-GFP-F_0 compared to inoculation with rNDV-F_0, while the opposite was observed for CFPAC cells.

Upon inoculation with rNDV-hIFNβ-F_0, all cells produced significant higher amounts of IFN compared to inoculation with rNDV-F_0, due to viral expression of the exogenous hIFNβ gene (Figure 3b). This exogenously expressed IFN did not induce upregulation of endogenous hIFNβ gene expression in SU.86.86, MIA PaCa-2 and, of course, Vero-118 cells (as seen in Figure 3a). Inoculation of HPACs and MRC-5 cells with rNDV-NS1-F_0 resulted in a significant decrease of IFN production in all these cells as compared to cells inoculated with rNDV-F_0 (Figure 3b).

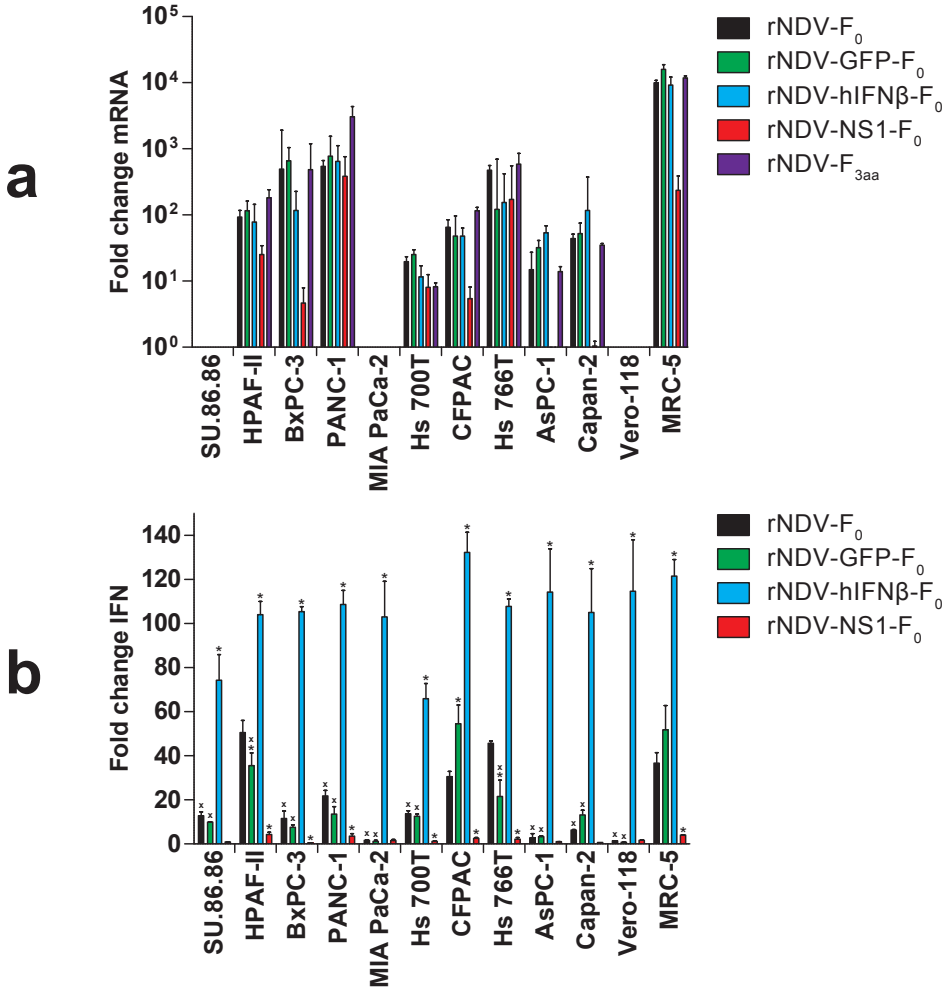


Figure 3. IFN responses upon inoculation with rNDVs. Cells were either mock inoculated or inoculated with the indicated rNDV at m.o.i. 3. After 24 hours: **(a)** RNA was isolated and quantitative real-time polymerase chain reaction (qRT-PCR) was performed for hIFNβ mRNA. Results are presented as fold change gene induction of treated versus mock treated cells calculated using the $2^{-\Delta\Delta C}{}_T$ method [194]. Means and ranges of triplicate experiments are plotted. **(b)** Supernatants were tested for functional IFN content using an ISRE-luc bioassay [204]. Results are presented as fold change in luminescence compared to mock inoculated cells. Means and standard deviations of triplicate experiments are plotted. x = $p < 0.05$ vs. MRC-5 (one-way ANOVA + Bonferroni post-test), * = $p < 0.05$ vs. rNDV-F_0 (one-way ANOVA + Bonferroni post-test).

In vitro cytotoxicity

To evaluate the effect of increasing the virulence or incorporation of hIFNβ or NS1 in the genome of rNDV on direct oncolysis, HPACs and MRC-5 fibroblasts were inoculated with serial dilutions of the viruses and the median lethal dose (LD_{50}) was determined for each rNDV-cell line combination. Mean LD_{50} levels for rNDV-F_0 and rNDV-GFP-F_0 were generally comparable between cell lines, although SU.86.86, Hs 700T, Capan-2 and MRC-5 demonstrated significant lower cytotoxicity upon inoculation with rNDV-GFP-F_0 compared to rNDV-F_0, while CFPAC cells demonstrated higher cytotoxicity for rNDV-GFP-F_0 compared to rNDV-F_0 (* above green bars in Figure 4). This indicates that incorporation of inert transgenes might have some effect on oncolytic efficacy, but with similar variation in reaction between the different cell lines.

Compared to rNDV-F_0, expression of NS1 from rNDV-NS1-F_0 did not improve the oncolytic effect for most cells. Equal LD_{50} values were detected for rNDV-F_0 and rNDV-NS1-F_0 in HPAF-II, Hs 700T, Hs 766T, and Capan-2 cells, with even higher LD_{50} values in the other cells. Compared to rNDV-F_0, expression of exogenous IFN from rNDV-hIFNβ-F_0 decreased the oncolytic effect significantly in SU.86.86 cells and Hs 766T cells, but increased oncolysis significantly in MIA PaCa-2, Capan-2 and MRC-5 cells. Although statistical analyses revealed differences in the oncolytic effect between rNDV-GFP-F_0, rNDV-NS1-F_0 and rNDV-hIFNβ-F_0, with variation in the response of the different cells, the results shown in Figure 4 demonstrate that inoculation with the more virulent rNDV-F_{3aa} results in a clear improvement of the oncolytic effect.

Inoculation with rNDV-F_{3aa} resulted in a significant increase in oncolysis in all HPACs when compared to inoculation with rNDV-F_0 (* above purple bars) for 7 out of 10 HPACs (SU.86.86, PANC-1, Hs 700T, CFPAC, Hs 766T, AsPC-1, Capan-2) when compared to rNDV-NS1-F_0 (^ above red bars), and for 7 out of 10 HPACs (SU.86.86, HPAF, BxPC-3, PANC-1, Hs 766T, AsPC-1 and Capan-2) when compared to rNDV-hIFNβ-F_0 (# above blue bars). Compared to rNDV-hIFNβ-F_0, which revealed improved oncolysis in MIA PaCa-2 and Capan-2 cells, rNDV-F_{3aa} had significantly increased oncolytic effect on Capan-2 cells but an equal effect on MIA PaCa-2 cells. In general, HPAF-II, MIA PaCa-2 and Hs 700T were most resistant to the oncolytic effects induced by the five viruses, including the virulent rNDV-F_{3aa}.

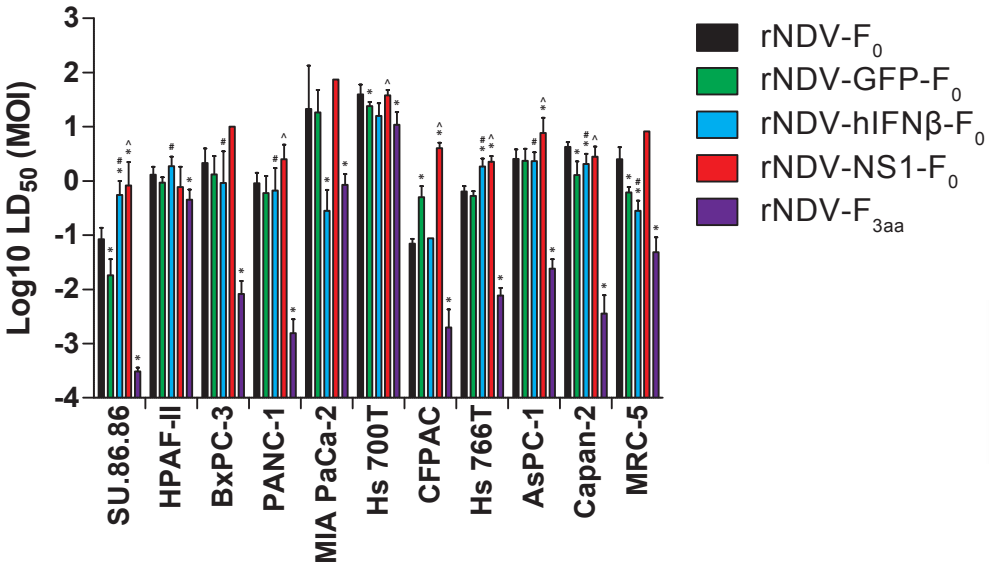


Figure 4. Median LD_{50} upon inoculation with rNDVs. Cells were either mock inoculated (not shown; set as 100% viable) or inoculated with rNDV at different m.o.i. (range 0.0001-100). Cytotoxicity was measured after 5 days by LDH assay and LD_{50} values were calculated. Means and ranges of LD_{50} calculations are plotted. * = $p < 0.05$ rNDV-GFP-F_0, rNDV-hIFNβ-F_0, rNDV-NS1-F_0 and rNDV-F_{3aa} as compared to rNDV-F_0. # = $p < 0.05$, rNDV-F_{3aa} compared to rNDV-hIFNβ-F_0. ^ = $p < 0.05$ rNDV-F_{3aa} compared to rNDV-NS1-F_0.

In vivo efficacy: rNDV-F3aa effective in multiple models

After having shown oncolytic and immunomodulating efficacy *in vitro*, we extended our evaluation of rNDVs to *in vivo* experiments in immune-deficient subcutaneous xenograft mouse models for pancreatic adenocarcinoma. Before starting treatment experiments, toxicity of all viruses was tested in small groups of mice (n=3), using per group escalating doses starting from 1 x 10^6 $TCID_{50}$ up to four times 5 x 10^7 $TCID_{50}$ injected intravenously or subcutaneously. None of the injected mice showed adverse effects or excessive weight loss during these experiments, and we concluded it was safe to inject mice with the highest dose. Also, based on a separate pilot experiment, we decided to inject mice intratumorally with rNDV since this gave better treatment responses compared to intravenous injection.

SU.86.86 tumor xenografts were resistant to all rNDV treatments, even to multiple injections with mesogenic rNDV-F_{3aa} (Figure 5). While BxPC-3 and MIA PaCa-2 tumor xenografts were resistant to injection with rNDV-F_0, rNDV-hIFNβ-F_0 and rNDV-NS1-F_0, they did respond to injection with rNDV-F_{3aa}. Treatment with rNDV-F_{3aa} resulted in tumor regression in 5 out of 6 animals and median tumor sizes were significantly smaller starting at day 13 and 10 after the first injection for BxPC-3 and MIA PaCa-2 tumor xenografts respectively ($P < 0.05$). Upon necropsy of these animals, only very small residual tumors were found, as opposed to the mostly large tumors in animals treated with either PBS or lentogenic rNDV injections.

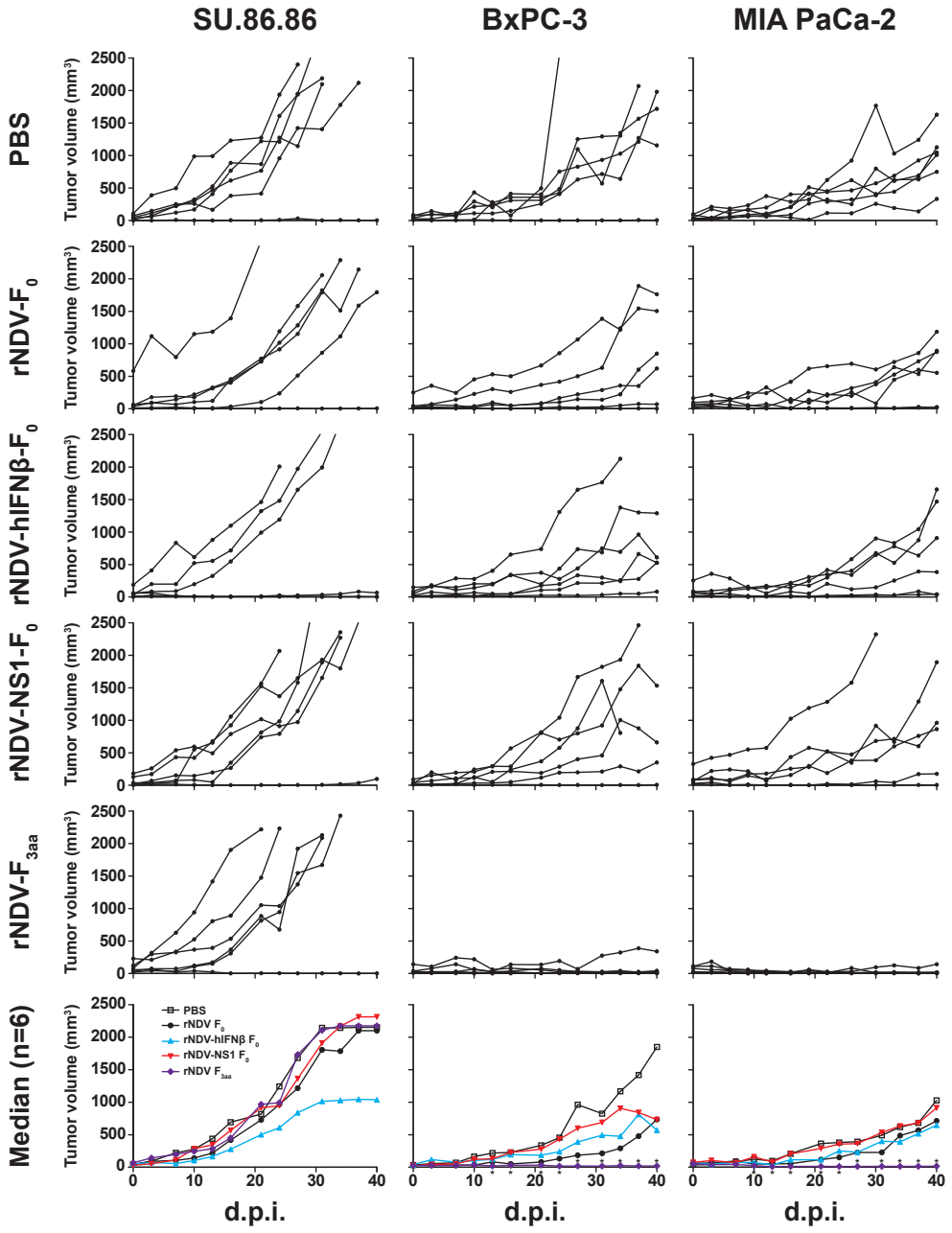


Figure 5. Efficacy of rNDV treatment in a mouse model using SU.86.86, BxPC-3 or MIA PaCa-2 subcutaneous tumor xenografts. Animals were treated and evaluated as described in the material and methods section. Graphs with specific xenograft-treatment combinations depict individual mouse tumor volumes with black lines and dots. Last observed tumor volumes were carried forward to calculate median volumes per group in the lowest plots. *: $P < 0.05$ as compared to PBS group. D.p.i.: days post first injection.

Discussion

In our efforts to further develop oncolytic virotherapy for pancreatic cancer, we focused our attention on the use of recombinant NDVs. In our previous study, we evaluated the efficacy of a wild type lentogenic oncolytic NDV strain in a panel of 11 HPACs. This demonstrated a high degree of variation between the cells in their response to inoculation with NDV, not only in oncolysis but also in activation of the IFN response [204]. We hypothesized that increasing the oncolytic effects of NDV would overcome this variability. Various strategies have been reported to improve the efficacy of oncolytic rNDVs: transfer of therapeutic or immunomodulating transgenes [114,115,120-122], targeting of tumor cells with modified attachment proteins [123,124], and increasing virulence by increasing the cleavability of the F protein [114-119]. As increased virulence can raise biosafety issues, we aimed to improve the direct oncolytic effect of non-virulent (lentogenic) rNDVs by expressing IFN modulating genes and we compared their efficacy with a more virulent (mesogenic) rNDV.

Expression of a non-modulating transgene (GFP) or the NS1 protein of the Influenza A virus from a lentogenic rNDV did not change the replication kinetics of rNDV-F_0 in a set of six HPACs, but expression of high levels of exogenous hIFNβ from rNDV-hIFNβ-F_0 hampered virus replication in susceptible HPACs. This was not surprising, since we showed earlier that HPACs have mostly intact IFN signaling pathways and replication of NDV is sensitive to IFN treatment [204]. In contrast to the lentogenic viruses, the mesogenic rNDV-F_{3aa} was capable of multicycle replication in all HPACs tested. This higher efficiency in replication correlated with higher cytotoxic effects for NDV-F_{3aa} in most HPACs, indicating this to be the oncolytic rNDV with the highest oncolytic efficacy. This is in agreement with other studies that reported on the oncolytic efficacy of mesogenic rNDVs [114-119]. However, three HPACs (HPAF-II, MIA PaCa-2, and Hs 700T) remained somewhat resistant to rNDV-F_{3aa}-induced cytotoxicity, and it remains to be determined why some cells are more resistant than others. Future experiments should elaborate more on the etiology of differences in susceptibility of HPACs for the oncolytic effects of NDV. These experiments should not only focus on innate immunity, but also on differences in apoptotic, necrotic, autophagy and/or immunogenic cell death pathways. Elucidating knowledge on the traits defining susceptibility to NDV induced oncolytic effects would allow improvement of oncolytic NDV to also attack relatively resistant tumor cells.

As previously reported for wild type NDV, inoculation with rNDV-F_0 resulted in IFN production by a number of HPACs, but this did not always correlate with virus replication kinetics or oncolytic effects induced by rNDV-F_0 [204]. Inoculation with rNDV-NS1-F_0 resulted in markedly reduced hIFNβ gene expression levels in most HPACs capable of expressing endogenous hIFNβ and, more interestingly, in almost complete absence of IFN production in almost all HPACs. Indeed, it is known that NS1 of influenza A is a potent blocker of IFN induction by, among other mechanisms, suppressing RIG-I receptor signaling, IRF3 dimerization and subsequent IFNβ promoter activation [183,184]. Expression of exogenous hIFNβ from rNDV-hIFNβ-F_0 did not lead to increased expression levels of the endogenous hIFNβ gene, but did result in production of high amounts of hIFNβ protein. This illustrates that lentogenic rNDV-F_0 is very suitable as a (transient) gene therapy vector, as the (additional) production of IFN can only be attributed to viral hIFNβ gene expression. Interestingly, the lower replication of rNDV-hIFNβ-F_0 did not always result in significant lower cytotoxicity in most HPACs (such as MIA Paca-2 cells), indicating that hIFNβ might have a cytotoxic effect by itself. A recent publication demonstrated

that most HPACs are susceptible to exogenous hIFNβ treatment, but SU.86.86 cells were found to be relatively resistant [111]. In line with these findings, inoculation of SU.86.86 cells with rNDV-hIFNβ-F_0 lead to lower cytotoxicity as compared to inoculation with rNDV-F_0. Apparently, rNDV-hIFNβ-F_0 replication and resulting cytotoxicity is hampered by the exogenous hIFNβ produced by inoculated SU.86.86 cells, while SU.86.86 cells are insensitive to the cytotoxic effects of hIFNβ. These findings are also in line with studies showing that incorporation of IFNβ genes into oncolytic viruses such as vesicular stomatitis virus or vaccinia virus leads to lower virus replication in cell lines with intact IFN signaling pathways [213-216], and, previously, we showed that most HPACs do have intact and functional innate immune pathways [204].

We observed up regulation of hIFNβ mRNA and functional IFN protein production in BxPC-3 cells, which was in conflict with the results of our previous study [204]. Other groups have also noted an unstable phenotype of BxPC-3 [217] and the difference between our findings in this study and the previously published one may be attributed to higher passage numbers of BxPC-3 cells used earlier.

Exogenous expression of influenza NS1 protein from rNDV-NS1-F_0 did not change replication kinetics in the inoculated HPACs. However, it did lead to less cytotoxicity in most HPACs, indicating again that IFN has a cytotoxic effect on these cells. This is in contrast with results reported for a mesogenic rNDV expressing NS1 (rNDV-NS1-F_{3aa}), which induced enhanced tumor cell killing due to inhibition of apoptosis, leading to increased syncytia formation [120].

In vivo experiments using a subcutaneous xenograft tumor model using BxPC-3 or MIA Paca-2 cells in immune-deficient mice demonstrated that intratumoral treatment with mesogenic rNDV-F_{3aa} induced tumor regression or stabilization. In contrast, SU.86.86 tumors, which are highly susceptible for oncolytic rNDV-F_{3aa} treatment *in vitro*, did not respond to injection with rNDV-F_{3aa} *in vivo*. SU.86.86 tumors showed a relatively aggressive growth rate in this mouse model when compared to the other tumor bearing groups, which might explain why rNDV-F_{3aa} treatment was not successful in achieving tumor regression or stabilization. These results illustrate again that variation in response and heterogeneity of tumors contributes to the efficacy of oncolytic virotherapy. Intratumoral treatment with the lentogenic viruses did not lead to direct oncolytic effects in this model, indicating that, for direct oncolytic effects, the mesogenic rNDV-F_{3aa} is most effective.

The immune system is thought to contribute to the efficacy of oncolytic viruses and to efficient clearance of the virus from healthy cells [125]. In this study we have focused on the direct oncolytic effects of the virus and the contribution of IFN to this, in absence of the immune system. It might well be possible that the transgenes expressed by the lentogenic viruses would increase the efficacy in an immune competent model, however based on our results we expect a higher efficacy of rNDV-F_{3aa} in these models. Evaluation of the beneficial effects of the immune system to the oncolytic effects of rNDV-F_{3aa} needs to be performed in an immune-competent model for pancreatic adenocarcinoma. Although transgenic animal models that mimic the natural development of pancreatic tumors have been created, at the moment these are limited and difficult to employ for evaluation of the efficacy of oncolytic viruses.

In conclusion, expression of exogenous IFN modulating genes from lentogenic rNDVs does not significantly enhance direct oncolysis induced by these viruses compared to those induced by a more virulent virus. However, increasing the virulence of rNDV by increasing cleavability of the F protein lead to a significant improvement of oncolytic activity of recombinant NDV. For further development of

virulent rNDV for oncolytic virotherapy, the biosafety risks of the virus for birds and poultry should be addressed. In addition, knowledge should be elucidated on the heterogeneity of pancreatic tumors and on the traits defining susceptibility to NDV induced oncolytic effects.

Chapter 4

Intravenously Injected Newcastle Disease Virus in Non-Human Primates Is Safe to Use for Oncolytic Virotherapy

Pascal R.A. Buijs
Geert van Amerongen
Stefan van Nieuwkoop
Theo M. Bestebroer
Peter R.W.A. van Run
Thijs Kuiken
Ron A.M. Fouchier
Casper H.J. van Eijck
Bernadette G. van den Hoogen

Cancer Gene Therapy 2014;21(11):463-471

Abstract

Newcastle disease virus (NDV) is an avian paramyxovirus with oncolytic potential. Detailed preclinical information regarding the safety of oncolytic NDV is scarce. In this study we evaluated the toxicity, biodistribution and shedding of intravenously injected oncolytic NDVs in non-human primates (*Macaca fascicularis*). Two animals were injected with escalating doses of either a non-recombinant vaccine strain, a recombinant lentogenic strain or a recombinant mesogenic strain. To study transmission, naive animals were co-housed with the injected animals. Injection with NDV did not lead to severe illness in the animals or abnormalities in hematologic or biochemistry measurements. Injected animals shed low amounts of virus, but this did not lead to seroconversion of the contact animals. Postmortem evaluation demonstrated no pathological changes or evidence of virus replication. This study demonstrates that NDV generated in embryonated chicken eggs is safe for intravenous administration to non-human primates. In addition, our study confirmed results from a previous report that naïve primate and human sera are able to neutralize egg-generated NDV. We discuss the implications of these results for our study and the use of NDV for virotherapy.

Introduction

Newcastle disease virus (NDV) is a single stranded negative-sense RNA virus belonging to the genus *Avulavirus* within the family of *Paramyxoviridae* [134]. NDV infections were first recognized early in the 20th century as a cause of high mortality and economic loss in the poultry industry [135]. Consequently, prophylactic vaccination of poultry is currently applied on a large scale, and (suspected) outbreaks are rigorously acted upon [138]. NDV strains vary widely in pathogenicity, which can be attributed mostly to the (multi)basic cleavage site of the fusion (F) protein [174,175]. Strains can be classified into three pathotypes: lentogenic (low virulent, no mortality in susceptible hosts), mesogenic (intermediate virulent, < 10% mortality) or velogenic (highly virulent viscerotropic or neurotropic, 10-100% mortality) [112,137].

Human infections with NDV have been observed after exposure to virus while handling infected birds or cadavers and lyophilized or aerosolized NDV vaccine. The associated symptoms have been described as mild: acute conjunctivitis and laryngitis, occasionally accompanied by low-grade fever and chills, with a rapid and spontaneous resolution [140-146]. Human-to-human transmission has never been reported.

During the last decade, NDV has become a renewed focus of research in the field of oncolytic viruses [83,113]. The tumor-specific replication and ability to induce a robust antiviral and antitumor immune response make the virus a good candidate for further development for virotherapy in cancer patients. Early clinical trials employing wildtype NDV strains for (intravenous) oncolytic therapy have shown NDV to be generally safe [91-93,97-101]. Several studies have demonstrated that increasing the virulence of recombinant NDV (rNDV) by editing the F protein cleavage site results in better oncolytic activity, but also in higher pathogenicity in chickens [114,116,195]. Surprisingly, detailed information on preclinical safety testing of oncolytic NDVs is scarce, and a comparison of different (non-)virulent strains in non-human primate models has never been described.

In our efforts to further translate oncolytic NDV to the clinic, we evaluated the toxicity, biodistribution and shedding of oncolytic NDV after injection of non-human primates with three different strains. Two recombinant strains (rNDV), one lentogenic and one mesogenic, were compared with a commercially available clonal vaccine strain, because this vaccine strain has a long history of safe usage in the poultry industry. Similar to clinical trials that employed wild-type NDV strains for intravenous oncolytic therapy, we generated the viruses in embryonated chicken eggs [91-93,97-101]. In the midst of our experiments it was reported that naive human serum is able to neutralize NDV generated in embryonated chicken eggs, in contrast to virus generated in human cells [218]. This was explained by the fact that egg-generated viruses do not express human regulators of complement activity (RCA; CD46 and CD55) on their viral envelope and are therefore susceptible to neutralization by complement when incubated with non-homologous (e.g. human or primate) serum. In the current study we confirm that non-heat inactivated (non-HI) primate and human sera neutralized NDVs generated in embryonated eggs *in vitro* and we discuss the implications of these results for our study and the use of NDV for virotherapy.

Material and Methods

Cell lines and culture conditions

BSR-T7, Vero clone 118 (Vero-118) and MRC-5 cells were cultured as described before [204,209]. All media and supplements were purchased from GIBCO (Life Technologies, Bleiswijk, the Netherlands).

Virus preparation

A full-length cDNA clone of lentogenic NDV strain La Sota (pNDV-F_0) and expression plasmids pCIneo-NP, pCIneo-P and pCIneo-L were kindly provided by Ben Peeters from the Central Veterinary Institute of Wageningen UR, the Netherlands [112]. To create a full length NDV with multibasic cleavage site in the fusion protein, the amino acid sequence of the protease cleavage site was changed from ^{112}GRQGR↓L^{117} (lentogenic) to ^{112}RRQRR↓F^{117} (mesogenic; pNDV-F^{3aa}) by means of site-directed mutagenesis as described earlier [114]. Recombinant viruses (rNDV-F_0 and rNDV-F_{3aa}) were rescued using a method adapted from the original method described previously [112]. Briefly, BSR-T7 cells were transfected with 5 µg pNDV-F_0 or pNDV-F_{3aa}, 2.5 µg pCIneo-NP, 1.25 µg pCIneo-P and 1.25 µg pCIneo-L using 10 µl lipofectamine (Life Technologies). Three days later, 200 µl BSR-T7 supernatant was injected into the allantoic cavity of 10 day old specified pathogen free (SPF) embryonated chicken eggs. After incubation in a humidified egg incubator at 37°C for two or three days (rNDV-F_{3aa} or rNDV-F_0, respectively), allantoic fluid was harvested and presence of virus demonstrated by hemagglutination assay, as described before [207]. Samples displaying hemagglutination were passaged once more in eggs to increase virus titer, and allantoic fluid was harvested after 2 or 3 days. Fresh allantoic fluid was purified and concentrated by ultracentrifugation at 27.000 rpm for two hours at 4°C using a 30%/60% sucrose gradient. Aliquots of purified rNDV were stored at -80°C. Recombinant virus stocks were sequenced to confirm the sequence of the protease cleavage site of the fusion protein (F_0/F_{3aa}). Stocks were titrated by end point dilution assay in Vero-118 cells, as described before [204].

Additional rNDV-F_0 and rNDV-F_{3aa} stocks were grown in Vero-118 and MRC-5 cells. To this end, cells were infected with MOI 0.1 in the presence of 20 µg ml^{-1} trypsin (Lonza, Breda, the Netherlands) and virus was harvested after 3 days.

Non-recombinant plaque purified (egg-generated) clonal vaccine strain AviPro ND C131 was obtained from Lohmann Animal Health (Cuxhaven, Germany). Vials containing 1 x 10^9 EID_{50} were reconstituted and diluted (if needed) in cold phosphate buffered saline (PBS) immediately before use, and vials were used only once.

Ethics statement

All experiments involving animals were conducted strictly according to European guidelines (EU directive on animal testing 86/609/EEC) and Dutch legislation (Experiments on Animals Act, 1997). The experimental protocol was reviewed and approved by an independent animal experimentation ethical review committee, not affiliated with Erasmus MC (DEC consult number EMC2921).

Animals and experimental design

Nine juvenile (average age 5 years), male NDV-seronegative cynomolgus macaques (*Macaca fascicularis*) were used. All animal handling was performed under light ketamine/medetomidine anesthesia, and

atipamezole was administered after handling to reverse the effect of medetomidine. Three weeks before the start of the experiment a Data Storage Tag centi-Temperature probe (Star-Oddi, Brussels, Belgium) was implanted intraperitoneally, set to register temperature every 10 minutes. Figure 1 displays a detailed experimental timeline. Animals were housed in groups of three per isolator (A: AviPro ND C131; B: rNDV-F_0; C: rNDV-F_{3aa}). Two animals (inject 1 & 2) per group were injected i.v. into the posterior tibial vein with escalating viral doses (day 0: 1 x 10^7; day 1: 1 x 10^8; day 2: 1 x 10^9 EID_{50} or $TCID_{50}$), the third animal (control) served as contact animal. Animal wellbeing was observed daily throughout the experiment, and the animals were weighed on day 0-4, 7, 14 and 21. Samples (see below) were collected just before and 2 hours after injection on day 0, 1 and 2, and also once on day 3, 4, 7, 14 and 21. The two injected animals per group were sacrificed on day 4, the contact animal on day 21, and all animals underwent full necropsy.

Samples

Eye, nose, throat and rectum swabs were collected in virus transport medium [219]. After vortexing, 200 µl medium was used for RNA isolation and residual medium was stored at -80°C for virus isolation later on.

Small volume blood samples were taken from an inguinal vein and collected into Vacuette Z Serum Sep Clot Activator and K_3EDTA tubes (both from Greiner Bio One, Alphen aan de Rijn, the Netherlands). Clotted blood samples were centrifuged and 100 µl separated serum was assayed with Piccolo BioChemistry Panel Plus Reagent Discs (Abaxis, Darmstadt, Germany), which were processed using a Piccolo Xpress chemistry analyzer (Abaxis) following the manufacturer's instructions. Measurements were obtained for glucose, BUN, creatinine, calcium, albumin, total protein, ALAT, ASAT, alkaline phosphatase, GGT, amylase and CRP. Reference values (if indicated) were obtained from the supplemental data of a publication by Xie et al [220].

100 µl EDTA blood (AviPro ND C131 and rNDV-F_0 groups only) was tested using a pocH-100iV automated counter (Sysmex, Etten-Leur, the Netherlands). Due to biosafety issues, these data could not be obtained for animals in the rNDV-F_{3aa} group. 500 µl EDTA blood was incubated with 10 ml Red Blood Cell Lysis Buffer (Roche, Woerden, the Netherlands) for 10 minutes, centrifuged, washed with PBS and centrifuged again. The cellular pellet, representing mostly WBCs, was resuspended in TRIzol (Life Technologies) and stored at -80°C. Remaining EDTA blood was centrifuged to separate plasma, of which 200 µl was used for RNA isolation.

Necropsy

Animals were sacrificed by exsanguination under deep anesthesia. All collected organ samples were transferred to tubes containing either 10% neutral-buffered formalin or RNA*later* (Life Technologies), or were frozen without additives at -80°C. Broncho-alveolar lavage (BAL) was performed by direct infusion of PBS into the right main bronchus. Recovered BAL fluid was centrifuged and the cellular pellet resuspended in TRIzol. Organ samples in RNA*later* were stored at -80°C, thawed later and transferred to tubes containing a quarter inch ceramic sphere in virus transport medium. After homogenization using a FastPrep 24 tissue homogenizer (MP Biomedicals, Eindhoven, the Netherlands), samples were centrifuged and cleared supernatant was used for RNA isolation.

RNA isolation and qRT-PCR

Samples stored in TRIzol were processed according to the manufacturer's instructions to isolate RNA. 200 µl of other samples (swabs, plasma and organ homogenates) was combined with 300 µl lysis buffer of the Total Nucleic Acid Isolation kit (Roche) and RNA was isolated in a volume of 50 µl using a MagNA Pure LC machine (Roche) following the manufacturer's instructions.

NDV specific qRT-PCR was performed using 5 µl (TRIzol samples) or 19,5 µl (MagNA Pure samples) RNA in an ABI PRISM 7000 Sequence Detection System using TaqMan Fast Virus 1-Step Master Mix (both from Life Technologies) in a total volume of 30 µl. The NDV specific primers used were described by Wise et al [221]. The RT step was 5 minutes at 50°C, followed by 95°C for 20 seconds. Cycling consisted of 45 cycles of 3 seconds denaturation at 95°C, 5 seconds annealing at 54°C and 31 seconds extension at 60°C.

Virus isolation

200 µl medium from collected swabs or 200 µl supernatant of homogenized dry frozen organs was injected in duplicate into 10 day old SPF embryonated chicken eggs. After 2 or 3 days (samples containing rNDV-F_{3aa} or Avipro ND C131 and rNDV-F_0, respectively), allantoic fluid was harvested and tested for presence of virus by hemagglutination assay.

NDV serology

Sera were tested for NDV specific antibodies by hemagglutination inhibition assay (HI) using SPF turkey erythrocytes [207]. To neutralize neuraminidase activity, serum samples were pretreated with cholera filtrate for 16 hours at 37°C, followed by inactivation for 1 hour at 56°C. Diluted chicken polyclonal anti-NDV antibody was used as positive control.

Histopathology

Samples for histologic examination were stored in 10% neutral-buffered formalin (lungs after inflation with formalin), embedded in paraffin, sectioned at 4 mm, and stained with hematoxylin and eosin for examination by light microscopy. The following tissues were examined by light microscopy for the presence of histopathological changes: lungs, primary bronchus, trachea, liver, spleen, kidney, nasal septum, nasal concha, eye and conjunctiva.

Additionally, separate sections were stained for NDV using an immunoperoxidase method. Tissue sections were mounted on coated slides (KliniPath, Duiven, the Netherlands), deparaffinised, rehydrated and endogenous peroxidase was blocked by incubating slides in 3% H_2O_2 in bidest for 10 minutes. Antigen was retrieved by boiling slides in Tris buffer (pH 9) for 15 minutes and sections were subsequently washed in PBS containing 0.05% Tween 20. Slides were incubated in PBS with 0.1% BSA (Aurion, Wageningen, the Netherlands) for 10 minutes at room temperature. After this, slides were incubated with monoclonal mouse anti-NDV antibody (HyTest Ltd, Turku, Finland) in PBS with 0.1% BSA for 1 hour at room temperature. Mouse IgG2a (R&D Systems, Abingdon, UK) was used as isotype control. After washing, slides were incubated with goat anti-mouse antibody (Southern Biotech, Birmingham, AL) labeled with horseradish peroxidase (HRP) for 30 minutes at room temperature. HRP activity was revealed by incubating slides in 3-amino-9-ethylcarbazole in N,N- dimethylformamide for 10 minutes. Slides were imbedded in Imsolmount and overlayed with Pertex (both from KliniPath). Brain tissue from a cormorant (*Phalacrorax auritus*) known to be infected with virulent NDV was used as positive control.

Virus neutralization assay

VN assays were performed as described previously [222]. Briefly, 3 human sera and 3 naive primate sera were left untreated (non-HI) or heat inactivated (HI) for 30 minutes at 56°C. Twofold serial dilutions of the sera starting at a 1:10 dilution were mixed 1:1 with 100 $TCID_{50}$ of rNDV. After incubation at 37°C for 1 hour, the serum–rNDV mixture was transferred to 96-wells plates containing Vero-118 cells. Plates were incubated for 1h at 37°C, and inoculum was replaced by 200 µL fresh medium. After six days, end-point dilutions were read by scoring cytopathic effect.

Results

To compare the safety of three different oncolytic NDVs, two macaques per group were injected i.v. with either lentogenic Avipro ND C131, lentogenic rNDV-F_0 or mesogenic rNDV-F_{3aa} (Figure 1). A dose escalation design was chosen to observe potential toxicity at different doses. To study transmission, naive animals were co-housed with the injected animals.

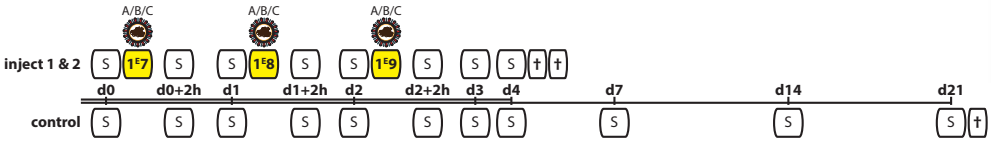


Figure 1: Timeline of experimental set up. Time is depicted in days (d) and hours (h). inject 1 & 2: inoculated animals 1 & 2; control: contact animal; S: sample time point; A: Avipro ND C131; B: rNDV-F0; C: rNDV-F3aa; †: euthanasia.

Clinical and vital signs

During the experiments, none of the injected animals displayed behavioral changes or overt signs of illness. Specifically, no conjunctivitis or ocular, oral or nasal discharge was observed. Animals were lively and displayed normal appetite similar to the period before the start of the experiment. No change in stool consistency or color was observed. All injected animals maintained their body weight (Figure 2a).

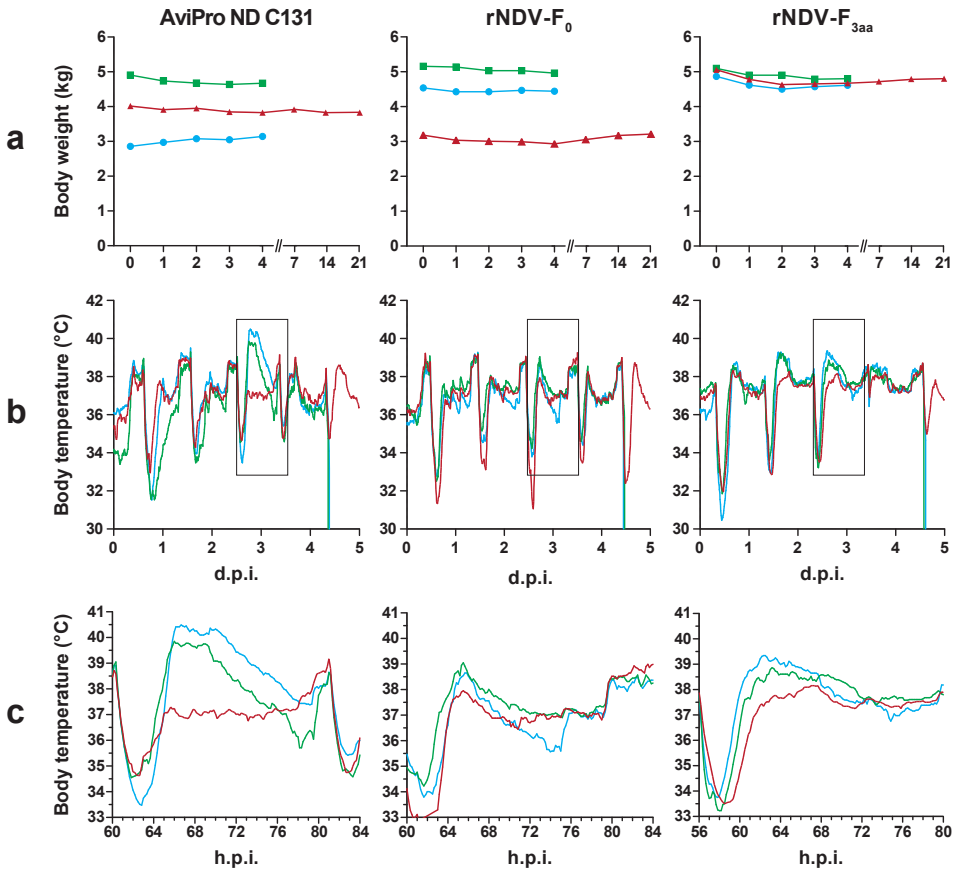


Figure 2: Clinical parameters recorded throughout the experiment. (a) Animals were weighed at indicated time points. (b) Body temperature was recorded using an intraperitoneally implanted probe (Star-Oddi). (c) Detailed view of body temperature just after third NDV injection. Blue circles & green squares: inoculated animals 1 & 2; red triangles: contact animal; d.p.i.: days post first inoculation; h.p.i.: hours post first inoculation; black box in (b): area of detailed view in (c).

Animals injected with Avipro ND C131 had a short-term fever peak higher than 40°C after injection with the highest dose (1 x 10^9 50% egg infectious dose (EID_{50}) on day 2, which decreased rapidly the same day (Figures 2b,c). Animals injected with the medium (1 x 10^8 50% tissue culture infectious dose ($TCID_{50}$) and highest dose (1 x 10^9 $TCID_{50}$) of rNDV-F_{3aa} also had a short term fever peak after injection on day 1 and 2 with a maximum around 39.5°C, which also decreased rapidly (Figures 2b,c). Sharp decreases in registered temperature during the first five days can be attributed to the general anesthesia animals underwent for sampling and injection.

Similar to the injected animals, none of the contact animals displayed signs of illness. All contact animals maintained their body weight up to 3 weeks after the start of the experiment (Figure 2a). In contrast to the injected animals, none of the contact animals had an abnormal increase of body temperature during the experiment (Fig. 2b,c; data after day 4 not shown).

Hematology and serum chemistry

Hemoglobin (Hb) concentration in all injected animals dropped from an average of 10.0 mmol l^{-1} at the start of the experiment, to an average of 7.1 mmol l^{-1} at day 4, which was still within normal limits (Figure 3a; reference value 6.7-9.7 mmol l^{-1})[220]. This decrease was probably due to the blood sampling during the first few days. Leukocyte counts were on the low side of normal and slightly lower than normal in one of the animals injected with rNDV-F_0, however no overt leukocytopenia or leukocytosis was observed (Figure 3b). Platelet counts did not show any abnormalities (Figure 3c).

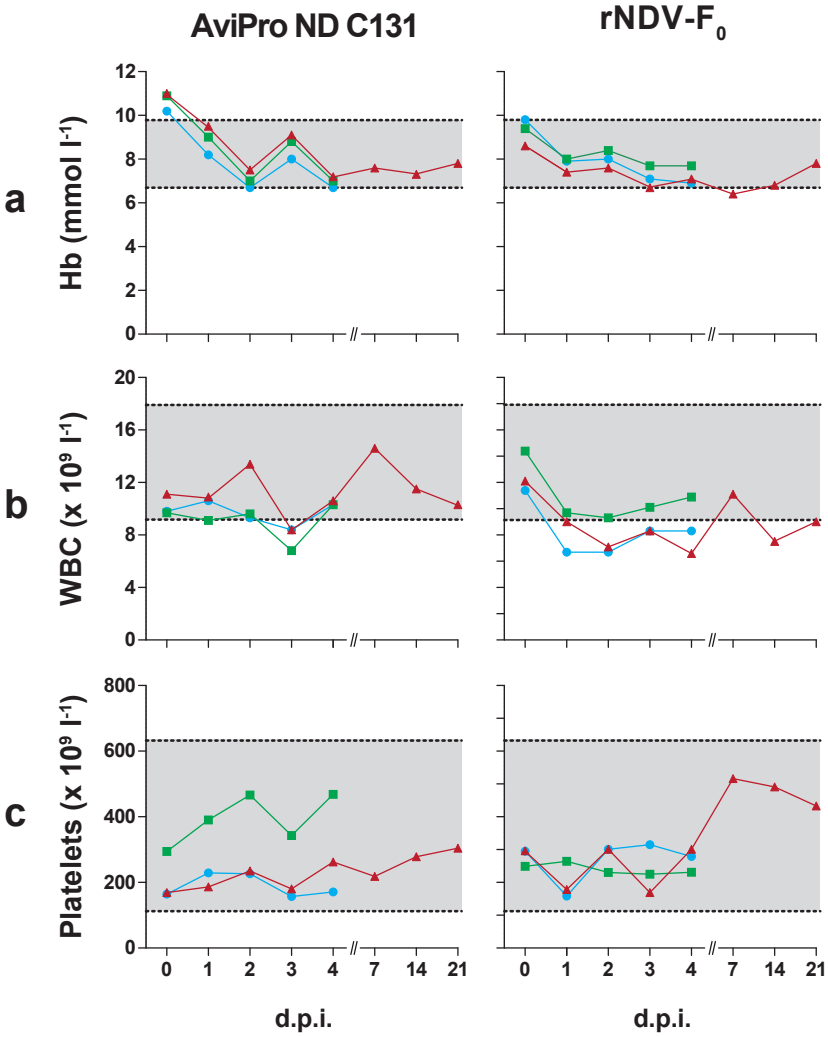
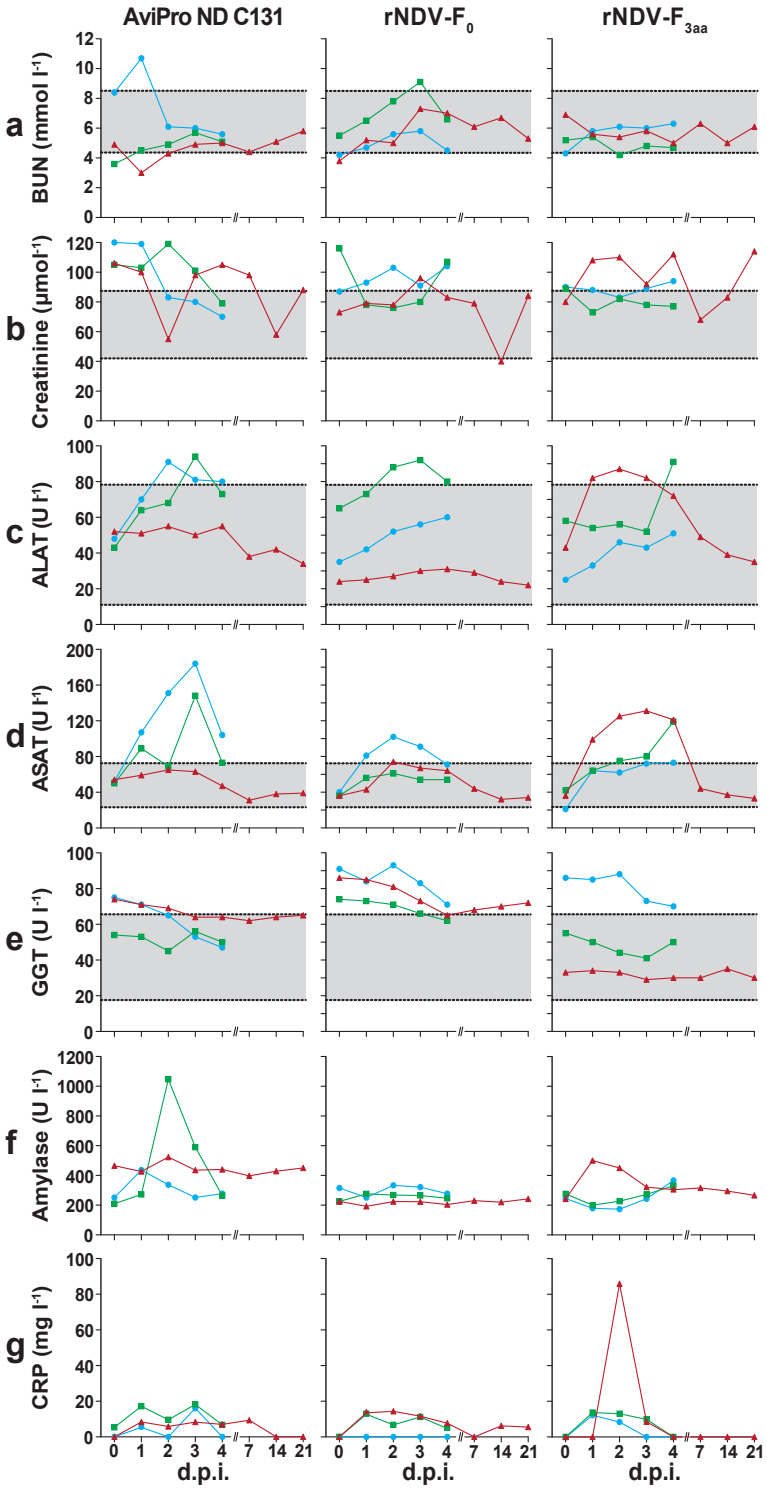


Figure 3: Hematological parameters for animals injected with lentogenic viruses and their co-housed contact animals. Due to biosafety issues, these parameters could not be obtained for animals injected with rNDV-F_{3aa}. EDTA blood samples were analyzed using an automated counter (Sysmex pocH-100iV) to obtain a basic blood profile. Blue circles & green squares: inoculated animals 1 & 2; red triangles: contact animal; d.p.i.: days post first inoculation. Reference values [220] are indicated by the grey area between the dotted lines.

In all injected animals kidney function, as measured by blood urea nitrogen (BUN) and creatinine concentration, did not deteriorate during the experiment (Figures 4a,b). Animals injected with Avipro ND C131 had slight increases of liver enzyme concentrations, with a maximum alanine aminotransferase (ALAT) of 94 U l^{-1} (reference value: 11-78 U l^{-1} [220]) and aspartate aminotransferase (ASAT) of 184 U l^{-1} (reference value: 23-71 U l^{-1} [220]), which seemed to decrease spontaneously on day 4 (Figures 4c,d). Animals injected with rNDV-F_0 or rNDV-F_{3aa} also had slight increases in liver enzyme concentrations, but much less than animals injected with Avipro ND C131. Gamma glutamyltransferase (GGT) values did not increase during the experiment, although they were generally higher than the reference values (Figure 4e; reference value: 18-65 U l^{-1} [220]). A peak in serum amylase concentration was detected in one animal injected with Avipro ND C131, while other injected animals had no abnormalities in their serum amylase concentrations (Figure 4f). C-reactive protein (CRP) was detected (detection limit >5 mg l^{-1}) in multiple serum samples of injected animals, but no peaks in concentration were observed (Figure 4g). Since there are no reference values for amylase or CRP measurements in cynomolgus macaques, it is difficult to evaluate the measurements obtained. Other serum chemistry markers (glucose, calcium, albumin, total protein and alkaline phosphatase) were not found to be abnormal during the course of the experiment (data not shown).

Hematologic parameters obtained for contact animals showed the same pattern as obtained from the injected animals: slight decreases in Hb concentration over time and normal leukocyte and platelet counts (Figure 3). Biochemistry values obtained for the contact animals revealed that BUN and creatinine, did not increase during the experiment (Figures 4a,b). In contrast with animals injected with Avipro ND C131, the contact animal did not show elevated liver enzymes (Figures 4c,d). The contact animal in the rNDV-F_{3aa} group showed slightly elevated liver enzymes, whereas injected animals did not (Figures 4c,d). GGT and amylase measurements did not show abnormalities (Figures 4e,f). Finally, there was a peak observed in CRP concentration in the contact animal in the rNDV-F_{3aa} group of 86 mg l^{-1} (Figure 4g).

Figure 4: Serum chemistry parameters in samples taken just before injection. Samples taken at indicated time points were assayed using an automated serum chemistry analyzer (Piccolo Xpress) to obtain a basic serum chemistry profile. Blue circles & green squares: inoculated animals 1 & 2; red triangles: contact animal; d.p.i.: days post first inoculation; BUN: blood urea nitrogen; ALAT: alanine aminotransferase; ASAT aspartate aminotransferase; GGT: gamma glutamyltransferase; CRP: c-reactive protein. Reference values [220] are indicated by the grey area between the dotted lines.


AviPro ND C131
rNDV-F0
rNDV-F3aa
a
BUN (mmol l-1)
b
Creatinine (µmol l-1)
c
ALAT (U l-1)
d
ASAT (U l-1)
e
GGT (U l-1)
f
Amylase (U l-1)
g
CRP (mg l-1)
0 1 2 3 4 7 14 21
d.p.i.

Virological data

All injected animals had detectable NDV-RNA in their plasma and WBCs immediately after injection of the lowest dose until euthanasia on day 4 (Figure 5a). Animals injected with either virus showed shedding of viral RNA from eyes, nose and throat, but not from their rectum, starting around the time of injection of the medium dose, with a peak after injection of the highest dose (Figure 5b). A low number of qRT-PCR positive swab samples (almost exclusively those taken on day 2) were also found positive in virus culture, without a correlation between Ct value and culture results.

A few samples collected from the eyes, nose and throat of the contact animals were found to be positive for NDV-RNA (Figure 5b). However, viable virus was isolated only once from a throat swab (rNDV-F_0, day 2+2h). Sporadically, the contact animals also had detectable NDV-RNA in plasma or WBC samples (Figure 5a).

Hemagglutination inhibition assay conducted on sera collected from the contact animals 21 days after exposure revealed that contact animals had not seroconverted (data not shown).

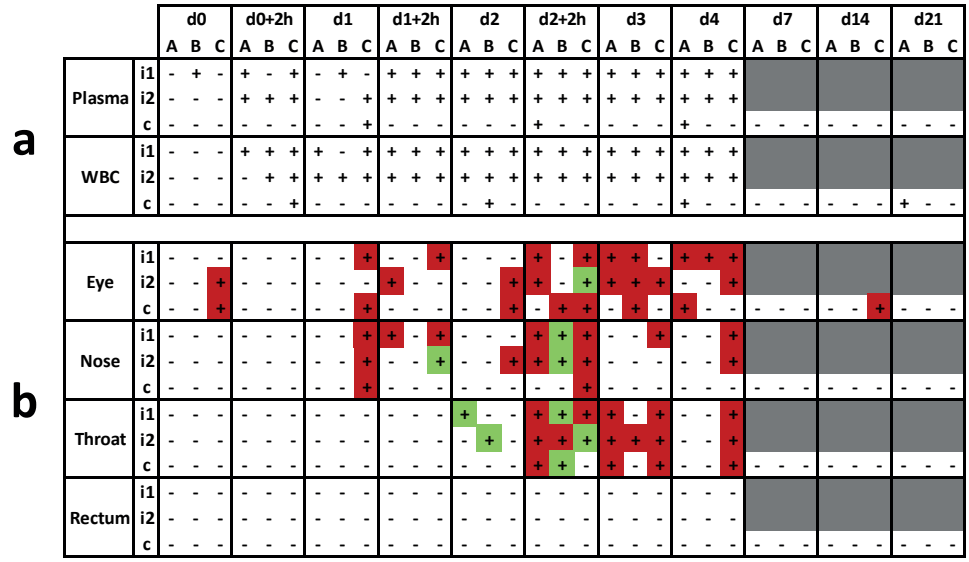


Figure 5: Virological parameters from samples taken from animals just before and 2 hours after injection. Blood (a) and swab (b) samples were collected at indicated time points. Total RNA was isolated and NDV specific qRT-PCR was performed on all samples (positive = +, negative = -). qRT-PCR positive swab samples were cultured in SPF embryonated chicken eggs (positive = green, negative = red, not cultured = blank). WBC: white blood cells; i1 & i2: inoculated animals; c: contact animal; A: Avipro ND C131; B: rNDV-F_0; C: rNDV-F_{3aa}; d: days post first inoculation; h: hours.

Necropsy & histopathology

Samples of organs collected upon necropsy were tested for the presence of NDV-RNA with qRT-PCR. I.v. injection with all three strains of NDV resulted in a systemic distribution of viral RNA mainly to the (upper and lower) respiratory tract, spleen, liver and kidney (Figure 6). No differences in distribution were observed between the three groups (Avipro ND C131 vs. rNDV-F_0 vs. rNDV-F_{3aa}). Virus was not isolated from the RNA positive samples.

	A			B			C		
	i1	i2	c	i1	i2	c	i1	i2	c
BAL	+	-	+	+	+	+	+	+	+
Lung RUL	+	+	-	+	-	-	-	+	-
Lung RLL	+	+	-	+	+	-	-	+	-
Trachea	-	-	-	-	-	-	-	+	-
Bronchus	+	+	-	-	-	-	-	-	-
TB LN	-	-	-	+	+	-	-	-	-
Mesenterial LN	-	-	-	+	+	-	-	-	-
Colon	-	-	-	-	-	-	-	-	-
Ileum	-	-	-	-	-	-	-	-	-
Heart	-	-	-	-	-	-	-	+	-
Liver	+	-	-	-	+	-	-	+	-
Spleen	+	+	-	+	+	-	+	+	-
Kidney	+	+	-	+	+	-	+	+	-
Adrenal	-	-	-	-	+	-	-	+	-
Pancreas	-	-	-	-	-	-	-	-	-
Brain	-	-	-	-	-	-	-	-	-
Liquor	-	-	-	-	-	-	-	+	-
Nasal septum	-	+	-	-	-	-	-	+	-
Nasal concha	+	+	-	-	-	-	-	-	-
Eye / conjunctiva	-	-	-	-	-	-	-	-	-

Figure 6: Virological parameters at necropsy. Organs were collected on day 4 (inoculated animals) or day 21 (contact animal) post first inoculation. Tissue samples were homogenized and supernatant was used to isolate total RNA. NDV specific qRT-PCR was performed on all samples (positive = +, negative = -). qRT-PCR positive swab samples were cultured in SPF embryonated chicken eggs (negative = red, not cultured = blank). A: Avipro ND C131; B: rNDV-F_0; C: rNDV-F_{3aa}; i1 & i2: inoculated animals; c: contact animal; BAL: bronchi-alveolar lavage; RUL: right upper lobe; RLL: right lower lobe; TB: tracheo-bronchiolar; LN: lymph node.

No gross pathological changes were observed at necropsy of injected animals. The following incidental lesions were detected upon light microscopy: mild acute focal suppurative bronchoadenitis in one animal injected with Avipro ND C131 and mild acute focal suppurative rhinitis with hemorrhage in one animal injected with rNDV-F_{3aa}. Otherwise, no microscopic lesions were detected in any of the evaluated tissues of any of the injected macaques. Immunohistochemic staining for NDV revealed no infected cells in tissues that were positive for NDV-RNA. None of the rare incidental lesions detected were considered to be related to the treatment.

One incidental lesion was detected in a contact animal by light microscopy: mild acute focal alveolar hemorrhage in the animal in the rNDV-F_0 group, considered not to be related to exposure to virus. Immunohistochemical staining for NDV again revealed no infected cells in evaluated tissues. None of the organs collected from contact animals on day 21 tested positive for NDV-RNA (Figure 6).

Virus neutralization (VN)

VN assays were conducted with virus stocks generated in embryonated chicken eggs and in human or primate cells. These viruses were tested against a panel of human and primate sera (Table 1). This experiment revealed that indeed non-HI naive human and primate sera, but not HI sera, neutralized rNDVs generated in embryonated chicken eggs. This was in contrast with rNDVs generated in primate (Vero-118) or human (MRC-5) cells, which managed to escape neutralization by non-HI naive human and primate sera.

Table 1: Virus neutralization assay using rNDVs generated in eggs, MRC-5 or Vero-118 cells. rNDVs were incubated with serum and titrated on Vero-118 cells. Values indicate VN-titers. V-118: Vero-118; HI: heat-inactivated.

			rNDV-F_0			rNDV-F_{3aa}		
			Egg	**MRC-5**	**V-118**	**Egg**	**MRC-5**	**V-118**
Primate	**Non-HI**	**1**	40	0	0	45	0	0
		2	20	0	5	20	0	0
		3	40	5	0	25	0	0
	HI	**1**	10	0	0	0	0	0
		2	0	0	0	0	0	0
		3	0	0	0	0	0	0
Human	**Non-HI**	**1**	40	15	15	60	10	0
		2	40	0	0	40	0	0
		3	30	0	0	60	0	0
	HI	**1**	0	0	0	0	0	0
		2	0	0	0	0	0	0
		3	0	0	0	10	0	0

Discussion

Limited information is available on preclinical biosafety regarding administration of oncolytic NDV in a human-like animal model. Therefore, we carried out a safety study comparing the toxicity, biodistribution and shedding of NDV strains differing in virulence in non-human primates.

A non-recombinant, commercially available vaccine strain was included, since this virus has a long history of safe usage in vaccinating poultry. Two other (recombinant) strains were tested as they are candidates for future virotherapy with oncolytic NDV, and are suitable for further development using reverse genetics. These two rNDVs differed in virulence as defined by their F protein cleavage site. Considering virulent NDV strains as potential oncolytic viruses, it is also important to know whether the virus is capable of spreading to the environment, as this could lead to accidental infection of susceptible hosts, and could thus also pose a serious threat to the poultry industry or domestic bird species.

We found that i.v. injection of a high dose of all three strains was safe for the animals. No clinical signs of illness were observed in the vaccine or recombinant virus injected animals, except short term fever in animal injected with the highest dose Avipro ND C131 and rNDV-F_{3aa}. Hematologic and serum chemistry parameters were mostly within normal range, with the exception of slightly elevated liver enzymes upon Avipro ND C131 injection. This could be related to the presence of preservatives and lyophilization products in vials of this vaccine strain, specifically peptone, magnesium sulphate, sucrose or gelatin. We hypothesize that other incidental findings of abnormalities in measured biochemistry parameters are most likely unrelated to the experiment. Due to biosafety issues, we were not able to measure hematologic parameters for animals injected with rNDV-F_{3aa}. However, it is unlikely that these parameters would differ much from the measurements obtained from animals injected with non-virulent strains, since there was no indication for increased pathogenicity (see below) of the virulent strain in this non-human primate model.

Analysis of the distribution of NDV showed that i.v. injection resulted in systemic spread, with most of the viral RNA ending up in the respiratory tract, spleen and liver. Histological examination however, did not reveal infected cells in any of the examined organs, indicating that substantial virus replication in these organs did not occur.

We observed shedding of virus from injected animals, irrespective of the strain used and we cannot exclude transmission of virus. However, productive infection of the contact animals did not occur, since no seroconversion was detected. Earlier studies of human infection with NDV reported mostly on workers in the poultry industry having contact with live virus either when handling infected or dead poultry or lyophilized or aerosolized vaccine strains [140-146]. The exposure of these human cases was probably to a higher dose than the contact animals in the present study, since they developed symptoms within a couple of days and seroconverted 1-2 weeks after exposure. We therefore argue that contact animals in the present study were not exposed to high concentrations of shed NDV, since they did not develop any symptoms nor did they seroconvert after 3 weeks of observation. Nonetheless, appropriate biosafety measures preventing environmental spread have to be considered when administrating high dose oncolytic NDV.

Preclinical studies employing NDV as a vaccine vector using subcutaneous, intranasal and/or intratracheal administration showed that NDV as vaccine vector is highly attenuated in non-human primates [127-132]. However, it is difficult to compare these studies with the one we present here, as

these animals were not i.v. inoculated and the viral doses administered in these vaccine studies were relatively low.

Several clinical phase I/II trials have been described previously in which high dose wild-type oncolytic NDV was injected i.v. into patients with advanced and/or metastatic solid cancer [91-93,97-101]. These trials employed naturally occurring, *in vitro* selected egg-grown oncolytic strains of NDV. Viral doses used in these clinical studies were comparable to the dose administered in the present study: up to 34-120 x 10^9 EID_{50} m^{-2} (body surface) as compared to an equivalent dose of 4 x 10^9 $TCID_{50}$ m^{-2} recombinant virus in our study, noting that egg titration is generally about tenfold more sensitive than tissue culture titration [92,99,223]. A study recruiting 14 patients suffering from glioblastoma multiforme showed that i.v. injection of a high dose of the lentogenic oncolytic strain NDV-HUJ was safe, with grade I-II fever as only adverse event [92]. Infectious virus was recovered from blood, saliva and urine samples up to 96 hours after injection. Two other phase I/II studies with a total of 95 patients showed that i.v. injection of a virulent NDV strain (PV701) was safe in patients with advanced or metastatic solid tumors [93,99]. Common side effects included fever, diarrhea and slight transient elevation of liver transaminases (but only in patients with hepatic metastases). Shedding of injected virus was also observed up to three weeks after injection. Measures to prevent environmental spread of this virulent NDV strain were not described [93,99]. Overall, the results of our present preclinical safety study with (recombinant) NDV strains corresponds well with the results previously reported on phase I/II clinical trials.

During the execution of our animal experiments it was reported that NDV generated in embryonated chicken eggs or non-human cells is susceptible to neutralization by human serum through complement binding and activation [218]. This was clearly an important finding related to our experiment. Upon testing, we confirmed that non-HI primate and human sera neutralized egg-generated NDV, in contrast to NDV generated in primate or human cells. All clinical trials thus far have used egg-generated virus stocks and still reported positive effects for NDV virotherapy as well as shedding of infectious virus, indicating that egg-generated NDV is still capable of replication [91-93,97-101]. The efficacy of NDV virotherapy might increase when using virus stocks generated in human or primate cells. However, neutralization by complement might also be important for safe administration of high doses of NDV, and abolishing this could result in more toxicity and a higher risk of shedding to the environment. More information should therefore be obtained on the safety and oncolytic efficacy of virotherapy with NDV stocks generated in human or primate cells.

Our data in a non-human primate model using recombinant avirulent and virulent NDV strains corroborates with the data from clinical trials with wild-type strains. Based on the lack of virus replication in organs, absence of NDV-related lesions and hematologic or biochemistry abnormalities, we conclude that the i.v. administration of oncolytic NDV generated in eggs is safe, even when using high doses of a virulent strain, when taking appropriate biosafety measures to prevent environmental spread. Future research has to elucidate whether NDV generated in human or primate cells has a similar safety profile and oncolytic efficacy.

Chapter 5

Pigeon Paramyxovirus Type 1 from a Fatal Human Case Induces Pneumonia in Cynomolgus Macaques (*Macaca Fascicularis*)

Pascal R.A. Buijs*
Thijs Kuiken*
Peter R.W.A. van Run
Geert van Amerongen
Sanela Svraka
Mya Breitbart
Marion P.G. Koopmans
Bernadette G. van den Hoogen

** = shared first author*

Abstract

Although Newcastle disease virus is known to cause mild transient conjunctivitis in humans, there are two recent reports of fatal respiratory disease in immunocompromised human patients infected with the pigeon lineage of the virus, pigeon paramyxovirus type 1 (PPMV-1). In order to evaluate the potential of PPMV-1 to cause respiratory tract disease in a non-human primate as a model for human disease, we inoculated 1.0 x 10^8 median tissue culture infectious dose of PPMV-1 into the trachea and nares and onto the conjunctiva of three cynomolgus macaques (*Macaca fascicularis*) and examined them by clinical, virological, and pathological assays. In all three macaques, PPMV-1 replication was restricted to the respiratory tract and caused pulmonary consolidation affecting up to 30% of the lung surface by 3 days post inoculation. Both alveolar and bronchiolar epithelial cells expressed viral antigen, which co-localized with areas of diffuse alveolar damage. The results of this study demonstrate that PPMV-1 is a primary respiratory pathogen in cynomolgus macaques, and support the conclusion that PPMV-1 may cause fatal respiratory disease in immunocompromised human patients.

Introduction

Newcastle disease virus (NDV), or avian paramyxovirus type 1 (APMV-1), is classified in the genus *Avulavirus*, subfamily *Paramyxovirinae*, family *Paramyxoviridae* [224]. Genetic and antigenic analysis have demonstrated that NDV strains cluster in 10 different genotypes, with different sublineages [149]. Based on their difference in reactivity in hemagglutination assays, and their host specifity for columbiform species, all pigeon paramyxoviruses (PPMV-1) cluster in lineage VIb/I of genotype VI chicken NDV strains [149-152].

NDV infection is known to cause mild disease in humans. Most commonly, human infection is associated with conjunctivitis, which resolves within one week. Less often, it is associated with transient fever or chills [225-228]. So far, only two cases of human infection with NDV associated with fatal disease have been reported, one in the USA [168] and one in the Netherlands (unpublished data). Both cases involved severe pneumonia and an immunosuppressive condition as an underlying comorbidity, and both virus isolates from the patients were typed as PPMV-1.

PPMV-1 was first detected in Southeastern Europe in the 1980s and became established in pigeon populations across Europe. From there, PPMV-1 has spread and continues to spread world-wide, including multiple invasion events into North America. PPMV-1 is now established in both domestic and wild pigeons and other columbiform species in Europe and North America, from where it regularly spreads to poultry, causing substantial outbreaks [149,153].

Strains of NDV and PPMV-1 can be categorized into three different groups based on the severity of disease they cause in birds: lentogenic (avirulent), mesogenic (intermediate virulent) and velogenic (virulent). These differences in virulence can be explained mostly by differences in the cleavage site in the fusion (F) protein, which is synthesized in an inactive form (F0) and needs to be proteolytically cleaved by host cellular proteases into active F1 and F2 forms in order to become functional. Activation of the F0 protein will result in fusogenic activity promoting virus-to-cell fusion and cell-to-cell fusion. Lentogenic viruses have a monobasic cleavage site, which can only be cleaved by trypsin-like proteases found in the respiratory and digestive tracts of birds. More virulent strains have a multibasic cleavage site, which can also be cleaved by furin-like proteases, found more abundantly in most organs and explaining why virulent strains can replicate systemically in birds [112]. Of note, virulent PPMV-1 strains possess a multibasic cleavage site and cause disease in columbiform species. However, some strains need further adaptation to become virulent in other bird species like poultry [164,165,229].

NDV strains have been tested in different animal models to evaluate their virulence for mammals. Inoculation of lentogenic, mesogenic, and velogenic strains of NDV induced conjunctivitis in rhesus macaques and rabbits, but only after mild mechanical abrasion of the conjunctival epithelium [230]. Intranasal combined with intratracheal inoculation of a mesogenic strain of NDV (Beaudette C) into African green monkeys resulted in a low level of replication at scattered locations in the lung, without overt disease signs: pathologic changes were not evaluated [127]. Intranasal inoculation of a lentogenic strain of NDV (La Sota) into BALB/c mice [231] or Syrian golden hamsters [232] resulted in body weight loss and, at 3 days post inoculation (dpi), necrotizing broncho-interstitial pneumonia associated with virus antigen expression in bronchial epithelium. Recently, evaluation of lentogenic and mesogenic recombinant NDV strains as oncolytic agents injected intravenously in high dose into non-human primates demonstrated no apparent toxicity [233]. However, the virulence of PPMV-1 in laboratory

mammals, as a model for human disease via a natural inoculation route has not been evaluated.
We used the PPMV-1 isolate from a fatal human case to investigate the potential of this isolate to cause respiratory tract disease in a non-human primate model. To this end, three cynomolgus macaques were inoculated and pathological, immunohistochemical and virological analyses were performed at 3 dpi. We demonstrate that PPMV-1 can cause severe virus-associated pneumonia in a non-human primate model.

Material and methods

Virus preparation

The PPMV-1 isolate was obtained from an adult female patient from the Netherlands with multiple myeloma, who died from respiratory failure after receiving allogenic bone marrow transplantation and immunosuppressive treatment (unpublished data). The virus isolate was passaged twice on human rhabdomyosarcoma cells and once on Vero cells. Prior to inoculation, the virus isolate was cultured in 10-day-old embryonated chicken eggs using standard techniques, and harvested after two days. Pooled fresh allantoic fluid was purified and concentrated by ultracentrifugation at 27.000 rpm for two hours at 4°C using a 30%/60% sucrose gradient. The virus stock was titrated by end point dilution assay in Vero clone 118 cells, as described before [204]. The infectious virus titer of this stock was 5×10^8 median tissue culture infective dose ($TCID_{50}$) per ml.

Experimental protocol

Three cynomolgus macaques, 3.5 yrs old, were colony-bred and had been maintained in group housing, where they were screened annually—and tested negative—for the following infections: simian virus 40, polyomavirus, tuberculosis, measles virus, mumps, simian immunodeficiency virus, simian retrovirus type D, and simian T-cell leukemia virus. Prior to inoculation, they were examined clinically and determined as healthy by a registered veterinarian. One month before the start of the experiment a Data Storage Tag centi-Temperature probe (Star-Oddi, Brussels, Belgium) was implanted intraperitoneally, set to register temperature every 10 minutes. One week before inoculation, the macaques were placed together in a negatively pressurized, HEPA-filtered isolator cage. They were provided with commercial food pellets and water ad libitum, and fruit samples twice daily. The macaques were inoculated with 1.0×10^8 $TCID_{50}$ of PPMV-1, which was suspended in 4.5 ml of phosphate-buffered saline (PBS). Approximately 3 ml was applied intratracheally by use of a catheter, 0.5 ml in each nare, and 0.25 ml on each of the conjunctivae. The macaques were observed daily for the occurrence of malaise, coughing, exudate from the eyes or nose, forced respiration, and any other signs of illness. The macaques were euthanized at 3 dpi by exsanguination under ketamine and medetomidine hydrochloride anesthesia. Just before inoculation and daily until euthanasia, the macaques were anesthetized with ketamine and medetomidine hydrochloride and pharyngeal, nasal, ocular, and rectal swabs were collected in 1 ml transport medium [219] and stored at –70 °C until RT-PCR and/or virus isolation. In addition, 4 ml blood was collected from an inguinal vein at each of these time points and collected in Vacuette Z Serum Sep Clot Activator tubes (Greiner Bio One, Alphen aan de Rijn, The Netherlands).

Ethics statement

All experiments involving animals were conducted strictly according to European guidelines (EU directive on animal testing 86/609/EEC) and Dutch legislation (Experiments on Animals Act, 1997). The experimental protocol was reviewed and approved by an independent animal experimentation ethical review committee, not affiliated with Erasmus MC (DEC consult number EMC2796).

Clinical biochemistry

Clotted blood samples were centrifuged and 100 µl separated serum was assayed with Piccolo BioChemistry Panel Plus Reagent Discs (Abaxis, Darmstadt, Germany), which were processed using a Piccolo Xpress chemistry analyzer (Abaxis) following the manufacturer's instructions. Measurements were obtained for glucose, blood urea nitrogen (BUN), creatinine, calcium, albumin, total protein, alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), alkaline phosphatase (AP), γ-glutamyl transpeptidase (GGT), amylase and C-reactive protein (CRP). Reference values (if indicated) were obtained from the supplemental data of a publication by Xie [220].

Autopsy and tissue sampling

Autopsies were carried out according to a standard protocol. Organs were examined for lesions and sampled for laboratory analyses. For histopathology and immunohistochemistry, samples of adrenal gland, brain stem, cerebellum, cerebrum, conjunctiva, ethmoid bone, eye, eyelid, heart (left and right ventricle), kidney, large intestine, lung (left and right; cranial, medial and caudal lobes), liver, mesenteric lymph node, nasal conchae, pancreas, small intestine, spleen, tonsil, and tracheo-bronchial lymph were collected in 10% neutral-buffered formalin (lungs after inflation with formalin). For RT-PCR, broncho-alveolar lavage (BAL) was performed by direct infusion of PBS into the right main bronchus. Recovered BAL fluid was centrifuged and the cellular pellet resuspended in TRIzol and stored at -80 °C until further analysis. In addition, samples of adrenal gland, brain, cerebrospinal fluid, conjunctiva, heart, kidney, mesenteric lymph node, lung (right cranial and caudal lobes), large intestine, liver, nasal concha, nasal septum, pancreas, primary bronchus, small intestine, spleen, trachea, and tracheo-bronchial lymph node were collected in RNA*later* (Life Technologies, Bleiswijk, The Netherlands) and stored at -80°C until further analysis. For virus isolation, samples from the same tissues as for RT-PCR were stored without additives at -80°C until further analysis.

Histopathology

Samples for histopathological analysis were embedded in paraffin, sectioned at 3 µm, and stained with hematoxylin and eosin (H & E) for examination by light microscopy. Tissue sections of a clinically healthy juvenile cynomolgus macaque that had not been infected with PPMV-1 were used as a negative control.

Immunohistochemistry

Formalin-fixed, paraffin-embedded, 3 µm-thick sections of the same tissues examined histopathologically were stained using an immunoperoxidase method. Tissue sections were mounted on coated slides (Klinipath, Duiven, The Netherlands), deparaffinized and rehydrated. Endogenous peroxidase was blocked by incubating sections in 3% H_2O_2 in PBS for 10 min at room temperature (RT). Antigen was retrieved by boiling buffer (pH 9) for 15 min. Sections were subsequently washed with PBS containing

0.05% Tween 20 (Fluka, Chemie AG, Buchs, Switzerland) and incubated in PBS with 0.1% BSA (Aurion, Wageningen, The Netherlands) for 10 min at RT. After this, sections were incubated in PBS with 0.1% BSA with a monoclonal mouse antibody IgG2a to NDV (dilution 1:100, MAb 6H12; La Sota strain, Hytest Ltd, Turku, Finland) or with a negative control isotype mouse monoclonal antibody (dilution 1:100, MAb 003, R&D System, Minneapolis, USA) for 1 hr at RT. After washing, sections were incubated with goat anti-mouse antibody (dilution 1:400, Southern Biotech, Birmingham, AL) labeled with horseradish peroxidase (HRP) for 1hr at RT. HRP activity was revealed by incubating the sections in 3-amino-9-ethylcarbazole (Sigma Chemical Co., St. Louis, USA) in N,N-dimethylformamide (Sigma Chemical Co.) solution for 10 min at RT, resulting in a red precipitate. Sections were counterstained with hematoxylin. Brain tissue sections from a cormorant (*Phalacrorax auritus*) known to be infected with NDV were used as positive control. Tissue sections of a clinically healthy juvenile cynomolgus macaque that had not been infected with NDV were used as a negative control.

RNA isolation and qRT-PCR assay

BAL samples stored in TRIzol were processed according to the manufacturer's instructions to isolate RNA. Tissue samples stored in RNA*later* were weighed, thawed, transferred to tubes containing a quarter-inch-diameter ceramic sphere in virus transport medium, and homogenized using a FastPrep 24 tissue homogenizer (MP Biomedicals, Eindhoven, The Netherlands). The homogenates were centrifuged, and the cleared supernatants were used for RNA isolation. 200 µl of the cleared supernatant of tissue samples, as well as 200 µl of other samples (transport medium of swabs, plasma) were combined with 300 µl lysis buffer of the Total Nucleic Acid Isolation kit (Roche, Woerden, The Netherlands) and RNA was isolated in a volume of 50 µl using a MagNA Pure LC machine (Roche) following the manufacturer's instructions.
NDV-specific qRT-PCR was performed on 5 µl (TRIzol samples) or 19.5 µl (MagNA Pure samples) RNA in an ABI PRISM 7000 Sequence Detection System using TaqMan Fast Virus 1-Step Master Mix (both from Life Technologies) in a total volume of 30 µl, as described earlier [233].

Virus isolation

200 µl transport medium from collected swabs or 200 µl supernatant from homogenized tissue samples was injected in duplicate into 10-day-old specific-pathogen-free embryonated chicken eggs. After 2 days allantoic fluid was harvested and tested for presence of virus by hemagglutination assay.

Results

Clinical findings of experimentally inoculated macaques

The body temperature of macaque #2 increased to 39.5 °C at 0.5 dpi, compared to 38.5 °C for macaque #1 and 38.0 °C for macaque #3 at that time point. Otherwise, no clinical signs were seen. Clinical chemical changes in the plasma were limited to creatinine and C-reactive protein (CRP) (Table 1). The creatinine concentration of Macaque #2 was slightly elevated at 1 dpi, but returned to normal concentrations at 2 and 3 dpi. The creatinine concentration of the other macaques remained within the normal range. The CRP concentration in all macaques was increased at 1 dpi, and returned below the level of detection at 2 and 3 dpi.

Table 1: Temporal course in clinical chemical values in the plasma of cynomolgus macaques inoculated with PPMV-1.

			Macaque #1				Macaque #2				Macaque #3			
	Unit	Normal range	D0	D1	D2	D3	D0	D1	D2	D3	D0	D1	D2	D3
Glucose	mmol/L	2.16-7.92	4.6	5.7	3.5	4.5	5.5	6.8	5.8	7.9	4.9	6.1	4.8	7.2
Blood Urea Nitrogen	mmol/L	4.36-9.28	4.9	7.6	5.6	5.2	5.1	5.9	5.3	5.8	5.8	7.2	6.7	6.6
Creatinine	µmol/L	30.57-72.37	57	60	49	70	59	93	65	64	49	68	53	51
Calcium	mmol/L	2.32-2.96	2.54	2.43	2.55	2.39	2.53	2.43	2.51	2.38	2.53	2.47	2.58	2.47
Albumin	g/L	28.31-50.79	27	23	25	24	31	30	29	28	32	29	31	31
Total Protein	g/L	61.91-88.11	65	62	61	61	65	66	64	63	67	64	67	66
Alanine aminotransferase	U/L	2.95-87.03	58	60	77	82	134	103	87	84	42	52	56	70
Aspartate aminotransferase	U/L	24.30-69.90	51	62	82	148	166	61	90	127	43	56	80	106
Alkaline phosphatase	U/L	121.22-727.22	175	159	158	149	335	307	310	322	374	334	334	318
Gamma glutamyltransferase	U/L	14.61-62.53	75	62	66	63	24	25	25	24	52	46	50	47
Amylase	U/L	n.a.	459	321	661	471	268	173	262	217	378	308	378	347
C-Reactive Protein	mg/L	n.a.	<5	10.1	<5	<5	<5	98.4	<5	<5	<5	7	<5	<5

n.a., not available.

Gross pathology

All three macaques had locally extensive or multifocal pulmonary lesions, which were red-purple, slightly raised, and firmer than normal (Figure 1). The estimated volume of affected lung tissue differed per macaque: 30% (macaque #1), 25% (macaque #2), and 1% (macaque #3). The only gross lesions outside the respiratory tract were in lymphoid organs. All three macaques had enlarged tracheobronchial lymph nodes and prominent white pulp of the spleen, while macaque #1 also had enlarged tonsils and mandibular lymph nodes, and multiple red areas in mesenteric and pancreatic lymph nodes.

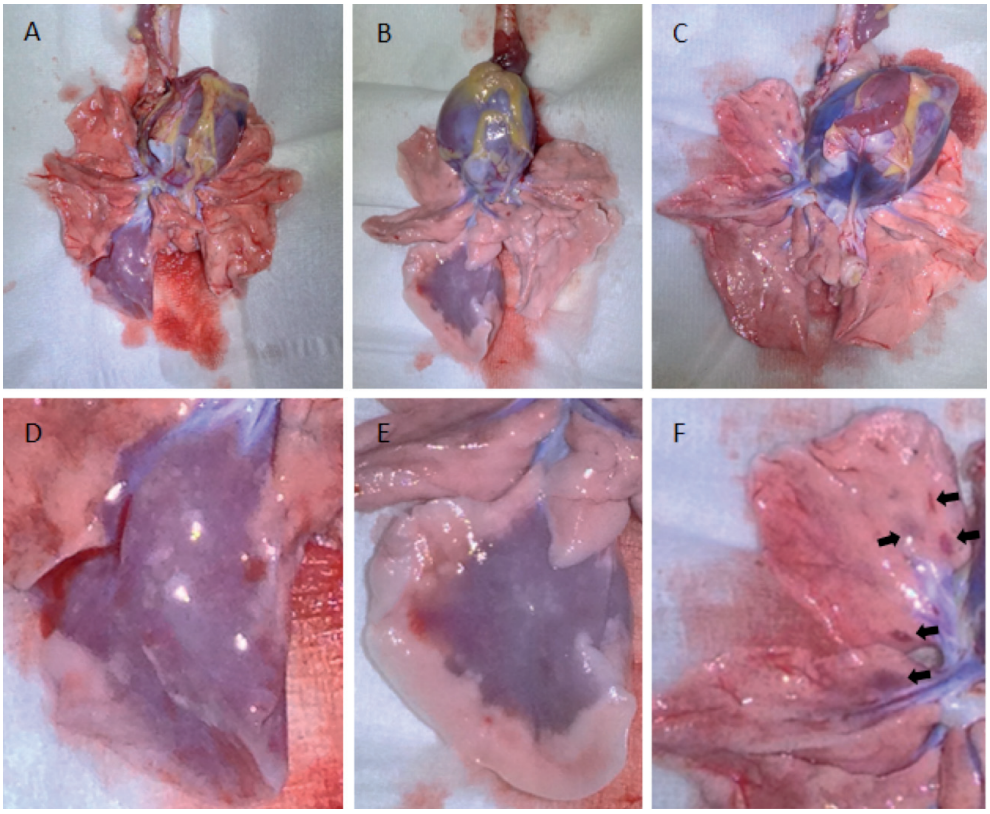


Figure 1. Gross pathology of experimental PPMV-1 inoculation in cynomolgus macaques. **A-C**: Overview of the ventral aspect of the lungs. **D-E**: Close-up of the right lower lung lobe. **F**: Close-up of the right upper and middle lung lobes. There is a locally extensive area of consolidation in the right lower lung lobe of macaques #1 (A,D) and #2 (B,E). There are multiple areas of consolidation (arrows)in the right upper and middle lung lobes of macaque #3 (C,F).

Histopathology

In the lung, all three macaques had multiple or coalescing foci of inflammation and necrosis that centered on the bronchioles (Figure 2). In these foci, the alveolar and bronchiolar lumina were filled with macrophages, lymphocytes, neutrophils, and rare eosinophils, mixed with edema fluid, fibrin, erythrocytes, and cellular debris. Alveolar and bronchiolar epithelia had evidence of both necrosis (denuded basement membranes, attenuated bronchiolar epithelial cells) and regeneration (type II pneumocyte hypertrophy and hyperplasia). The alveolar and bronchiolar walls were moderately thickened by the presence of mononuclear cells, neutrophils, and edema fluid. Some alveolar walls were lined by hyaline membranes. The bronchi were only mildly affected. There was moderate accumulation of lymphocytes around branches of both pulmonary arteries and pulmonary veins, and mild infiltration of mononuclear cells and neutrophils in pulmonary vein walls. The pleura overlying the inflammatory foci was infiltrated with mononuclear cells, neutrophils, and eosinophils.

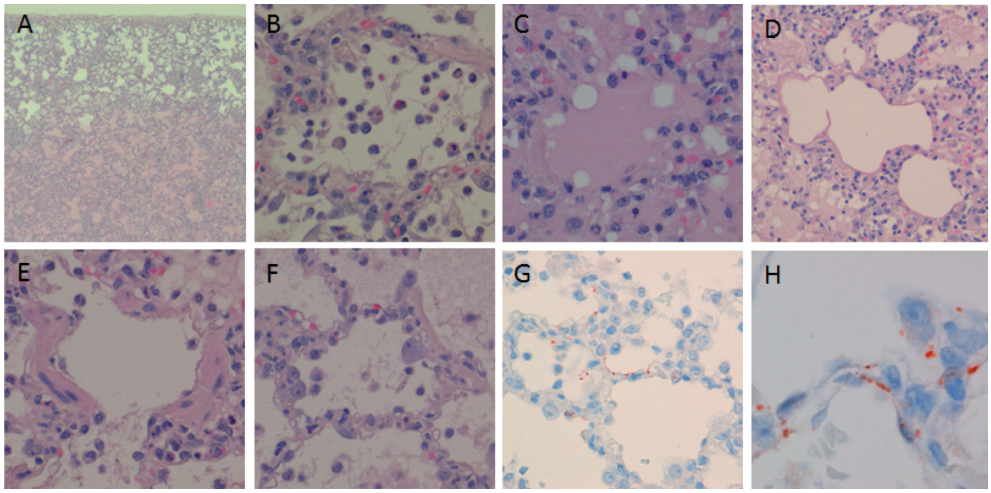


Figure 2. Pulmonary histopathology and immunohistochemistry of experimental PPMV-1 inoculation in cynomolgus macaques. **A**: Pulmonary section of macaque #1. Diffuse alveolar damage. There is edema fluid and inflammatory cells in the alveolar lumina (H&E. Original magnification X2). **B**: Pulmonary section of macaque #1. Diffuse alveolar damage. There are macrophages, lymphocytes, neutrophils, and rare eosinophils, mixed with fibrin strands, in the alveolar lumen (H&E. Original magnification X40). **C**: Pulmonary section of macaque #1. Diffuse alveolar damage. In addition to inflammatory cells, there is abundant edema fluid in in the alveolar lumen (H&E. Original magnification X40). **D**: Pulmonary section of macaque #1. Diffuse alveolar damage. Hyaline membranes lining the alveolar walls (H&E. Original magnification X20). **E**: Pulmonary section of macaque #1. Diffuse alveolar damage. There is loss of epithelial cells from the bronchiolar wall (H&E. Original magnification X40). **F**: Pulmonary section of macaque #2. Diffuse alveolar damage. Hypertrophy of type II pneumocytes lining the alveolar wall (H&E. Original magnification X40). **G**: Pulmonary section of macaque #2. Diffuse alveolar damage. Expression of NDV in epithelial cells of the alveoli (Immunoperoxidase stain for PPMV-1. Original magnification X40). **H**: Pulmonary section of macaque #1. Diffuse alveolar damage. Expression of NDV in type I pneumocytes and type II pneumocytes (Immunoperoxidase stain for PPMV-1. Original magnification X100).

The lymph nodes and spleen had benign lymphoid hyperplasia, characterized by increased number of lymphocytes and expansion of germinal centers. In addition, the medullary sinuses in the pancreatic and mesenteric lymph nodes of macaque #1 contained many erythrocytes; furthermore, the colon of this macaque had multiple small hemorrhages in the lamina propria. Macaques #1 and #2 had a mild superficial tonsillitis, characterized by the presence of neutrophils and bacterial colonies in the lumen and epithelium of the tonsillar crypt. Macaque #3 had a mild superficial conjunctivitis, characterized by the presence of neutrophils, lymphocytes, plasma cells, and expansile lymphoid follicles in the subconjunctival connective tissue, and migration of neutrophils through the conjunctiva into the lumen (Figure 3).

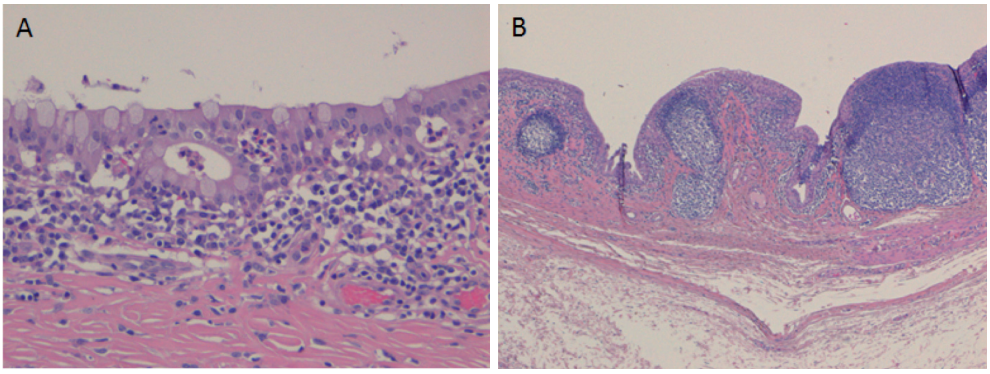


Figure 3. Conjunctival histopathology of experimental PPMV-1 inoculation in cynomolgus macaques. **A**: Conjunctival section of macaque #3. Conjunctivitis. There are neutrophils in the subconjunctival connective tissue and conjunctiva (H&E. Original magnification X20). **B**: Conjunctival section of macaque #3. Conjunctivitis. Enlarged lymphoid follical in the subconjunctival connective tissue (H&E. Original magnification X2).

Immunohistochemistry

Expression of NDV was visible as red-brown granular cytoplasmic staining in alveolar and bronchiolar epithelial cells in affected areas of the lung in all three macaques (Figure 2). Neither unaffected areas of the respiratory tract nor extra-respiratory organs showed NDV expression. NDV expression was present in the positive control tissue and absent in the isotype control and negative control tissues.

qRT-PCR and virus isolation

By qRT-PCR, the highest levels of PPMV-1 RNA were detected in eye, nose, and throat swabs of all three macaques at 1 dpi (Figure 4). The amount of RNA declined on subsequent days, but RNA was still detectable at 3 dpi in one (nose swab) or two out of three macaques (eye and throat swabs). Virus isolation conducted with PPMV RNA-positive swabs, demonstrated the presence of infectious virus only at 1 dpi in the throat swabs of macaques #2 (titer: 1.0×10^1 $TCID_{50}$/ml) and #3 (2.3×10^2 $TCID_{50}$/ml) and the eye swab of macaque #3 (7.4×10^2 $TCID_{50}$/ml).

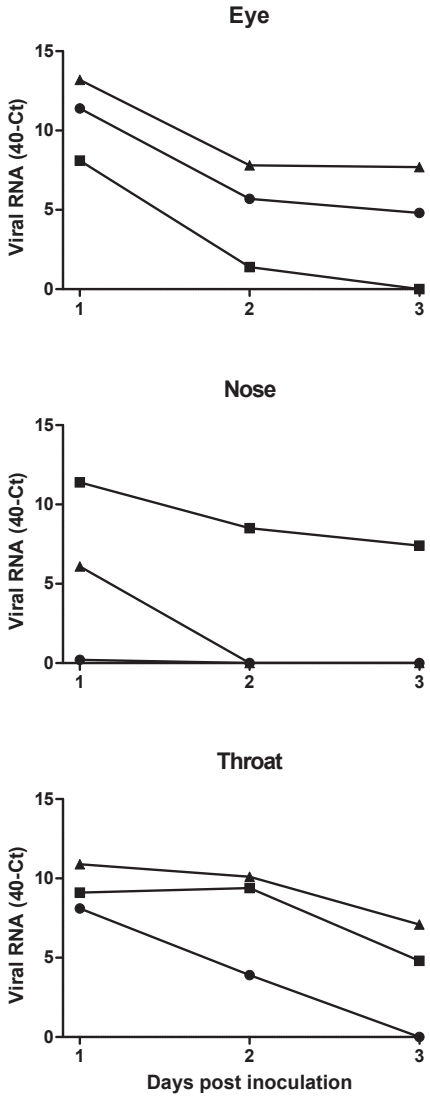


Figure 4. Detection of PPMV-1 by qRT-PCR in experimentally inoculated cynomolgus macaques. Circle: macaque #1, square: macaque #2, triangle: macaque #3.

At autopsy, PPMV-1 was detected by qRT-PCR and virus isolation in the lung of all three macaques (Table 2). PPMV-1 also was detected by qRT-PCR but not by virus isolation in other respiratory tract tissues: nasal turbinate, trachea, or bronchus. Except for the tracheo-bronchial lymph node, which was positive by qRT-PCR but not by virus isolation, all extra-respiratory organs were negative for PPMV-1 by qRT-PCR.

Table 2: Detection of PPMV-1 by qRT-PCR and virus isolation in respiratory tract organs and tracheo-bronchial lymph nodes of cynomolgus macaques at 3 dpi. Adrenal gland, brain, cerebrospinal fluid, conjunctiva, heart, kidney, mesenteric lymph node, large intestine, liver, nasal septum, pancreas, small intestine, and spleen tested negative by qRT-PCR for PPMV-1.

	Nasal concha		Trachea		Bronchus		Upper lung lobe		Lower lung lobe		BAL fluid		TB LN	
Macaque no.	qRT-PCR	VI	qRT-PCR	VI	qRT-PCR	VI	qRT-PCR	VI	qRT-PCR	VI	qRT-PCR	VI	qRT-PCR	VI
1	-	nd	34.9	-	35.8	-	-	nd	29.7	1.2×10^3	25.1	nd	-	nd
2	36.4	-	37.0	-	37.3	-	38.8	1.6×10^1	27.2	8.4×10^1	22.8	nd	-	nd
3	32.2	-	38.7	-	-	nd	29.7	-	32.2	1.3×10^3	23.3	nd	34.2	-

VI, virus isolation; nd, not done; -, negative; BAL, broncho-alveolar lavage; TB LN, tracheo-bronchial lymph node

Discussion

This experimental inoculation shows that PPMV-1 can cause severe pneumonia in a non-human primate model. Histological lesions in PPMV-1-infected macaques consisted of diffuse alveolar damage, affecting up to 30% of the lungs. The co-localization of these lesions with specific expression of PPMV-1 antigen, together with the re-isolation of PPMV-1 from lung samples at autopsy, support our conclusion that PPMV-1 was the cause of pneumonia.

These findings largely correspond with the presentation of PPMV-1 infection in two human fatal cases from New York [168] and The Netherlands (unpublished data). Both human patients also developed pneumonia. The pathological characterization of the pneumonia in our macaques was nearly identical to that in the New York patient: "a subacute, severe, diffuse interstitial pneumonia with flooding of alveoli by fibrin, protein-rich fluid, red blood cells, sloughed pneumocytes, and a few neutrophils" [168]. The lower number of neutrophils in the New York patient is likely due to the later stage of disease and treatment with a nonmyeloablative regimen for peripheral blood stem cell transplantation. The cell tropism also appeared to be the same in our macaques (pneumocytes and bronchioloar epithelial cells) and the New York patient, where NDV antigen expressing cells "appeared to be mostly sloughed pneumocytes" [168]. This sloughing may have been due to a prolonged interval between death and autopsy; it also would preclude definite differentiation between alveoli and adjacent bronchioles as the source of the sloughed epithelial cells. While it was suggested that the New York patient had systemic infection based on PPMV-1-positive urine and stool samples, we did not detect PPMV-1 in any extra-respiratory tissues—including kidney, small and large intestine, pancreas and liver—of our macaques, either by qRT-PCR or immunohistochemistry. However, it cannot be excluded that under conditions of immunosuppression, the virus would be able to spread systemically in macaques.

It remains to be determined whether PPMV-1, and particularly these isolates from fatal human cases, are more virulent for people than other NDV strains. NDV is able to selectively replicate in cancer cells and therefore is a potential therapeutic agent in cancer therapy. The virulence of PPMV-1—a pigeon-specific lineage of NDV—for humans and macaques suggests that the strain used for cancer therapy should be carefully selected. However, during application of different NDV strains by different routes—locally, subcutaneously, intravenously, by inhalation—in thousands of people, severe adverse effects have never been recorded [234]. Also, preclinical studies employing NDV as a vaccine vector using subcutaneous, intranasal and/or intratracheal administration, or even employing NDV as a high dose intravenous oncolytic agent, all demonstrated that oncolytic NDV is highly attenuated in non-human primates [127-132,233].

chapter 9

Summarizing Discussion

adapted from:

Oncolytic Viruses: from Bench to Bedside with a Focus on Safety

Pascal R.A. Buijs
Judith H.E. Verhagen
Casper H.J. van Eijck
Bernadette G. van den Hoogen

Human Vaccines & Immunotherapeutics 2015
Jul 3;11(7):1573-84
COGEM onderzoeksproject:
Inventarisatie preklinische gentherapiestudies

In this thesis, experiments are presented that were undertaken to develop oncolytic NDV for the treatment of pancreatic adenocarcinoma. Oncolytic viruses (OVs), reported first halfway the previous century, have undergone a tremendous evolution from anecdotal experimental and clinical efficacy to state-of-the-art clinical trials employing recombinant viruses in the last decade. With the advent of reverse engineering techniques, modifications attributing to efficacy and safety have marked the introduction of new generations of recombinant OVs.

In the following, an overview is given of the current state of affairs concerning oncolytic virotherapy, including different OV platforms that are being developed towards clinical trials or that are used in clinical trials. An overview of discussed OVs is given in **table 1**.

Table 1. Characteristics of oncolytic virus platforms

Virus	Packaging capacity	Oncoselectivity	Optimized with		Prime examples	Status clinical trials	Tox	Shed	Mutation
			Arming	Targeting					
HSV	large	dividing cells (UL39) PKR response (γ_1-34.5) downregulation MHC-I (α47)	yes	yes	talimogene laherparepvec rQNestin34.5 NV1020, G207	pending FDA/ EMA	low	limited	seldom
HAdV	large	E1B-55kDa late viral RNA export Rb pathway defects overactive KRAS pathway	yes	yes	ONYX-015, Gendicine Oncorine, Delta24 ColoAd1, Telomelysin	phase III	low	yes	no
MeV	1-2 (large) transgenes	CD46 receptor innate immunity defects	yes	yes	MeV-CEA, MeV-NIS	phase II	low	no	no
NDV	1-2 transgenes	innate immunity defects apoptosis dysregulation	yes	yes	MTH68, 73-T, Ulster PV701, HUJ, rNDV F_{3aa}	phase II	low	yes	no
VSV	1-2 transgenes	type I IFN response defects abnormal translation machinery	yes	yes	VSV-hIFNβ	phase I	low	no	yes
CVA	N/A*	ICAM-1/DAF receptor overexpression	N/A*	N/A*	CAVATAK	phase II	low	no info	yes
PV	N/A	CD155 receptor overexpression	no	no	PVS-RIPO	phase I	low	no	yes
SVV	1 transgene	Unknown, neuroendocrine tumors	no	no	recombinant SVV-001	phase II	low	no	yes
VV	1-3 transgenes	overactive EGF-R pathway cellular tk expression type I IFN response defects	yes	no	Pexa-vec, vvDD, GL-ONC1	phase II	low	yes	no
mORV	N/A	overactive KRAS pathway	yes	no	mORV-T3D REOLYSIN	phase II	low	yes	yes
MuLV	1 transgene	dividing cells	yes	no	Toca 511	phase II	low	no	no

Overview of different oncolytic virus platforms and their characteristics. Prime examples include specific viruses that have been evaluated extensively in clinical trials or have promising potential based on preclinical experiments. Mutation describes the possibility of viruses to revert to wildtype, to recombine with wildtype virus, or mutate to more virulent (quasi)species. Abbreviations: HSV, Herpes simplex virus; HAdV, Human mastadenovirus; MeV, Measles virus; NDV, Newcastle disease virus; VSV, vesicular stomatitis virus; CVA, Coxsackievirus A; PV, Poliovirus; SVV, Seneca Valley virus; VV, Vaccinia virus; mORV, Mammalian orthoreovirus; MuLV, Murine leukemia virus; N/A, non-applicable; tox, toxicity; shed, shedding. * = CVA does not have a reverse genetics system.

Current status of (pre)clinical research on oncolytic virus platforms

Herpes simplex virus 1 (HSV)

HSV-1 was one of the first viruses to be developed into a recombinant oncolytic virotherapeutic vector. The large HSV genome is easy to manipulate and allows insertions of multiple additional transgenes. Furthermore, HSV infects and replicates in most tumor cell types and spreads throughout the tumor. If needed, viral replication can be hampered with anti-HSV medication (Acyclovir). Because HSV is neurotropic and causes a latent infection, most genetic modifications of oncolytic HSV (oHSV) have first focused on this potential safety issue. To generate tumor-specific oHSVs, three main strategies have been used [235]. These strategies include first attenuation by conditional replication in tumor cells by means of deletion of viral genes that are essential for viral replication in non-dividing cells (UL39) [236], counteract the protein kinase R (PKR) response (γ_1-34.5) [237-242], or contribute to immune evasion (α47) [243,244]. Secondly, to increase the oncolytic efficacy, oHSVs have been armed with immune stimulatory genes to boost local cytotoxic immune responses or other therapy enhancing transgenes [235]. Thirdly, targeting by tropism or transcription specificity has been applied.

First generation oHSVs harboring the aforementioned genetic deletions have shown to be safe based on their attenuation in normal cells. However, they are also attenuated in tumor cells and thus less cytotoxic. Recent strategies have focused on improving the targeting of less attenuated oHSVs by changing tropism or transcription specificity. Glycoprotein D is the receptor binding protein of HSV-1, and fusion of this glycoprotein with a heterologous ligand can retarget the virus to the tumor-specific receptor of choice. This tumor specific targeting is enhanced by detargeting the normal receptor [245,246]. Examples include IL-13Rα2, human epidermal growth factor receptor 2 (HER2), and epidermal growth factor receptor (EGF-R) [247-249]. Using transcriptional targeting, tumor specificity has been enhanced by placing viral genes under the control of tumor-specific promoters. A promising example is rQNestin34.5, which has the expression of the γ_1-34.5 gene under the control of the glioma-specific nestin promoter, which restores viral replication and cytotoxicity only in glioma tumors [250].

Several early generation oHSVs (talimogene laherparepvec, HSV1716, NV1020, G207, M032, rRp450 and others) have already been evaluated in clinical trials [235]. Most of these trials have demonstrated a good safety profile of HSV and treatment benefits were also observed. Talimogene laherparepvec (oHSV with deletions in γ_1-34.5 and α47, armed with granulocyte-macrophage colony-stimulating factor (GM-CSF) has recently undergone evaluation in a phase III clinical trial in patients with advanced or metastatic melanoma. Probably this will be the first oncolytic virus to obtain Food and Drug Administration (FDA) approval, while a marketing authorization application for the European Union has just been submitted to the European Medicines Agency (EMA) [86].

Human mastadenovirus (HAdV)

Because of its association with mild disease and a relatively easy to manipulate genome, most work on HAdV as vector for (cancer) gene therapy has been done with serotype 5 of species C. HAdVs have distinct advantages as a gene transfer vector, including high transfection efficiency of cells irrespective of their growth status. The genome of HAdVs is easy to manipulate for retargeting and insertion of transgenes, and efficient production of high titer virus stocks is possible. Disadvantages include high immunogenicity of prevalent serotypes with pre-existing immunity, and transient expression of the

transgene due to dilution of HAdV episomes upon cellular division [251].
Replication of oncolytic HAdV vectors is thought to be advantageous because of direct cancer cell killing induced by viral replication, and due to which the number of administrations needed for effective treatment can be reduced. Efforts have been made to develop conditionally replicating HAdVs (crHAdVs), with specific and higher replication in cancer cells. Early examples are ONYX-015 (*dl1520*) and H101, which have a deletion of E1B-55kDa (and a deletion in E3 for H101), normally responsible for p53 binding and inactivation [252]. The tumor specific replication of ONYX-015 was later shown to be due to loss of E1B-55kDa-mediated late viral RNA export, rather than p53-inactivation [253]. In a similar approach, newer crHAdVs have been created exploiting the defects in retinoblastoma (Rb) pathways in cancer cells by deleting the Rb-binding E1A-CR2 region, creating *dl922-947*, also known as Delta24 [254,255]. Additional modifications in Delta24 have been created and successfully evaluated for oncolytic efficacy [256-259], as well as oncolytic crHAdVs which target cells with an (hyper)active Kirsten rat sarcoma viral oncogene homolog (KRAS) pathway [260], or cells with YB-1 overexpression [261-264]. A different approach for creating crHAdVs is using cancer- or tissue-specific promoters to limit expression of essential early HAdV genes to specific cell types and/or tissues [265,266].
Like other oncolytic viruses that have undergone extensive development, crHAdVs have also been armed with transgenes, often under the control of a tissue/cancer-specific promoter. Examples include i.e. immunomodulatory, pro-apoptotic or pro-drug converting enzyme genes [267-270].
Despite their capacity to achieve tumor infection in animal models, the therapeutic efficacy of crHAdVs in clinical trials has thus far been disappointing [271,272]. The discrepancy between preclinical and clinical studies using crHAdV-5 could be explained by the differences in expression of the Coxsackie Adenovirus receptor (CAR) in primary tumors compared to established laboratory cell lines [273]. In addition, off-target toxicity by sequestration in mainly the liver is a serious concern, even when crHAdVs are blinded for CAR [274-277]. Hexon mutations or even complete exchange of hexons have been shown to reduce liver sequestration and transduction dramatically [278-280]. Other strategies used to circumvent liver sequestration include polyethylene glycol (PEG)-ylation or polymer/dendrimer coating of crHAdV virions, and cell-based or magnetic/liposomal nanoparticle delivery [281-323].
To circumvent the limitation of low CAR expression in (tumor) cells, retargeting has also been applied to crHAdVs, permitting CAR-independent infection [324]. The retargeting strategy can also circumvent existing humoral immunity for HAdV-5 in the general population, and contributes to the prevention of liver sequestration as described above. Examples include conjugation with anti-knob or anti-penton/hexon antibodies or adapters with retargeting ligands, pseudotyping or xenotyping with (chimeric) fiber knobs or capsids, peptide presentation (arginylglycylaspartic acid (Arg-Gly-Asp / RGD) or other), Affibody targeting, knob-less HAdVs and genetically modified capsids and/or fiber knobs [251]. More recently, efforts have also been made to develop crHAdVs fully based on other serotypes, most notably HAdV-3, or even non-human AdVs [315,325-339]. Using 'directed evolution' or 'accelerated evolution' strategies, ColoAd1 and other crHAdVs have been generated which appear to be more potent than their parental HAdV-5 based vectors [340-343].
A total of 458 clinical trials employing HAdV-mediated gene therapy have been reported to date. ONYX-015, H101 (Oncorine) and other first-generation oncolytic crHAdVs have gone through several phase I/II trials without relevant signs of toxicity but also without significant therapeutic effects, resulting in discontinuation of further trails [344]. More recent clinical trials employing new generations

of crHAdVs like RGD retargeted oncolytic crHAdVs [251,345,346], crHAdV-5/3 chimeric vectors [269,347-350], ColoAd1 [351], human telomerase reverse transcriptase (hTERT)-promoter driven crHAdV-5 vector Telomelysin [352], E2F-1-promoter driven CG0070 [270], Rb-targeted crHAdV expressing hyaluronidase (VCN-01) [353], and crHAdV vectors expressing immunomodulating genes [269,270,349,354-357] have shown safety with some promising preliminary results.

Measles virus (MeV)

Most (pre) clinical research with oncolytic MeVs have used the attenuated vaccine Edmonston strain [358,359]. The cancer selectivity of MeV stems from overexpression of the MeV receptor CD46 on malignant cells [360]. Recombinant MeV can accommodate and maintain large sizes of foreign genetic material with good genetic stability *in vitro* and *in vivo* [361-364], and MeVs expressing transgene(s) have shown good genetic stability upon passaging. Both arming and targeting strategies have been used to improve efficacy of MeV in a wide array of malignancies [358].

Completed and ongoing clinical trials in patients with T cell lymphoma, ovarian cancer or glioblastoma multiforme have first used wild type MeV and later recombinant MeV expressing marker genes carcinoembryonic antigen (CEA) and sodium/iodide symporter (NIS) [359,365,366].

Vesicular stomatitis virus (VSV)

Compared with other oncolytic viruses, VSV has some distinct advantages: first of all a well-studied biology with relative replication independency of cell cycle and a specific receptor. Secondly, VSV produces high virus yields in a wide range of cell types, it replicates intracytoplasmatic without risk of genomic integration, it harbors a small and easy to manipulate genome, and there is no pre-existing immunity in humans [367]. VSV infection in humans is generally asymptomatic and limited to people having direct contact with VSV [367]. A single case of VSV strain Indiana related encephalitis in humans has been reported [368]. VSV oncoselectivity is based largely on defective or reduced type I interferon (IFN) responses in tumor cells [369-372], although abnormal translation machinery and other cellular proteins have also been shown to play a role [373-376]. All three strategies previously described (conditional replication, arming, and targeting) have been employed to increase efficacy of VSV [367]. Furthermore, combination therapy has been described with different other therapies. Finally, optimizing delivery and distribution of oncolytic VSVs has been evaluated using cell-based carriers and aptamer or PEGylation of virions [377-380].

Coxsackievirus (CVA)

Similarly to rhinoviruses, CVA-21 binds to intercellular adhesion molecule 1 (ICAM-1) and additionally needs decay accelerating factor (DAF)-attachment for productive viral infection [381]. Given that ICAM-1 and DAF are overexpressed in melanoma cells, efforts to evaluate the oncolytic potential of wildtype CVA-21 (and other coxsackieviruses) have mainly focused on this disease [382].

Currently ongoing phase I/II clinical trials employing intratumoral injection of wildtype CVA-21 (CAVATAK) in Australian patients with advanced melanoma are showing promising preliminary results [383]. All (pre) clinical work so far has been conducted with wildtype CVA, while no progress has been made regarding conditional replication, arming or targeting.

Poliovirus (PV)

Overexpression of CD155, one of the natural receptors for PV, has been shown in (neuro) ectodermal tumors, and transcriptional upregulation has been linked to signaling pathways commonly affected in malignancy, including Raf-Erk-Mnk signaling [384,385]. The neuropathogenicity of PV is dependent on the neuronal cell-type-specific function of its internal ribosome entry site (IRES) element, which assures initiation of translation in a 5' end- and cap-independent manner. Mutations in the IRES genomic region or exchange with other viral IRES counterparts result in markedly neuro-attenuation in CD155-transgenic mice and non-human primates, without reducing the cytopathogenicity in malignant cell types that express CD155 [386].

Most preclinical research has been performed with PVS-RIPO, a recombinant PV type 1 (Sabin vaccine) strain with the IRES element of human rhinovirus type 2. PVS-RIPO has shown oncolytic efficacy in immune-deficient xenograft rat and mouse models of malignant glioma [387]. Currently a phase I clinical trial is ongoing with intratumoral infusion of PVS-RIPO in patients with recurrent glioblastoma multiforme showing durable responses [388].

Seneca Valley virus (SVV)

SVV does not infect humans but does propagate in tumor cells with neuroendocrine features [389,390]. Since its introduction as an oncolytic virus in 2007, SVV has shown preclinical efficacy in nude mouse xenograft models for several malignancies [389-394].

In a phase I clinical trial employing an intravenous dose escalation in patients with neuroendocrine tumors, SVV had (marginal) treatment benefits without causing serious adverse events when administered even in high dose (10^{11} viral particles/kg) [395]. A phase II randomized clinical trial in patients with extensive stage non-small cell lung carcinoma and a phase I dose escalation trial in pediatric patients with neuroblastoma, rhabdomyosarcoma or rare tumors with neuroendocrine features are currently underway [396].

Vaccinia virus (VV)

VV infection induces a strong cytotoxic T lymphocyte response and neutralizing antibodies without causing significant disease in humans [397,398]. As an oncolytic virus, VV has the advantage of fast replication and cell lysis with a broad cell/tumor tropism [399,400]. Furthermore, it lacks genomic integration, and shields extracellular enveloped virus VV virions from host immunity resulting in capability of (systemic) spreading between tumors [400-404]. Lastly, it also harbors a large genome packaging accommodation [404].

Several strategies have been described to target oncolytic VV to tumor cells. The VV protein Vaccinia growth factor (VGF) is homologous to cellular growth factor EGF and transforming growth factor alpha (TGFα) and can stimulate the cell for enhanced viral replication through EGF-R [405-407]. Deletion of the VGF gene will result in a VV that is targeted to cells with inherent EGF-R pathway activity, which is often observed in cancer cells [70,400,408]. J2R gene (encoding for viral thymidine kinase (tk) deletion similarly results in a VV that is dependent on overexpression of cellular tk, which is also often observed in cancer cells [409-412]. The combination of VGF and tk gene deletion is known as vvDD and results in an even more selective oncolytic VV [400,401]. VV gene B18R binds to the IFN receptor and can thereby inhibit the cellular antiviral innate immune response [413,414]. Deletion of B18R thus leads to

selectivity for IFN-deficient cells [216]. A56R gene encodes for hemagglutinin and its deletion from the VV genome results in severe (neuro)-attenuation [415].
Arming of VV has also been described, e.g. with immune stimulators, apoptotic proteins, anti-angiogenic antibodies/proteins, extracellular matrix proteases and prodrug-converting enzymes.
Early clinical trials employing non-recombinant vaccine strains of VV have shown safety when injected superficially into melanoma tumors, while local control of bladder cancer was also noted [416-418]. JX-594 (tk gene deleted, GM-CSF expressing VV Wyeth; Pexa-Vec)[419] has been evaluated in phase I-II clinical trials for patients with metastatic melanoma, (primary) liver tumors, lung, colorectal and various other solid cancer types [419-428]. GLV-1h68 (GL-ONC1) is currently being investigated in several phase I clinical trials [429,430].

Mammalian orthoreovirus type 3 Dearing (mORV-T3D)

mORV-T3D replicates in cells with dysfunctional cell signaling cascades, most importantly (but not exclusively) KRAS-overexpression and subsequent PKR inhibition, making it an inherent oncolytic virus [431-448]. A multitude of cancer types have been shown to respond to mORV-T3D treatment in (animal) models [435-437,443,449-483]. Cellular immunity has been found to be important for increasing anti-tumor efficacy [464,471,475,483-487]. The absence or inaccessibility of the JAM-A/1 receptor is perceived as a possible limitation for mORV-T3D infection of tumor cells [454,456,488]. As such, bio-selection through passaging has been attempted to retarget mORV-T3D to other receptors, although this strategy is probably limited by the quasispecies presence in mORV-T3D isolates [489-493]. Only one study using recombinant oncolytic mORV-T3D has been described thus far [494]. More studies with recombinant mORV-T3D can be expected in the near future, probably focusing on receptor retargeting and expression of therapeutic or imaging transgenes.
At this time, 16 clinical trials employing intratumoral or intravenous injection of wildtype mORV-T3D (REOLYSIN®: pelareorep) have been conducted and more are currently underway or planned to start in the near future [484,495]. These trials have shown safety of mORV-T3D administration to patients with various solid tumors without dose limiting toxicities, while having some appreciable anti-tumor effects in phase II/III trials.

Murine leukemia virus (MuLV)

MuLV development for cancer therapy has been focusing on non-replicating, as well as more recently, replication competent retroviral (RCR) oncolytic vectors. The capacity of MuLV and other retroviruses to integrate into the host genome of dividing cells carries the risk of insertional mutagenesis/oncogenesis. Reducing this risk has been an important goal in designing retroviral vectors. The replication capacity of RCR-MuLV is considered to be beneficial for optimizing gene expression in tumors. Recent RCR-MuLV vector genomes consist of an intact viral genome including an IRES-transgene immediately after the stop codon of the *env* gene, which results in more genetic stability, while retaining good replication capacity [496-498]. The fact that RCR-MuLVs can only infect and integrate in dividing cells results in an inherent onco-selectivity. In contrast to most other oncolytic viruses, the oncolytic activity of RCR-MuLVs depends solely on the transgene that is carried by the virus, since infection itself is not cytolytic. To date the transgene of choice has mostly been cytosine deaminase (CD), which converts the antifungal drug 5-fluorocytosine into active chemotherapeutic agent 5-fluorouracil. Oncolytic

activity of RCR-MuLV-CD (Toca 511) has been evaluated in preclinical (animal) models for breast cancer, glioblastoma multiforme and mesothelioma [499-504]. Toca 511 has a modified backbone and a codon-optimized and heat-stabilized CD gene and has been shown to be highly genomically stable while maintaining oncolytic efficacy upon passaging [500,505].

Newcastle disease virus (NDV)

NDV has been shown to be very safe in tumor models using mice or rats, even when used in high dose and injected intravenously, and NDV also appeared to be safe for high dose administration in humans, with no serious adverse events noted in early clinical trials [96]. Several wildtype NDV strains have shown (limited) antitumor activity without major side effects in phase I–II clinical trials for patients with various types of solid cancer [96].

Using recombinant NDVs, the oncolytic efficacy has been improved by increasing the virulence of the virus and the expression of immunomodulating or apoptotic transgenes. In addition, tumor cells are targeted with modified attachment proteins and combinations with other treatment modalities, most recently immune checkpoint blockade [114-117,119,120,125,506]. Clinical trials with these improved viruses have not yet been described, but pre-clinical data indicate efficacy in multiple tumor models for several solid malignancies, including pancreatic adenocarcinoma [114-117,119,120,125,204,506].

Work presented in this thesis regarding oncolytic NDV

Despite progress in several fields (e.g. surgery and peri-operative care, chemotherapy, radiotherapy, immunotherapy) in treatment of a multitude of different malignancies, the treatment of PDAC remains a very difficult one with little progress over the last decades. In our search for new therapies for pancreatic adenocarcinoma, we have focused our attention on the use of NDV as an oncolytic agent. For a rational design of a recombinant oncolytic NDV to treat patients with pancreatic tumors, the effect of wild-type NDV infection on human pancreatic adenocarcinoma cell lines (HPACs) *in vitro* was evaluated in **chapter 2**. Variations in NDV replication in the different HPACs generally coincided with differences in induced cytotoxic effects, with cell lines displaying more cytotoxic effects upon better NDV replication. This suggests that virus replication itself is a critical factor for NDV induced cytotoxicity, as was described in earlier studies in other cells [102-107,187,196-198]. This would argue for increasing the fusogenicity of an optimized recombinant NDV for treatment of pancreatic tumors.

It is thought that replication of oncolytic NDV is tumor specific based on defects in the innate immune responses, however, discrepancies for this theory have also been reported [107-109]. Most studies focusing on defective IFN production as a reason for tumor-specific NDV susceptibility, have only studied limited sets of tumor cell lines, which might have resulted in non-representative findings [105,106]. Using a more elaborate panel of HPACs we have demonstrated that IFN production upon NDV infection by these HPACs did not correlate with NDV susceptibility. Besides the lack of IFN production, defective IFN signaling through the JAK-STAT pathway has been indicated to be responsible for tumor specific replication of NDV [103-105]. Although our results with HPACs showed a highly variable and generally low anti-viral gene expression pattern after IFN stimulation, we also have shown that most HPACs still have an intact IFN signaling pathway, as IFN pretreatment protected the cells for NDV

replication and virus induced cytotoxicity. Few other groups have demonstrated that IFN production and signaling are not the only factors to consider. Cells over-expressing anti-apoptotic proteins like Rac1, Livin, and Bcl-xL have also shown markedly increased susceptibility to NDV replication and virus mediated killing, irrespective of IFN production and signaling [107-109].

Based on the results in chapter 2, we hypothesized that an oncolytic lentogenic rNDV would benefit from increased replication, or from the capacity to antagonize the innate immune pathways. In **chapter 3**, lentogenic rNDVs with immunomodulating potential (either stimulating innate immunity with human IFNβ (rNDV-hIFNβ-F_0) or antagonizing with the influenza virus NS1 protein (rNDV-NS1-$F_{0)}$ or increased virulence through increased cleavability of F protein (rNDV-F_{3aa}) were evaluated in a panel of 6 representative HPACs to test these hypotheses. Immunomodulation with lentogenic rNDVs resulted in markedly changed early IFN responses of HPACs as compared to parental rNDV-F_0 *in vitro*. Upon inoculation of HPACs with rNDV-hIFNβ-F_0 exogenous hIFNβ was produced in high amounts. Conversely, inoculation with rNDV-NS1-F_0 resulted in almost complete absence of IFNβ production in most HPACs, showing that immunomodulation using lentogenic rNDVs was successful. Interestingly, lower replication rates of rNDV-hIFNβ-F*0* (compared to parental rNDV-F_0) resulted in similar cytotoxicity in most HPACs, indicating that hIFNβ had a cytotoxic effect by itself. Blocking of the IFN production by exogenous expression of influenza NS1 protein from lentogenic rNDV-NS1-F_0 lead to decreased cytotoxicity (compared to parental rNDV-F_0) in most HPACs, indicating again that IFN has a cytotoxic effect on these cells. This is in contrast with results reported for a mesogenic rNDV expressing NS1 (rNDV-NS1-F_{3aa}), which induced enhanced tumor cell killing due to inhibition of apoptosis, leading to increased syncytia formation [120]. Hence, the expression of IFN-antagonists from either lentogenic or mesogenic rNDVs can have different outcomes for oncolytic efficacy. In our study, mesogenic rNDV-F_{3aa} was capable of multicycle replication in all tested cells, in contrast to lentogenic rNDVs. Testing the cytotoxicity of rNDV-F_{3aa} revealed increased oncolytic efficacy in most HPACs as compared to rNDV-F_0, indicating this to be the oncolytic rNDV with superior efficacy. This is in agreement with other studies that reported on the oncolytic efficacy of mesogenic rNDVs [114-119]. However, in 2 out of 6 HPACs (HPAF-II and Hs 700T) no significant differences were observed in cytotoxicity induced by rNDV-F_0 or rNDV-F_{3aa}. Specifically Hs 700T cells were very resistant to rNDV-F_{3aa}-induced cytotoxicity and it remains to be determined why some cells are more resistant to oncolytic rNDV than others. In addition, *in vivo* experiments using a subcutaneous xenograft tumor model in immune-deficient mice demonstrated that intratumoral treatment with mesogenic rNDV-F*3aa* induced tumor regression or stabilization, whereas treatment with lentogenic (immunomodulating) rNDVs did not result in significant anti-tumor effects.

Safety of NDV for use in virotherapy

Early (pre)clinical trials employing wildtype oncolytic NDV have shown good safety with low toxicity in laboratory animals and human patients. However, information on environmental safety is relatively scarce. In the following, we discuss our findings on safety of oncolytic rNDV.
Virulent NDV strains pose an environmental risk, as birds (specifically poultry) are very susceptible to

infection with mesogenic or velogenic strains. Limited information is available on preclinical biosafety regarding administration of oncolytic NDV in a human-like animal model. Therefore, we carried out a safety study comparing the toxicity, biodistribution and shedding of NDV strains differing in virulence (commercially available clonal NDV vaccine strain Avipro ND C131, recombinant lentogenic rNDV-F_0 and recombinant mesogenic rNDV-F_{3aa}) in non-human primates, which is presented in **chapter 4**. Intravenous injection of a high dose of (a)virulent NDV was safe with regard to toxicity for the animals. No clinical signs of illness were observed in the vaccine or recombinant virus injected animals, except short-term fever in animals injected with the highest dose Avipro ND C131 and rNDV-F_{3aa}. Hematologic and serum chemistry parameters were mostly within normal range. Analysis of the distribution of NDV showed that i.v. injection resulted in systemic spread, with most of the viral RNA ending up in the respiratory tract, spleen and liver. Histological examination however, did not reveal infected cells in any of the examined organs, indicating that substantial virus replication in these organs did not occur. Shedding of virus from injected animals was observed, irrespective of the strain used and transmission of virus could not be excluded. However, productive infection of the contact animals did not occur, since no seroconversion was detected. Earlier studies of human infection with NDV reported mostly on workers in the poultry industry having contact with live virus either when handling infected or dead poultry or lyophilized or aerosolized vaccine strains [140-146]. The exposure of these human cases was probably to a higher dose than the contact animals in the present study, since the human cases developed symptoms within a couple of days and seroconverted 1-2 weeks after exposure. We therefore argue that contact animals in the present study were not exposed to high concentrations of shed NDV, since they did not develop any symptoms nor did they seroconvert after 3 weeks of observation. Nonetheless, appropriate biosafety measures preventing environmental spread have to be considered when administrating high dose oncolytic NDV.

Preclinical studies employing NDV as a vaccine vector using subcutaneous, intranasal and/or intratracheal administration showed that NDV as vaccine vector was highly attenuated in non-human primates [127-132]. However, it is difficult to compare the results of these studies with those described in **chapter 4**, as these animals were not i.v. inoculated and the viral doses administered in these vaccine studies were relatively low.

Several clinical phase I/II trials have been described in which high dose wild-type oncolytic NDV was injected i.v. into patients with advanced and/or metastatic solid cancer [91-93,97-101]. These trials employed naturally occurring, *in vitro* selected oncolytic strains of NDV. Viral doses used in these clinical studies were comparable to the dose administered in our primate study: up to 34-120 x 10^9 EID_{50} m^{-2} (body surface) as compared to an equivalent dose of 4 x 10^9 $TCID_{50}$ m^{-2} recombinant virus in our study. A study recruiting 14 patients suffering from glioblastoma multiforme showed that i.v. injection of a high dose of the lentogenic oncolytic strain NDV-HUJ was safe, with grade I-II fever as only adverse event [92]. Infectious virus was recovered from blood, saliva and urine samples up to 96 hours after injection. Two other phase I/II studies with a total of 95 patients showed that i.v. injection of a virulent NDV strain (PV701) was safe in patients with advanced or metastatic solid tumors [93,99]. Common side effects included fever, diarrhea and slight transient elevation of liver transaminases (but only in patients with hepatic metastases). Shedding of injected virus was also observed up to three weeks after injection. Measures to prevent environmental spread of this virulent NDV strain were not described [93,99].

In chapter 4, we demonstrated that high dose i.v. oncolytic NDV is safe in a non-human primate model, and previous clinical studies have reported safety as well. However, Pigeon Paramyxovirus type 1 (PPMV-1), a pigeon-specific strain of NDV, has been shown to cause lethal disease with severe pneumonia in at least two human cases [168]. In **chapter 5**, we investigated whether PPMV-1 isolated from a fatal human case had zoonotic potential in a non-human primate model. We demonstrated that experimental infection resulted in severe pneumonia. These findings largely correspond with the presentation of PPMV-1 infection in two human fatal cases from New York [168] and The Netherlands (unpublished data). In contrast to what is reported for a PPMV-1 positive patient from New York, who had systemic infection based on PPMV-1-positive urine and stool samples, in our non-human primates no PPMV-1 was detected in any extra-respiratory tissues—including kidney, small and large intestine, pancreas and liver—of our macaques, either by qRT-PCR or immunohistochemistry. However, it cannot be excluded that under conditions of immunosuppression, the virus would be able to spread systemically.

The virulence of PPMV-1 for humans and macaques suggests that the safety of NDV as an oncolytic agent needs to be evaluated properly for different strains. The results presented in chapter 5, together with two fatal human cases of PPMV-1 infection, indicate that some variants of NDV can cause severe pneumonia in people under certain conditions, such as immunosuppression. It remains to be determined whether PPMV-1, and particularly these isolates from fatal human cases, is more virulent for people than other NDV strains.

Safety of other oncolytic virus platforms

As described earlier, several oncolytic viruses have made their way into clinical trials. Also, for these virus platforms, information regarding their safety, especially environmental, is limited. The following presents a discussion of findings from a literature study.

Herpes simplex virus 1 (HSV)

Preclinical evaluation employing intra-organ (brain or prostate) injection with oHSV in non-human primates demonstrated no shedding of virus, which points to limitation of oHSV replication to injection sites [507,508]. This was confirmed by early clinical trials in patients injected intratumorally (glioma) with oHSVs: sporadic shedding of HSV in saliva was noted, but this was shown to be wildtype virus as opposed to the injected oHSV [509,510]. Other studies have observed limited leakage of oHSV from injection sites up to 2 weeks post treatment, without other excreta containing viable oHSV [511-513]. Intra-arterial hepatic injection did not result in detectable environmental shedding either [514]. A possible concern is that an oHSV recombines with a wildtype endogenous virus. This however, is an unlikely event, given that the two viruses would have to infect the same cell in (near) equal genome copy numbers. If the oHSV carries heterologous genes, the recombinant would have to arise by illegitimate recombination – an extremely rare event that cannot be replicated *in vitro* [515]. Spontaneous reversion of oHSVs with deleted viral genes to wildtype virus is not possible. However, compensatory mutations can arise, but so far resulting HSV mutants have been highly attenuated [516]. These compensatory mutations can be of concern when evaluating the safety of newer generation oHSVs.

Human mastadenovirus (HAdV)

In general, the use of early generation oncolytic crHAdVs appears to be reasonably safe when administered locally and at lower doses systemically. However, the development of newer generations of crHAdVs expressing transgenes, having altered capsids or different promoters can dramatically alter this perceived safety. Shedding of crHAdVs from injection sites and patient excretions, although not always reported, has been observed in several (pre) clinical trials, and increases with higher doses and systemic administration [345,517-523]. Homologous recombination between AdVs of the same subgroup occurs with high efficiency during growth in co-infected cultured cells, and there is evidence of recombination events in humans as well [524-526]. Theoretically, homologous recombination between wildtype AdVs and recombinant crHAdVs could lead to new wildtype AdVs that e.g. possess transgenes, or worse, have expanded tissue tropism due to retargeting strategies. However, to date such recombination has never been detected in any clinical trial.

Measles virus (MeV)

Intratumoral, intraperitoneal and intravenous administration has been reported using doses up to 10^9 infectious viral particles without dose limiting toxicity or MeV induced immunosuppression [359,365,366]. Although wildtype MeV can cause potentially serious disease, attenuated MeV vaccine strains like Edmonston have an excellent safety record [527]. In clinical trials with rMeV-CEA, no evidence was seen of shedding in sputum and urine samples of patients who were intraperitoneally injected [365]. Spread of oncolytic MeV in the general population is highly unlikely since most individuals in industrialized countries are immunized, although herd immunity is currently waning with declining vaccination percentages.

Vesicular stomatitis virus (VSV)

A recent study in purpose-bred beagle dogs showed that a dose up to 10^{10} $TCID_{50}$ of VSV-hIFNβ was well tolerated, with mild adverse events with the exception of one dog that received 10^{11} $TCID_{50}$ which developed severe hepatotoxicity and shock leading to euthanasia [528]. A following study testing VSV-hIFNβ in rhesus macaques via intrahepatic injection did not result in neurological symptoms and is considered to be safe enough to proceed into phase I clinical trials, which are currently ongoing in humans and pet dogs [529,530]. No VSV RNA was detected in buccal swabs taken from non-human primates after intrahepatic injection with VSV-hIFNβ [529]. Theoretically, VSV mutants harboring mutations in their M or G gene (making them oncoselective and abolishing neurotropism) could revert to wildtype VSV upon passaging. Also, VSVs expressing attenuating transgenes like hIFNβ can acquire mutations in this transgene, which has been shown in several studies [531-533]. Furthermore, oncolytic VSVs have been shown to optimize targeting to glycoproteins upon passaging in tumor cells [534], and to mutate expressed transgenes to optimize replication [535]. These examples have strangely not been perceived as a safety problem.

Coxsackievirus (CVA)

Clinical trials thus far have not led to serious adverse events. No information is available regarding shedding. When considering non- or low-pathogenic coxsackieviruses for oncolytic virotherapy, environmental risks are considered to be low. However, when using viruses that do cause (severe) disease in humans, care should be taken to evaluate and/or attenuate these new vectors.

Poliovirus (PV)

Extensive evaluation in non-human primates has shown PVS-RIPO to be safe for intraspinal and intrathalamic injection, without observations of extraneural replication or shedding [386,536]. No serious adverse events have been observed so far in an ongoing phase I clinical trial [388]. One of the biggest concerns with PV is the inherent genomic instability of picornaviruses and thus the possible reversion to wildtype pathogenicity. PVS-RIPO has been evaluated extensively for genomic instability by e.g. serial passaging *in vitro* and *in vivo* and it was shown that escape mutants reverting to neuropathogenic virulence in the CD155-transgenic mouse model do arise [537]. Similar mutants have not been observed in other animal models, which makes it unclear what the importance of this preclinical finding is in relation to currently ongoing clinical trials in humans.

Seneca Valley virus (SVV)

Recent reports indicated that, although the natural host is still uncertain, this virus seems safe for use as oncolytic virotherapy in (pediatric) patients [395]. Analysis of samples obtained from researchers in close contact with phase I clinical trial patients revealed the absence of neutralizing antibody titers, which points to lack of viral shedding by the treated patients [395]. However, detailed evaluation of shedding was not performed, and should be determined in future clinical trials.

Vaccinia virus (VV)

Clinical trials with oncolytic VV have thus far reported good safety with minor side effects like transient low-grade fever and local pain [425,538,539]. Commonly, live vaccinia virus is shed from skin injection sites after vaccination [539]. Also, in clinical trials, live JX-594 was detected in throat swabs and skin pustules of patients up to one week after administration [428]. Theoretically, recombination between oncolytic recombinant VV and wildtype VV is possible, however, since VV vaccination is not practiced on a large scale anymore, this is highly unlikely. In addition, spontaneous mutation rates for VV have been shown to be very low [540].

Mammalian orthoreovirus type 3 Dearing (mORV-T3D)

High mORV titers injected intravenously have been shown to be reasonably safe, even in combination with standard therapies like chemo- or radiotherapy, as well as in combination with transient immune suppression [484,495]. Limited mORV shedding has been observed in clinical trials in patient samples of urine, saliva and feces, mostly with high intravenous administrations [484]. As an RNA virus with a viral RNA polymerase, mORV genome replication is prone to errors, which can lead to mutations in offspring. Furthermore, since wild-type isolates are in use, these probably represent several quasispecies [541]. Even so, since mORV-T3D does not seem to cause disease in human subjects, the relevance of this mutation rate is low.

Murine leukemia virus (MuLV)

Toca 511 is being investigated in clinical trials in the United States in subjects with recurrent high-grade glioma. Up to now, over 70 patients have been treated without dose limiting toxicity and with evidence of clinical oncolytic efficacy [505]. Since RCR-MuLV vectors are capable of genomic integration, germline transmission is a theoretical risk of these vectors, and should be taken into consideration when designing clinical trials.

Conclusions

Oncolytic virus platforms

The field of oncolytic virus research has seen a tremendous progression of several first and second generation vectors towards clinical trials. Most current strategies used in oncolytic virotherapy focus on the use of second and third generation of more virulent conditionally replicating viruses, armed with immune stimulating, anti-tumor or tracking transgenes. Also, immune evasion is still sought after to optimize vector delivery. There seems to be a general agreement that direct cell killing by an oncolytic virus might not be as important as once thought, and that indirect immunologic effects ultimately define the success of oncolytic virotherapy. With the first oncolytic virus (talimogene laherparepvec) now on the break of FDA and EMA approval, we can expect an even greater interest for this relatively young field of oncologic research in the near future.

Safety of oncolytic virus platforms

The newer generation of oncolytic viruses has been evaluated extensively for their efficacy in preclinical trials, and they have shown to be more effective than first generation vectors on many occasions. However, although some evidence has been gathered regarding their safety in terms of toxicity in laboratory animals, safety studies focusing on environmental shedding and possible recombination of these new oncolytic agents with wildtype viruses are scarce.

Future perspective

To further develop oncolytic recombinant NDV as treatment modality for pancreatic adenocarcinoma, there are a few key issues to address.

First and foremost, experiments in an immune-competent animal model of PDAC should be undertaken, since immune responses could dramatically alter the outcome (for better or worse) of oncolytic NDV treatment, especially with immunomodulating viruses or in combination with other (immuno)therapies [125]. Although transgenic animal models that mimic the natural development of PDAC have been created, these are also difficult to employ for evaluation of the efficacy of OVs. Mostly because the transgenic animal model for PDAC is a very difficult model to maintain with a lot of surplus animals needed for the breeding of specific genetic alterations. In addition, the tumors arising in this model have been shown to be very aggressive, which makes it difficult to evaluate therapies. Syngeneic models, either with orthotopic (in the pancreas or liver for local or metastatic disease respectively) or subcutaneous allografts, might be used to circumvent these disadvantages. These are less difficult to maintain in a breeding colony as the recipient animals do not need to have the same genetic alterations, only the same genetic background. Also, in these animals the PDAC tumors tend to develop more slowly, allowing a better evaluation of treatment over time. These syngeneic models could provide "naturally grown" mouse PDAC tumors in an immunocompetent animal, although it remains a model with artificially created tumors and an immune system that is not completely translatable to the human equivalent.

Secondly, future experiments should elaborate more on the etiology of differences in susceptibility of HPACs for the oncolytic effect of NDV. These experiments should not only focus on innate immunity, but also on differences in apoptotic, necrotic, autophagy and/or immunogenic cell death pathways. Elucidating knowledge on the traits defining susceptibility to NDV induced oncolytic effects would allow improvement of oncolytic NDV to also attack relatively resistant tumor cells.

Thirdly, during the execution of our safety experiments with oncolytic NDVs in non-human primates, it was reported that NDV generated in embryonated chicken eggs was susceptible to neutralization by (human) serum through complement binding and activation [218]. We also confirmed that non-heat inactivated primate and human sera neutralized egg-generated NDV, in contrast to NDV generated in primate or human cells. All clinical trials thus far have been using egg-generated virus stocks and still reported positive effects for NDV virotherapy, as well as shedding of infectious virus, indicating that egg-generated NDV is still capable of replication [91-93,97-101]. The efficacy of NDV virotherapy might increase when using virus stocks generated in human or primate cells. However, neutralization by complement might also be important for safe administration of high doses of NDV, and abolishing this could result in more toxicity and a higher risk of shedding to the environment. More information should therefore be obtained on the safety and oncolytic efficacy of virotherapy with NDV stocks generated in human or primate cells.

Finally, the revival of oncolytic virus research has led to the investigation of new (recombinant) viruses without proper safety evaluations, especially environmental safety. A new OV should not only be tested for efficacy, but also for (environmental) safety, as this combination of efficacy and safety truly defines a promising new agent. Without a thorough environmental risk assessment, new agents will not be accepted by the regulatory agencies like EMA and FDA for marketing as new therapies. Especially with the latest oncolytic agents becoming more virulent, and with the possibility of expressing transgenes that alter the nature of the virus, any possibility of environmental shedding and recombination with wild type virus should be excluded. More studies evaluating the environmental safety of promising OVs (including NDV) should therefore be conducted and reported.

Chapter 7

Nederlandse Samenvatting

Pancreascarcinoom is een zeer agressieve vorm van kanker met een slechte prognose en bijna iedere patiënt met pancreascarcinoom zal uiteindelijk aan zijn of haar ziekte overlijden. Dit wordt weerspiegeld in de incidentie (338.000 patiënten) en sterftecijfers (330.000 gevallen) wereldwijd per jaar, waarvan in Nederland in 2014 3000 patiënten. Er worden verschillende vormen van pancreascarcinoom onderscheiden, waarvan het ductaal type de meest voorkomende is. Pancreascarcinomen worden klinisch ingedeeld als zijnde operabel of inoperabel op basis van lokale (door)groei en uitzaaiingen. Helaas komt slechts 15 procent van de patiënten die wordt gediagnosticeerd met pancreascarcinoom in aanmerking voor een curatieve operatie middels pancreaticoduodenectomie (Whipple procedure) of pancreasstaartresectie. Voor de overige 85 procent van de patiënten rest slechts palliatieve chemotherapie. De prognose van een operabel pancreascarcinoom is ondanks verbeteringen in (peri-) operatieve zorg en adjuvante therapieën nog steeds slecht: de vijfjaarsoverleving bedraagt 20 procent, met een mediane overleving van 24 maanden. Voor patiënten met een inoperabel pancreascarcinoom is het toekomstperspectief nog somberder, de mediane overleving bedraagt voor deze patiënten slechts 6 maanden.

Oncolytische virussen (OVs) zijn virussen die selectief kwaadaardige tumoren kunnen infecteren en beschadigen, zonder dat gezonde weefsels ernstige schade oplopen. Het gebruik van OVs voor de behandeling van kanker wordt oncolytische virotherapie genoemd. Veel kwaadaardige tumoren hebben in hun ontwikkeling eigenschappen verworven waardoor ze minder gevoelig zijn voor surveillance van het immuunsysteem, ze ongecontroleerd en ongeremd groeien en ze inductie van natuurlijke celdood tegengaan. Deze eigenschappen zorgen er tevens voor dat OVs deze cellen makkelijker kunnen infecteren. OVs maken gebruik van de cellulaire bronnen van kankercellen om zich te vermenigvuldigen en deze infectie leidt tot lysis van de cellen, waardoor deze kankercellen uiteindelijk te gronde gaan. De introductie van en de vooruitgang in genetische modificatie technieken heeft een enorme impuls gegeven aan het onderzoeksveld van de oncolytische virotherapie. Het is nu mogelijk om de eigenschappen van OVs zodanig aan te passen dat ze selectiever kankercellen infecteren en dus veiliger zijn. Bovendien kunnen OVs voorzien worden van additionele transgenen, waardoor de effectiviteit van de behandeling vergroot kan worden, de aflevering van OVs ter plaatse van tumoren kan worden verbeterd, en er bijvoorbeeld betere beeldvorming verricht kan worden.

Newcastle disease virus (NDV) is een van de veelbelovende OVs die verder ontwikkeld worden voor het gebruik in oncolytische virotherapie. De eerste klinische onderzoeken met wild type NDV stammen zijn verricht in de jaren '50-'60 van de vorige eeuw. Sindsdien zijn er meerdere klinische onderzoeken ondernomen in patiënten met diverse vormen van kanker, die in ieder geval hebben aangetoond dat het gebruik van wild type NDV in oncolytische virotherapie veilig is voor mensen. Het is niet geheel duidelijk waarom oncolytisch NDV selectief is voor kankercellen, hoewel verscheidene redenen zoals verschillen in aangeboren immuniteit of afwijkingen in de mechanismen voor groei en celdood van kankercellen zijn aangedragen. Sinds het mogelijk is om NDV genetisch te modificeren, is er meer aandacht gekomen voor het verder ontwikkelen van recombinant oncolytisch NDV voor het gebruik in oncolytische virotherapie. Dit heeft tot op heden nog niet geleid tot nieuwe klinische onderzoeken in patiënten. Een van de belangrijkste redenen hiervoor is dat hoog virulente stammen van NDV een gevaar kunnen vormen voor het milieu. Met name kippen en ander gevogelte zijn zeer gevoelig voor infectie en een uitbraak van NDV in een (commerciële) kippen populatie kan gepaard gaan met een hoge mortaliteit. Mensen kunnen ook geïnfecteerd worden met NDV, maar dit leidt slechts tot lichte

symptomen als conjunctivitis en laryngitis, welke spontaan weer voorbijgaan. Er zijn één tot twee casussen beschreven waarin immuun gecompromitteerde patiënten zijn overleden aan de gevolgen van een infectie met een speciale duivenstam van NDV, ook wel Pigeon Paramyxovirus type 1 (PPMV-1) genoemd.

In dit proefschrift worden studies gepresenteerd die zijn ondernomen om oncolytisch NDV verder te ontwikkelen als nieuwe behandeling van pancreascarcinoom.
In **hoofdstuk 2** wordt een studie beschreven naar de effecten voor pancreascarcinoom cellen van inoculatie met wild type laag virulent NDV voor wat betreft immuun respons en celdood. Hoewel alle 11 verschillende cellijnen gevoelig waren voor infectie met NDV, was er wel verschil tussen de cellijnen in de mate van ondersteuning van replicatie van NDV en de geïnduceerde celdood. Over het algemeen leidde een betere replicatie van NDV tot meer celdood. Normale cellen reageren op een NDV infectie met het produceren van interferon (IFN) wat vervolgens virus replicatie remt, en er werd altijd verondersteld dat tumor cellen een defect in dit mechanisme hebben waardoor zij gevoeliger zijn voor NDV infectie. Wij hebben echter laten zien dat een aantal van de 11 onderzochte pancreascarcinoom cellijnen nog altijd IFN produceert, en dat deze IFN productie geen invloed heeft op de geïnduceerde celdood door het virus. Daarnaast hebben wij laten zien dat het voorbehandelen van pancreascarcinoom cellen met het aangeboren immuunsysteem stimulerend IFN bèta leidde tot een duidelijke bescherming tegen infectie en replicatie van oncolytisch NDV in de meeste cellen. Dit duidt er op dat er geen correlatie is tussen defecten in aangeboren immuniteit en gevoeligheid van cellen voor replicatie van NDV.
Gebaseerd op de bevindingen van hoofdstuk 2, beschrijft **hoofdstuk 3** het ontwikkelen van verbeterde recombinante NDVs om de oncolytische effectiviteit te vergroten. Hierbij is gekozen om laag virulente NDVs met ingebouwde immuun modulerende eigenschappen te vergelijken met een hoog virulent NDV zonder deze eigenschappen. Het bleek mogelijk om de immuunrespons van pancreascarcinoom cellen te beïnvloeden door middel van de virale expressie van IFN-stimulerende of IFN-remmende eiwitten. Dit leidde overigens niet tot een duidelijke verbetering van het oncolytisch effect in celkweken of in een immuun deficiënt muismodel voor pancreascarcinoom. Dit in tegenstelling tot hoog virulent recombinant NDV, dat een duidelijke verbetering van oncolytische effectiviteit liet zien in de meeste geteste modellen, zowel *in vitro* als *in vivo*.

Naast effectiviteit is veiligheid een belangrijke eigenschap van een oncolytisch virus. **Hoofdstuk 4** beschrijft een studie naar de veiligheid van oncolytisch NDV in non-humane primaten. Deze dieren werden intraveneus geïnjecteerd met een hoge doses van verschillende stammen van (recombinant) NDV en vervolgens onderzocht op toxische effecten en op eventuele uitscheiding van het virus, om een inschatting te kunnen maken wat betreft risico's voor mens en milieu. Deze studie toonde aan dat intraveneuze injectie van de geteste stammen van (recombinant) NDV niet leidde tot toxiciteit in non-humane primaten. De weinige uitscheiding van NDV die werd aangetoond, zorgde niet voor infectie van contactdieren. Deze studie heeft laten zien dat NDV veilig gebruikt kan worden in oncolytische virotherapie.
Hoewel NDV niet bekend staat als een veroorzaker van een ernstige zoönose in mensen, zijn er toch één à twee ernstige infecties in immuun-gecompromitteerde patiënten beschreven met een speciale

duiven stam van NDV, te weten PPMV-1. Een isolaat van één van deze casussen is gebruikt om in een non-humane primaten model te onderzoeken of dit virus inderdaad in staat is om zoogdieren te infecteren. De resultaten van deze studie staan beschreven in **hoofdstuk 5**. Hierbij werden non-humane primaten geïnfecteerd via de natuurlijke route (intra-tracheaal, intra-nasaal en conjunctivaal) en enkele dagen later onderzocht op mogelijke PPMV-1 gerelateerde afwijkingen. Deze studie toonde dat non-humane primaten inderdaad gevoelig zijn voor virale pneumonie veroorzaakt door PPMV-1, wat in overeenstemming lijkt met de beschreven menselijke casussen.

De toekomst van oncolytische virotherapie, met in het bijzonder oncolytisch NDV, is veelbelovend, maar zit ook vol met uitdagingen. Zo bestaat nog steeds de vraag waarom NDV nu precies selectief is voor kankercellen. Een antwoord hierop zou de ontwikkeling naar de kliniek kunnen versnellen. Daarbij zal er ook onderzocht moeten worden welke van de recombinante stammen van NDV het meest geschikt is voor ontwikkeling richting klinische onderzoeken: laag virulente NDVs met immuun modulerende of andere versterkende eigenschappen of hoog virulente NDVs, waarbij middels genetische modificatie de replicatie wordt beperkt tot humane tumoren om schade aan het milieu te voorkomen. Onderzoeken in immuun competente muismodellen voor pancreascarcinoom zullen moeten worden ondernomen om een antwoord op deze vraag te krijgen. Hoe dan ook, het hier beschreven onderzoek heeft laten zien dat oncolytisch NDV de potentie heeft om uit te groeien tot een klinisch toepasbare modaliteit voor de behandeling van pancreascarcinoom en waarschijnlijk ook andere vormen van kanker.

chapter 8

Reference list

1. Klimstra, D.S. Nonductal neoplasms of the pancreas. ***Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc*** **2007**, *20 Suppl 1*, S94-112.
2. Bosman, F.T.; Carneiro, F.; Hruban, R.H.; Theise, N.D. ***WHO Classification of Tumours of the Digestive System***. 4th ed.; IARC: 2010; Vol. 3.
3. Feig, C.; Gopinathan, A.; Neesse, A.; Chan, D.S.; Cook, N.***, et al.*** The pancreas cancer microenvironment. ***Clin Cancer Res*** **2012**, *18*, 4266-4276.
4. Hruban, R.H.; van Mansfeld, A.D.; Offerhaus, G.J.; van Weering, D.H.; Allison, D.C. K-ras oncogene activation in adenocarcinoma of the human pancreas. A study of 82 carcinomas using a combination of mutant-enriched polymerase chain reaction analysis and allele-specific oligonucleotide hybridization. ***Am J Pathol*** **1993**, *143*, 545-554.
5. Jones, S.; Zhang, X.; Parsons, D.W.; Lin, J.C.; Leary, R.J.***, et al.*** Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. ***Science*** **2008**, *321*, 1801-1806.
6. Almoguera, C.; Shibata, D.; Forrester, K.; Martin, J.; Arnheim, N.***, et al.*** Most human carcinomas of the exocrine pancreas contain mutant c-K-ras genes. ***Cell*** **1988**, *53*, 549-554.
7. Redston, M.S.; Caldas, C.; Seymour, A.B.; Hruban, R.H.; da Costa, L.***, et al.*** p53 mutations in pancreatic carcinoma and evidence of common involvement of homocopolymer tracts in DNA microdeletions. ***Cancer Res*** **1994**, *54*, 3025-3033.
8. Rozenblum, E.; Schutte, M.; Goggins, M.; Hahn, S.A.; Panzer, S.***, et al.*** Tumor-suppressive pathways in pancreatic carcinoma. ***Cancer Res*** **1997**, *57*, 1731-1734.
9. Caldas, C.; Hahn, S.A.; da Costa, L.T.; Redston, M.S.; Schutte, M.***, et al.*** Frequent somatic mutations and homozygous deletions of the p16 (MTS1) gene in pancreatic adenocarcinoma. ***Nature genetics*** **1994**, *8*, 27-32.
10. Schutte, M.; Hruban, R.H.; Geradts, J.; Maynard, R.; Hilgers, W.***, et al.*** Abrogation of the Rb/p16 tumor-suppressive pathway in virtually all pancreatic carcinomas. ***Cancer Res*** **1997**, *57*, 3126-3130.
11. Hahn, S.A.; Schutte, M.; Hoque, A.T.; Moskaluk, C.A.; da Costa, L.T.***, et al.*** DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. ***Science*** **1996**, *271*, 350-353.
12. Goggins, M.; Offerhaus, G.J.; Hilgers, W.; Griffin, C.A.; Shekher, M.***, et al.*** Pancreatic adenocarcinomas with DNA replication errors (RER+) are associated with wild-type K-ras and characteristic histopathology. Poor differentiation, a syncytial growth pattern, and pushing borders suggest RER+. ***Am J Pathol*** **1998**, *152*, 1501-1507.
13. Wilentz, R.E.; Goggins, M.; Redston, M.; Marcus, V.A.; Adsay, N.V.***, et al.*** Genetic, immunohistochemical, and clinical features of medullary carcinoma of the pancreas: A newly described and characterized entity. ***Am J Pathol*** **2000**, *156*, 1641-1651.
14. Hruban, R.H.; Goggins, M.; Parsons, J.; Kern, S.E. Progression model for pancreatic cancer. ***Clin Cancer Res*** **2000**, *6*, 2969-2972.
15. Kanda, M.; Matthaei, H.; Wu, J.; Hong, S.M.; Yu, J.***, et al.*** Presence of somatic mutations in most early-stage pancreatic intraepithelial neoplasia. ***Gastroenterology*** **2012**, *142*, 730-733 e739.
16. Hustinx, S.R.; Leoni, L.M.; Yeo, C.J.; Brown, P.N.; Goggins, M.***, et al.*** Concordant loss of MTAP and p16/CDKN2A expression in pancreatic intraepithelial neoplasia: evidence of homozygous deletion in a noninvasive precursor lesion. ***Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc*** **2005**, *18*, 959-963.
17. Ferlay, J.; Soerjomataram, I.; Dikshit, R.; Eser, S.; Mathers, C.***, et al.*** Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. ***Int J Cancer*** **2015**, *136*, E359-386.
18. Siegel, R.; Ma, J.; Zou, Z.; Jemal, A. Cancer statistics, 2014. ***CA Cancer J Clin*** **2014**, *64*, 9-29.
19. Tersmette, A.C.; Petersen, G.M.; Offerhaus, G.J.; Falatko, F.C.; Brune, K.A.***, et al.*** Increased risk of incident pancreatic cancer among first-degree relatives of patients with familial pancreatic cancer. ***Clin Cancer Res*** **2001**, *7*, 738-744.
20. Jacobs, E.J.; Chanock, S.J.; Fuchs, C.S.; Lacroix, A.; McWilliams, R.R.***, et al.*** Family history of cancer and risk of pancreatic cancer: a pooled analysis from the Pancreatic Cancer Cohort Consortium (PanScan). ***Int J Cancer*** **2010**, *127*, 1421-1428.
21. Rebours, V.; Boutron-Ruault, M.C.; Schnee, M.; Ferec, C.; Maire, F.***, et al.*** Risk of pancreatic adenocarcinoma in patients with hereditary pancreatitis: a national exhaustive series. ***The American journal of gastroenterology*** **2008**, *103*, 111-119.
22. Howes, N.; Lerch, M.M.; Greenhalf, W.; Stocken, D.D.; Ellis, I.***, et al.*** Clinical and genetic characteristics of

hereditary pancreatitis in Europe. ***Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association*** **2004**, *2*, 252-261.
23. Roberts, N.J.; Jiao, Y.; Yu, J.; Kopelovich, L.; Petersen, G.M.***, et al.*** ATM mutations in patients with hereditary pancreatic cancer. ***Cancer discovery*** **2012**, *2*, 41-46.
24. Thompson, D.; Easton, D.F.; Breast Cancer Linkage, C. Cancer Incidence in BRCA1 mutation carriers. ***J Natl Cancer Inst*** **2002**, *94*, 1358-1365.
25. Iqbal, J.; Ragone, A.; Lubinski, J.; Lynch, H.T.; Moller, P.***, et al.*** The incidence of pancreatic cancer in BRCA1 and BRCA2 mutation carriers. ***Br J Cancer*** **2012**, *107*, 2005-2009.
26. Murphy, K.M.; Brune, K.A.; Griffin, C.; Sollenberger, J.E.; Petersen, G.M.***, et al.*** Evaluation of candidate genes MAP2K4, MADH4, ACVR1B, and BRCA2 in familial pancreatic cancer: deleterious BRCA2 mutations in 17%. ***Cancer Res*** **2002**, *62*, 3789-3793.
27. Hahn, S.A.; Greenhalf, B.; Ellis, I.; Sina-Frey, M.; Rieder, H.***, et al.*** BRCA2 germline mutations in familial pancreatic carcinoma. ***J Natl Cancer Inst*** **2003**, *95*, 214-221.
28. Su, G.H.; Hruban, R.H.; Bansal, R.K.; Bova, G.S.; Tang, D.J.***, et al.*** Germline and somatic mutations of the STK11/LKB1 Peutz-Jeghers gene in pancreatic and biliary cancers. ***Am J Pathol*** **1999**, *154*, 1835-1840.
29. Lynch, H.T.; Fusaro, R.M. Pancreatic cancer and the familial atypical multiple mole melanoma (FAMMM) syndrome. ***Pancreas*** **1991**, *6*, 127-131.
30. Vasen, H.F.; Gruis, N.A.; Frants, R.R.; van Der Velden, P.A.; Hille, E.T.***, et al.*** Risk of developing pancreatic cancer in families with familial atypical multiple mole melanoma associated with a specific 19 deletion of p16 (p16-Leiden). ***Int J Cancer*** **2000**, *87*, 809-811.
31. Bosetti, C.; Lucenteforte, E.; Silverman, D.T.; Petersen, G.; Bracci, P.M.***, et al.*** Cigarette smoking and pancreatic cancer: an analysis from the International Pancreatic Cancer Case-Control Consortium (Panc4). ***Ann Oncol*** **2012**, *23*, 1880-1888.
32. Lynch, S.M.; Vrieling, A.; Lubin, J.H.; Kraft, P.; Mendelsohn, J.B.***, et al.*** Cigarette smoking and pancreatic cancer: a pooled analysis from the pancreatic cancer cohort consortium. ***American journal of epidemiology*** **2009**, *170*, 403-413.
33. Ahlgren, J.D. Epidemiology and risk factors in pancreatic cancer. ***Seminars in oncology*** **1996**, *23*, 241-250.
34. Aune, D.; Greenwood, D.C.; Chan, D.S.; Vieira, R.; Vieira, A.R.***, et al.*** Body mass index, abdominal fatness and pancreatic cancer risk: a systematic review and non-linear dose-response meta-analysis of prospective studies. ***Ann Oncol*** **2012**, *23*, 843-852.
35. Ben, Q.; Xu, M.; Ning, X.; Liu, J.; Hong, S.***, et al.*** Diabetes mellitus and risk of pancreatic cancer: A meta-analysis of cohort studies. ***Eur J Cancer*** **2011**, *47*, 1928-1937.
36. Lowenfels, A.B.; Maisonneuve, P.; Cavallini, G.; Ammann, R.W.; Lankisch, P.G.***, et al.*** Pancreatitis and the risk of pancreatic cancer. International Pancreatitis Study Group. ***The New England journal of medicine*** **1993**, *328*, 1433-1437.
37. Ekbom, A.; McLaughlin, J.K.; Karlsson, B.M.; Nyren, O.; Gridley, G.***, et al.*** Pancreatitis and pancreatic cancer: a population-based study. ***J Natl Cancer Inst*** **1994**, *86*, 625-627.
38. Bang, U.C.; Benfield, T.; Hyldstrup, L.; Bendtsen, F.; Beck Jensen, J.E. Mortality, cancer, and comorbidities associated with chronic pancreatitis: a Danish nationwide matched-cohort study. ***Gastroenterology*** **2014**, *146*, 989-994.
39. Duell, E.J.; Lucenteforte, E.; Olson, S.H.; Bracci, P.M.; Li, D.***, et al.*** Pancreatitis and pancreatic cancer risk: a pooled analysis in the International Pancreatic Cancer Case-Control Consortium (PanC4). ***Ann Oncol*** **2012**, *23*, 2964-2970.
40. Modolell, I.; Guarner, L.; Malagelada, J.R. Vagaries of clinical presentation of pancreatic and biliary tract cancer. ***Ann Oncol*** **1999**, *10 Suppl 4*, 82-84.
41. Porta, M.; Fabregat, X.; Malats, N.; Guarner, L.; Carrato, A.***, et al.*** Exocrine pancreatic cancer: symptoms at presentation and their relation to tumour site and stage. ***Clinical & translational oncology : official publication of the Federation of Spanish Oncology Societies and of the National Cancer Institute of Mexico*** **2005**, *7*, 189-197.
42. Chari, S.T.; Leibson, C.L.; Rabe, K.G.; Ransom, J.; de Andrade, M.***, et al.*** Probability of pancreatic cancer following diabetes: a population-based study. ***Gastroenterology*** **2005**, *129*, 504-511.
43. Canto, M.I.; Harinck, F.; Hruban, R.H.; Offerhaus, G.J.; Poley, J.W.***, et al.*** International Cancer of the Pancreas Screening (CAPS) Consortium summit on the management of patients with increased risk for familial pancreatic cancer. ***Gut*** **2013**, *62*, 339-347.

44. Valls, C.; Andia, E.; Sanchez, A.; Fabregat, J.; Pozuelo, O.*, et al.* Dual-phase helical CT of pancreatic adenocarcinoma: assessment of resectability before surgery. *AJR Am J Roentgenol* **2002**, *178*, 821-826.
45. Bronstein, Y.L.; Loyer, E.M.; Kaur, H.; Choi, H.; David, C.*, et al.* Detection of small pancreatic tumors with multiphasic helical CT. *AJR Am J Roentgenol* **2004**, *182*, 619-623.
46. Wong, J.C.; Raman, S. Surgical resectability of pancreatic adenocarcinoma: CTA. *Abdominal imaging* **2010**, *35*, 471-480.
47. Brambs, H.J.; Claussen, C.D. Pancreatic and ampullary carcinoma. Ultrasound, computed tomography, magnetic resonance imaging and angiography. *Endoscopy* **1993**, *25*, 58-68.
48. Karlson, B.M.; Ekbom, A.; Lindgren, P.G.; Kallskog, V.; Rastad, J. Abdominal US for diagnosis of pancreatic tumor: prospective cohort analysis. *Radiology* **1999**, *213*, 107-111.
49. Maringhini, A.; Ciambra, M.; Raimondo, M.; Baccelliere, P.; Grasso, R.*, et al.* Clinical presentation and ultrasonography in the diagnosis of pancreatic cancer. *Pancreas* **1993**, *8*, 146-150.
50. Niederau, C.; Grendell, J.H. Diagnosis of pancreatic carcinoma. Imaging techniques and tumor markers. *Pancreas* **1992**, *7*, 66-86.
51. Wang, W.; Shpaner, A.; Krishna, S.G.; Ross, W.A.; Bhutani, M.S.*, et al.* Use of EUS-FNA in diagnosing pancreatic neoplasm without a definitive mass on CT. *Gastrointest Endosc* **2013**, *78*, 73-80.
52. Chen, J.; Yang, R.; Lu, Y.; Xia, Y.; Zhou, H. Diagnostic accuracy of endoscopic ultrasound-guided fine-needle aspiration for solid pancreatic lesion: a systematic review. *Journal of cancer research and clinical oncology* **2012**, *138*, 1433-1441.
53. Puli, S.R.; Bechtold, M.L.; Buxbaum, J.L.; Eloubeidi, M.A. How good is endoscopic ultrasound-guided fine-needle aspiration in diagnosing the correct etiology for a solid pancreatic mass?: A meta-analysis and systematic review. *Pancreas* **2013**, *42*, 20-26.
54. Martin, R.C., 2nd; Scoggins, C.R.; Egnatashvili, V.; Staley, C.A.; McMasters, K.M.*, et al.* Arterial and venous resection for pancreatic adenocarcinoma: operative and long-term outcomes. *Archives of surgery (Chicago, Ill. : 1960)* **2009**, *144*, 154-159.
55. Konstantinidis, I.T.; Warshaw, A.L.; Allen, J.N.; Blaszkowsky, L.S.; Castillo, C.F.*, et al.* Pancreatic ductal adenocarcinoma: is there a survival difference for R1 resections versus locally advanced unresectable tumors? What is a "true" R0 resection? *Ann Surg* **2013**, *257*, 731-736.
56. Oettle, H.; Neuhaus, P.; Hochhaus, A.; Hartmann, J.T.; Gellert, K.*, et al.* Adjuvant chemotherapy with gemcitabine and long-term outcomes among patients with resected pancreatic cancer: the CONKO-001 randomized trial. *JAMA* **2013**, *310*, 1473-1481.
57. Neoptolemos, J.P.; Stocken, D.D.; Bassi, C.; Ghaneh, P.; Cunningham, D.*, et al.* Adjuvant chemotherapy with fluorouracil plus folinic acid vs gemcitabine following pancreatic cancer resection: a randomized controlled trial. *JAMA* **2010**, *304*, 1073-1081.
58. Regine, W.F.; Winter, K.A.; Abrams, R.; Safran, H.; Hoffman, J.P.*, et al.* Fluorouracil-based chemoradiation with either gemcitabine or fluorouracil chemotherapy after resection of pancreatic adenocarcinoma: 5-year analysis of the U.S. Intergroup/RTOG 9704 phase III trial. *Ann Surg Oncol* **2011**, *18*, 1319-1326.
59. Klinkenbijl, J.H.; Jeekel, J.; Sahmoud, T.; van Pel, R.; Couvreur, M.L.*, et al.* Adjuvant radiotherapy and 5-fluorouracil after curative resection of cancer of the pancreas and periampullary region: phase III trial of the EORTC gastrointestinal tract cancer cooperative group. *Ann Surg* **1999**, *230*, 776-782; discussion 782-774.
60. Further evidence of effective adjuvant combined radiation and chemotherapy following curative resection of pancreatic cancer. Gastrointestinal Tumor Study Group. *Cancer* **1987**, *59*, 2006-2010.
61. Neoptolemos, J.P.; Stocken, D.D.; Friess, H.; Bassi, C.; Dunn, J.A.*, et al.* A randomized trial of chemoradiotherapy and chemotherapy after resection of pancreatic cancer. *The New England journal of medicine* **2004**, *350*, 1200-1210.
62. Sultana, A.; Smith, C.T.; Cunningham, D.; Starling, N.; Neoptolemos, J.P.*, et al.* Meta-analyses of chemotherapy for locally advanced and metastatic pancreatic cancer. *J Clin Oncol* **2007**, *25*, 2607-2615.
63. Burris, H.A., 3rd; Moore, M.J.; Andersen, J.; Green, M.R.; Rothenberg, M.L.*, et al.* Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. *J Clin Oncol* **1997**, *15*, 2403-2413.
64. Conroy, T.; Desseigne, F.; Ychou, M.; Bouche, O.; Guimbaud, R.*, et al.* FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. *The New England journal of medicine* **2011**, *364*, 1817-1825.
65. Von Hoff, D.D.; Ervin, T.; Arena, F.P.; Chiorean, E.G.; Infante, J.*, et al.* Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. *The New England journal of medicine* **2013**, *369*, 1691-1703.

66. Chauffert, B.; Mornex, F.; Bonnetain, F.; Rougier, P.; Mariette, C.*, et al.* Phase III trial comparing intensive induction chemoradiotherapy (60 Gy, infusional 5-FU and intermittent cisplatin) followed by maintenance gemcitabine with gemcitabine alone for locally advanced unresectable pancreatic cancer. Definitive results of the 2000-01 FFCD/SFRO study. *Ann Oncol* **2008**, *19*, 1592-1599.
67. Loehrer, P.J., Sr.; Feng, Y.; Cardenes, H.; Wagner, L.; Brell, J.M.*, et al.* Gemcitabine alone versus gemcitabine plus radiotherapy in patients with locally advanced pancreatic cancer: an Eastern Cooperative Oncology Group trial. *J Clin Oncol* **2011**, *29*, 4105-4112.
68. Carpelan-Holmstrom, M.; Nordling, S.; Pukkala, E.; Sankila, R.; Luttges, J.*, et al.* Does anyone survive pancreatic ductal adenocarcinoma? A nationwide study re-evaluating the data of the Finnish Cancer Registry. *Gut* **2005**, *54*, 385-387.
69. Russell, S.J.; Peng, K.W. Viruses as anticancer drugs. *Trends in pharmacological sciences* **2007**, *28*, 326-333.
70. Hanahan, D.; Weinberg, R.A. Hallmarks of cancer: the next generation. *Cell* **2011**, *144*, 646-674.
71. O'Neill, L.A.; Bowie, A.G. Sensing and signaling in antiviral innate immunity. *Curr Biol* **2010**, *20*, R328-333.
72. Takeuchi, O.; Akira, S. Innate immunity to virus infection. *Immunol Rev* **2009**, *227*, 75-86.
73. Randall, R.E.; Goodbourn, S. Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. *J Gen Virol* **2008**, *89*, 1-47.
74. Cattaneo, R.; Miest, T.; Shashkova, E.V.; Barry, M.A. Reprogrammed viruses as cancer therapeutics: targeted, armed and shielded. *Nature reviews. Microbiology* **2008**, *6*, 529-540.
75. Dorer, D.E.; Nettelbeck, D.M. Targeting cancer by transcriptional control in cancer gene therapy and viral oncolysis. *Adv Drug Deliv Rev* **2009**, *61*, 554-571.
76. Naik, S.; Russell, S.J. Engineering oncolytic viruses to exploit tumor specific defects in innate immune signaling pathways. *Expert Opin Biol Ther* **2009**, *9*, 1163-1176.
77. Kelly, E.J.; Russell, S.J. MicroRNAs and the regulation of vector tropism. *Mol Ther* **2009**, *17*, 409-416.
78. Kelly, E.; Russell, S.J. History of oncolytic viruses: genesis to genetic engineering. *Mol Ther* **2007**, *15*, 651-659.
79. Southam, C.M. Present status of oncolytic virus studies. *Transactions of the New York Academy of Sciences* **1960**, *22*, 657-673.
80. Asada, T. Treatment of human cancer with mumps virus. *Cancer* **1974**, *34*, 1907-1928.
81. Russell, S.J.; Peng, K.W.; Bell, J.C. Oncolytic virotherapy. *Nat Biotechnol* **2012**, *30*, 658-670.
82. Liu, T.C.; Galanis, E.; Kirn, D. Clinical trial results with oncolytic virotherapy: a century of promise, a decade of progress. *Nat Clin Pract Oncol* **2007**, *4*, 101-117.
83. Bell, J. Oncolytic viruses: an approved product on the horizon? *Mol Ther* **2010**, *18*, 233-234.
84. Rowan, K. Oncolytic viruses move forward in clinical trials. *J Natl Cancer Inst* **2010**, *102*, 590-595.
85. Eager, R.M.; Nemunaitis, J. Clinical development directions in oncolytic viral therapy. *Cancer Gene Ther* **2011**, *18*, 305-317.
86. Amgen. Amgen Submits Marketing Authorization Application For Talimogene Laherparepvec To The European Medicines Agency. http://www.amgen.com/media/media_pr_detail.jsp?releaseID=1962767 (23-05-2015)
87. Russell, S.J. Replicating vectors for cancer therapy: a question of strategy. *Semin Cancer Biol* **1994**, *5*, 437-443.
88. Moore, A.E.; Diamond, L.C.; Mackay, H.H.; Sabachewsky, L. Influence of hemagglutinating viruses on tumor cell suspensions. II. Newcastle disease virus and Ehrlich carcinoma. *Proc Soc Exp Biol Med* **1952**, *81*, 498-501.
89. Prince, A.M.; Ginsberg, H.S. Studies on the cytotoxic effect of Newcastle disease virus (NDV) on Ehrlich ascites tumor cells. II. The mechanism and significance of in vitro recovery from the effect of NDV. *J Immunol* **1957**, *79*, 107-112.
90. Prince, A.M.; Ginsberg, H.S. Studies on the cytotoxic effect of Newcastle disease virus (NDV) on Ehrlich ascites tumor cells. I. Characteristics of the virus-cell interaction. *J Immunol* **1957**, *79*, 94-106.
91. Lorence, R.M.; Roberts, M.S.; O'Neil, J.D.; Groene, W.S.; Miller, J.A.*, et al.* Phase 1 clinical experience using intravenous administration of PV701, an oncolytic Newcastle disease virus. *Curr Cancer Drug Targets* **2007**, *7*, 157-167.
92. Freeman, A.I.; Zakay-Rones, Z.; Gomori, J.M.; Linetsky, E.; Rasooly, L.*, et al.* Phase I/II trial of intravenous NDV-HUJ oncolytic virus in recurrent glioblastoma multiforme. *Mol Ther* **2006**, *13*, 221-228.
93. Pecora, A.L.; Rizvi, N.; Cohen, G.I.; Meropol, N.J.; Sterman, D.*, et al.* Phase I trial of intravenous administration of PV701, an oncolytic virus, in patients with advanced solid cancers. *J Clin Oncol* **2002**, *20*, 2251-2266.
94. Schirrmacher, V. Clinical trials of antitumor vaccination with an autologous tumor cell vaccine modified by virus infection: improvement of patient survival based on improved antitumor immune memory. *Cancer*

Immunol Immunother **2005**, *54*, 587-598.
95. Cassel, W.A.; Murray, D.R. A ten-year follow-up on stage II malignant melanoma patients treated postsurgically with Newcastle disease virus oncolysate. *Med Oncol Tumor Pharmacother* **1992**, *9*, 169-171.
96. Sinkovics, J.G.; Horvath, J.C. Newcastle disease virus (NDV): brief history of its oncolytic strains. *J Clin Virol* **2000**, *16*, 1-15.
97. Csatary, L.K.; Bakacs, T. Use of Newcastle disease virus vaccine (MTH-68/H) in a patient with high-grade glioblastoma. *JAMA* **1999**, *281*, 1588-1589.
98. Csatary, L.K.; Gosztonyi, G.; Szeberenyi, J.; Fabian, Z.; Liszka, V.*, et al.* MTH-68/H oncolytic viral treatment in human high-grade gliomas. *J Neurooncol* **2004**, *67*, 83-93.
99. Laurie, S.A.; Bell, J.C.; Atkins, H.L.; Roach, J.; Bamat, M.K.*, et al.* A phase 1 clinical study of intravenous administration of PV701, an oncolytic virus, using two-step desensitization. *Clin Cancer Res* **2006**, *12*, 2555-2562.
100. Wagner, S.; Csatary, C.M.; Gosztonyi, G.; Koch, H.C.; Hartmann, C.*, et al.* Combined treatment of pediatric high-grade glioma with the oncolytic viral strain MTH-68/H and oral valproic acid. *APMIS : acta pathologica, microbiologica, et immunologica Scandinavica* **2006**, *114*, 731-743.
101. Hotte, S.J.; Lorence, R.M.; Hirte, H.W.; Polawski, S.R.; Bamat, M.K.*, et al.* An optimized clinical regimen for the oncolytic virus PV701. *Clin Cancer Res* **2007**, *13*, 977-985.
102. Reichard, K.W.; Lorence, R.M.; Cascino, C.J.; Peeples, M.E.; Walter, R.J.*, et al.* Newcastle disease virus selectively kills human tumor cells. *J Surg Res* **1992**, *52*, 448-453.
103. Fiola, C.; Peeters, B.; Fournier, P.; Arnold, A.; Bucur, M.*, et al.* Tumor selective replication of Newcastle disease virus: association with defects of tumor cells in antiviral defence. *Int J Cancer* **2006**, *119*, 328-338.
104. Krishnamurthy, S.; Takimoto, T.; Scroggs, R.A.; Portner, A. Differentially regulated interferon response determines the outcome of Newcastle disease virus infection in normal and tumor cell lines. *J Virol* **2006**, *80*, 5145-5155.
105. Wilden, H.; Fournier, P.; Zawatzky, R.; Schirrmacher, V. Expression of RIG-I, IRF3, IFN-beta and IRF7 determines resistance or susceptibility of cells to infection by Newcastle Disease Virus. *Int J Oncol* **2009**, *34*, 971-982.
106. Biswas, M.; Kumar, S.R.; Allen, A.; Yong, W.; Nimmanapalli, R.*, et al.* Cell-type-specific innate immune response to oncolytic Newcastle disease virus. *Viral immunology* **2012**, *25*, 268-276.
107. Mansour, M.; Palese, P.; Zamarin, D. Oncolytic specificity of Newcastle disease virus is mediated by selectivity for apoptosis-resistant cells. *J Virol* **2011**, *85*, 6015-6023.
108. Puhlmann, J.; Puehler, F.; Mumberg, D.; Boukamp, P.; Beier, R. Rac1 is required for oncolytic NDV replication in human cancer cells and establishes a link between tumorigenesis and sensitivity to oncolytic virus. *Oncogene* **2010**, *29*, 2205-2216.
109. Lazar, I.; Yaacov, B.; Shiloach, T.; Eliahoo, E.; Kadouri, L.*, et al.* The oncolytic activity of Newcastle disease virus NDV-HUJ on chemoresistant primary melanoma cells is dependent on the proapoptotic activity of the inhibitor of apoptosis protein Livin. *J Virol* **2010**, *84*, 639-646.
110. Vitale, G.; van Eijck, C.H.; van Koetsveld Ing, P.M.; Erdmann, J.I.; Speel, E.J.*, et al.* Type I interferons in the treatment of pancreatic cancer: mechanisms of action and role of related receptors. *Ann Surg* **2007**, *246*, 259-268.
111. Booy, S.; van Eijck, C.H.; Dogan, F.; van Koetsveld, P.M.; Hofland, L.J. Influence of type-I Interferon receptor expression level on the response to type-I Interferons in human pancreatic cancer cells. *J Cell Mol Med* **2014**, *18*, 492-502.
112. Peeters, B.P.; de Leeuw, O.S.; Koch, G.; Gielkens, A.L. Rescue of Newcastle disease virus from cloned cDNA: evidence that cleavability of the fusion protein is a major determinant for virulence. *J Virol* **1999**, *73*, 5001-5009.
113. Zamarin, D.; Palese, P. Oncolytic Newcastle disease virus for cancer therapy: old challenges and new directions. *Future microbiology* **2012**, *7*, 347-367.
114. Vigil, A.; Park, M.S.; Martinez, O.; Chua, M.A.; Xiao, S.*, et al.* Use of reverse genetics to enhance the oncolytic properties of Newcastle disease virus. *Cancer Res* **2007**, *67*, 8285-8292.
115. Zamarin, D.; Vigil, A.; Kelly, K.; Garcia-Sastre, A.; Fong, Y. Genetically engineered Newcastle disease virus for malignant melanoma therapy. *Gene Ther* **2009**, *16*, 796-804.
116. Altomonte, J.; Marozin, S.; Schmid, R.M.; Ebert, O. Engineered newcastle disease virus as an improved oncolytic agent against hepatocellular carcinoma. *Mol Ther* **2010**, *18*, 275-284.
117. Silberhumer, G.R.; Brader, P.; Wong, J.; Serganova, I.S.; Gonen, M.*, et al.* Genetically engineered oncolytic

Newcastle disease virus effectively induces sustained remission of malignant pleural mesothelioma. ***Mol Cancer Ther*** **2010**, *9*, 2761-2769.
118. Song, K.Y.; Wong, J.; Gonzalez, L.; Sheng, G.; Zamarin, D.***, et al.*** Antitumor efficacy of viral therapy using genetically engineered Newcastle disease virus [NDV(F3aa)-GFP] for peritoneally disseminated gastric cancer. ***Journal of molecular medicine (Berlin, Germany)*** **2010**, *88*, 589-596.
119. Li, P.; Chen, C.H.; Li, S.; Givi, B.; Yu, Z.***, et al.*** Therapeutic effects of a fusogenic newcastle disease virus in treating head and neck cancer. ***Head Neck*** **2011**, *33*, 1394-1399.
120. Zamarin, D.; Martinez-Sobrido, L.; Kelly, K.; Mansour, M.; Sheng, G.***, et al.*** Enhancement of oncolytic properties of recombinant newcastle disease virus through antagonism of cellular innate immune responses. ***Mol Ther*** **2009**, *17*, 697-706.
121. Zhao, H.; Janke, M.; Fournier, P.; Schirrmacher, V. Recombinant Newcastle disease virus expressing human interleukin-2 serves as a potential candidate for tumor therapy. ***Virus Res*** **2008**, *136*, 75-80.
122. Bai, F.; Niu, Z.; Tian, H.; Li, S.; Lv, Z.***, et al.*** Genetically engineered Newcastle disease virus expressing interleukin 2 is a potential drug candidate for cancer immunotherapy. ***Immunology letters*** **2014**, *159*, 36-46.
123. Bian, H.; Fournier, P.; Moormann, R.; Peeters, B.; Schirrmacher, V. Selective gene transfer in vitro to tumor cells via recombinant Newcastle disease virus. ***Cancer Gene Ther*** **2005**, *12*, 295-303.
124. Shobana, R.; Samal, S.K.; Elankumaran, S. Prostate-specific antigen-retargeted recombinant newcastle disease virus for prostate cancer virotherapy. ***J Virol*** **2013**, *87*, 3792-3800.
125. Zamarin, D.; Holmgaard, R.B.; Subudhi, S.K.; Park, J.S.; Mansour, M.***, et al.*** Localized oncolytic virotherapy overcomes systemic tumor resistance to immune checkpoint blockade immunotherapy. ***Science translational medicine*** **2014**, *6*, 226ra232.
126. Wei, D.; Sun, N.; Nan, G.; Wang, Y.; Liu, H.Q.***, et al.*** Construction of recombinant Newcastle disease virus Italien strain for oncolytic virotherapy of tumors. ***Hum Gene Ther*** **2012**, *23*, 700-710.
127. Bukreyev, A.; Huang, Z.; Yang, L.; Elankumaran, S.; St Claire, M.***, et al.*** Recombinant newcastle disease virus expressing a foreign viral antigen is attenuated and highly immunogenic in primates. ***J Virol*** **2005**, *79*, 13275-13284.
128. DiNapoli, J.M.; Kotelkin, A.; Yang, L.; Elankumaran, S.; Murphy, B.R.***, et al.*** Newcastle disease virus, a host range-restricted virus, as a vaccine vector for intranasal immunization against emerging pathogens. ***Proc Natl Acad Sci U S A*** **2007**, *104*, 9788-9793.
129. DiNapoli, J.M.; Yang, L.; Suguitan, A., Jr.; Elankumaran, S.; Dorward, D.W.***, et al.*** Immunization of primates with a Newcastle disease virus-vectored vaccine via the respiratory tract induces a high titer of serum neutralizing antibodies against highly pathogenic avian influenza virus. ***J Virol*** **2007**, *81*, 11560-11568.
130. DiNapoli, J.M.; Ward, J.M.; Cheng, L.; Yang, L.; Elankumaran, S.***, et al.*** Delivery to the lower respiratory tract is required for effective immunization with Newcastle disease virus-vectored vaccines intended for humans. ***Vaccine*** **2009**, *27*, 1530-1539.
131. DiNapoli, J.M.; Yang, L.; Samal, S.K.; Murphy, B.R.; Collins, P.L.***, et al.*** Respiratory tract immunization of non-human primates with a Newcastle disease virus-vectored vaccine candidate against Ebola virus elicits a neutralizing antibody response. ***Vaccine*** **2010**, *29*, 17-25.
132. DiNapoli, J.M.; Nayak, B.; Yang, L.; Finneyfrock, B.W.; Cook, A.***, et al.*** Newcastle disease virus-vectored vaccines expressing the hemagglutinin or neuraminidase protein of H5N1 highly pathogenic avian influenza virus protect against virus challenge in monkeys. ***J Virol*** **2010**, *84*, 1489-1503.
133. Wu, Y.; Zhang, X.; Wang, X.; Wang, L.; Hu, S.***, et al.*** Apoptin enhances the oncolytic properties of Newcastle disease virus. ***Intervirology*** **2012**, *55*, 276-286.
134. Mayo, M.A. A summary of taxonomic changes recently approved by ICTV. ***Arch Virol*** **2002**, *147*, 1655-1663.
135. Kraneveld, F.C. Over een in Ned.-Indië heerschende ziekte onder het pluimvee. ***Nederlands-Indische bladen voor Diergeneeskunde*** **1926**, *38*, 448-450.
136. Doyle, T.M. A hitherto unrecorded disease of fowls due to a filter-passing virus. ***J Comp Pathol*** **1927**, *40*, 144-169.
137. OIE: World Organisation for Animal Health. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.03.14_NEWCASTLE_DIS.pdf, 2012.
138. Council of the European Communities. Council Directive 92/66/EEC of 14 July 1992 introducing Community measures for the control of Newcastle disease.
139. OIE: World Organisation for Animal Health. WAHID 2014: http://www.oie.int/wahis_2/public/wahid.php/Wahidhome/Home. (October 5)

140. Shimkin, N.I. Conjunctival haemorrhage due to an infection of Newcastle virus of fowls in man; laboratory and contact infection. ***The British journal of ophthalmology*** **1946**, *30*, 260-264.
141. Keeney, A.H.; Hunter, M.C. Human infection with the Newcastle virus of fowls. ***A.M.A. archives of ophthalmology*** **1950**, *44*, 573-580.
142. Nelson, C.B.; Pomeroy, B.S.; Schrall, K.; Park, W.E.; Lindeman, R.J. An outbreak of conjunctivitis due to Newcastle disease virus (NDV) occurring in poultry workers. ***Am J Public Health Nations Health*** **1952**, *42*, 672-678.
143. Quinn, R.W.; Hanson, R.P.; Brown, J.W.; Brandly, C.A. Newcastle disease virus in man; results of studies in five cases. ***The Journal of laboratory and clinical medicine*** **1952**, *40*, 736-743.
144. Evans, A.S. Pathogenicity and immunology of Newcastle disease virus (NVD) in man. ***Am J Public Health Nations Health*** **1955**, *45*, 742-745.
145. Trott, D.G.; Pilsworth, R. Outbreaks of conjunctivitis due to the Newcastle disease virus among workers in chicken-broiler factories. ***British medical journal*** **1965**, *2*, 1514-1517.
146. Hales, R.H.; Ostler, H.B. Newcastle disease conjunctivitis with subepithelial infiltrates. ***The British journal of ophthalmology*** **1973**, *57*, 694-697.
147. Alexander, D.J.; Russell, P.H.; Collins, M.S. Paramyxovirus type 1 infections of racing pigeons: 1 characterisation of isolated viruses. ***The Veterinary record*** **1984**, *114*, 444-446.
148. Meulemans, G.; van den Berg, T.P.; Decaesstecker, M.; Boschmans, M. Evolution of pigeon Newcastle disease virus strains. ***Avian pathology : journal of the W.V.P.A*** **2002**, *31*, 515-519.
149. Alexander, D.J. Newcastle disease in the European Union 2000 to 2009. ***Avian pathology : journal of the W.V.P.A*** **2011**, *40*, 547-558.
150. Ballagi-Pordany, A.; Wehmann, E.; Herczeg, J.; Belak, S.; Lomniczi, B. Identification and grouping of Newcastle disease virus strains by restriction site analysis of a region from the F gene. ***Arch Virol*** **1996**, *141*, 243-261.
151. Czegledi, A.; Herczeg, J.; Hadjiev, G.; Doumanova, L.; Wehmann, E.***, et al.*** The occurrence of five major Newcastle disease virus genotypes (II, IV, V, VI and VIIb) in Bulgaria between 1959 and 1996. ***Epidemiol Infect*** **2002**, *129*, 679-688.
152. Lomniczi, B.; Wehmann, E.; Herczeg, J.; Ballagi-Pordany, A.; Kaleta, E.F.***, et al.*** Newcastle disease outbreaks in recent years in western Europe were caused by an old (VI) and a novel genotype (VII). ***Arch Virol*** **1998**, *143*, 49-64.
153. Chong, Y.L.; Lam, T.T.; Kim, O.; Lu, H.; Dunn, P.***, et al.*** Successful establishment and global dispersal of genotype VI avian paramyxovirus serotype 1 after cross species transmission. ***Infect Genet Evol*** **2013**, *17*, 260-268.
154. Abolnik, C.; Gerdes, G.H.; Kitching, J.; Swanepoel, S.; Romito, M.***, et al.*** Characterization of pigeon paramyxoviruses (Newcastle disease virus) isolated in South Africa from 2001 to 2006. ***The Onderstepoort journal of veterinary research*** **2008**, *75*, 147-152.
155. Alexander, D.J.; Manvell, R.J.; Frost, K.M.; Pollitt, W.J.; Welchman, D.***, et al.*** Newcastle disease outbreak in pheasants in Great Britain in May 1996. ***The Veterinary record*** **1997**, *140*, 20-22.
156. Alexander, D.J.; Morris, H.T.; Pollitt, W.J.; Sharpe, C.E.; Eckford, R.L.***, et al.*** Newcastle disease outbreaks in domestic fowl and turkeys in Great Britain during 1997. ***The Veterinary record*** **1998**, *143*, 209-212.
157. Alexander, D.J.; Wilson, G.W.; Russell, P.H.; Lister, S.A.; Parsons, G. Newcastle disease outbreaks in fowl in Great Britain during 1984. ***The Veterinary record*** **1985**, *117*, 429-434.
158. Irvine, R.M.; Aldous, E.W.; Manvell, R.J.; Cox, W.J.; Ceeraz, V.***, et al.*** Outbreak of Newcastle disease due to pigeon paramyxovirus type 1 in grey partridges (Perdix perdix) in Scotland in October 2006. ***The Veterinary record*** **2009**, *165*, 531-535.
159. Liu, H.; Wang, Z.; Son, C.; Wang, Y.; Yu, B.***, et al.*** Characterization of pigeon-origin Newcastle disease virus isolated in China. ***Avian Dis*** **2006**, *50*, 636-640.
160. Werner, O.; Romer-Oberdorfer, A.; Kollner, B.; Manvell, R.J.; Alexander, D.J. Characterization of avian paramyxovirus type 1 strains isolated in Germany during 1992 to 1996. ***Avian pathology : journal of the W.V.P.A*** **1999**, *28*, 79-88.
161. Alexander, D.J.; Parsons, G. Avian paramyxovirus type 1 infections of racing pigeons: 2 pathogenicity experiments in pigeons and chickens. ***The Veterinary record*** **1984**, *114*, 466-469.
162. Collins, M.S.; Strong, I.; Alexander, D.J. Evaluation of the molecular basis of pathogenicity of the variant Newcastle disease viruses termed "pigeon PMV-1 viruses". ***Arch Virol*** **1994**, *134*, 403-411.
163. Collins, M.S.; Strong, I.; Alexander, D.J. Pathogenicity and phylogenetic evaluation of the variant Newcastle disease viruses termed "pigeon PMV-1 viruses" based on the nucleotide sequence of the fusion protein gene. *Arch Virol* **1996**, *141*, 635-647.

164. Dortmans, J.C.; Fuller, C.M.; Aldous, E.W.; Rottier, P.J.; Peeters, B.P. Two genetically closely related pigeon paramyxovirus type 1 (PPMV-1) variants with identical velogenic fusion protein cleavage sites but with strongly contrasting virulence. ***Vet Microbiol*** **2010**, *143*, 139-144.
165. Dortmans, J.C.; Rottier, P.J.; Koch, G.; Peeters, B.P. Passaging of a Newcastle disease virus pigeon variant in chickens results in selection of viruses with mutations in the polymerase complex enhancing virus replication and virulence. ***J Gen Virol*** **2011**, *92*, 336-345.
166. Fuller, C.M.; Collins, M.S.; Easton, A.J.; Alexander, D.J. Partial characterisation of five cloned viruses differing in pathogenicity, obtained from a single isolate of pigeon paramyxovirus type 1 (PPMV-1) following passage in fowls' eggs. ***Arch Virol*** **2007**, *152*, 1575-1582.
167. Kaleta, E.F.; Alexander, D.J.; Russell, P.H. The first isolation of the avian PMV-1 virus responsible for the current panzootic in pigeons ? ***Avian pathology : journal of the W.V.P.A*** **1985**, *14*, 553-557.
168. Goebel, S.J.; Taylor, J.; Barr, B.C.; Kiehn, T.E.; Castro-Malaspina, H.R.***, et al.*** Isolation of avian paramyxovirus 1 from a patient with a lethal case of pneumonia. ***J Virol*** **2007**, *81*, 12709-12714.
169. Czegledi, A.; Ujvari, D.; Somogyi, E.; Wehmann, E.; Werner, O.***, et al.*** Third genome size category of avian paramyxovirus serotype 1 (Newcastle disease virus) and evolutionary implications. ***Virus Res*** **2006**, *120*, 36-48.
170. de Leeuw, O.; Peeters, B. Complete nucleotide sequence of Newcastle disease virus: evidence for the existence of a new genus within the subfamily Paramyxovirinae. ***J Gen Virol*** **1999**, *80 (Pt 1)*, 131-136.
171. Huang, Y.; Wan, H.Q.; Liu, H.Q.; Wu, Y.T.; Liu, X.F. Genomic sequence of an isolate of Newcastle disease virus isolated from an outbreak in geese: a novel six nucleotide insertion in the non-coding region of the nucleoprotein gene. Brief Report. ***Arch Virol*** **2004**, *149*, 1445-1457.
172. Krishnamurthy, S.; Samal, S.K. Nucleotide sequences of the trailer, nucleocapsid protein gene and intergenic regions of Newcastle disease virus strain Beaudette C and completion of the entire genome sequence. ***J Gen Virol*** **1998**, *79 (Pt 10)*, 2419-2424.
173. Steward, M.; Vipond, I.B.; Millar, N.S.; Emmerson, P.T. RNA editing in Newcastle disease virus. ***J Gen Virol*** **1993**, *74 (Pt 12)*, 2539-2547.
174. Collins, M.S.; Bashiruddin, J.B.; Alexander, D.J. Deduced amino acid sequences at the fusion protein cleavage site of Newcastle disease viruses showing variation in antigenicity and pathogenicity. ***Arch Virol*** **1993**, *128*, 363-370.
175. Dortmans, J.C.; Koch, G.; Rottier, P.J.; Peeters, B.P. Virulence of Newcastle disease virus: what is known so far? ***Veterinary research*** **2011**, *42*, 122.
176. Garten, W.; Berk, W.; Nagai, Y.; Rott, R.; Klenk, H.D. Mutational changes of the protease susceptibility of glycoprotein F of Newcastle disease virus: effects on pathogenicity. ***J Gen Virol*** **1980**, *50*, 135-147.
177. Huang, Z.; Krishnamurthy, S.; Panda, A.; Samal, S.K. Newcastle disease virus V protein is associated with viral pathogenesis and functions as an alpha interferon antagonist. ***J Virol*** **2003**, *77*, 8676-8685.
178. Park, M.S.; Garcia-Sastre, A.; Cros, J.F.; Basler, C.F.; Palese, P. Newcastle disease virus V protein is a determinant of host range restriction. ***J Virol*** **2003**, *77*, 9522-9532.
179. Park, M.S.; Shaw, M.L.; Munoz-Jordan, J.; Cros, J.F.; Nakaya, T.***, et al.*** Newcastle disease virus (NDV)-based assay demonstrates interferon-antagonist activity for the NDV V protein and the Nipah virus V, W, and C proteins. ***J Virol*** **2003**, *77*, 1501-1511.
180. Mebatsion, T.; Verstegen, S.; De Vaan, L.T.; Romer-Oberdorfer, A.; Schrier, C.C. A recombinant newcastle disease virus with low-level V protein expression is immunogenic and lacks pathogenicity for chicken embryos. ***J Virol*** **2001**, *75*, 420-428.
181. Morak, M.J.; van Koetsveld, P.M.; Kanaar, R.; Hofland, L.J.; van Eijck, C.H. Type I interferons as radiosensitisers for pancreatic cancer. ***Eur J Cancer*** **2011**, *47*, 1938-1945.
182. Levy, D.E.; Garcia-Sastre, A. The virus battles: IFN induction of the antiviral state and mechanisms of viral evasion. ***Cytokine Growth Factor Rev*** **2001**, *12*, 143-156.
183. van de Sandt, C.E.; Kreijtz, J.H.; Rimmelzwaan, G.F. Evasion of influenza A viruses from innate and adaptive immune responses. ***Viruses*** **2012**, *4*, 1438-1476.
184. Kochs, G.; Garcia-Sastre, A.; Martinez-Sobrido, L. Multiple anti-interferon actions of the influenza A virus NS1 protein. ***J Virol*** **2007**, *81*, 7011-7021.
185. Ferlay, J.; Shin, H.R.; Bray, F.; Forman, D.; Mathers, C.***, et al.*** Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. ***Int J Cancer*** **2010**, *127*, 2893-2917.
186. Chiocca, E.A. Oncolytic viruses. ***Nat Rev Cancer*** **2002**, *2*, 938-950.

187. Elankumaran, S.; Chavan, V.; Qiao, D.; Shobana, R.; Moorkanat, G.*, et al.* Type I interferon-sensitive recombinant newcastle disease virus for oncolytic virotherapy. *J Virol* **2010**, *84*, 3835-3844.
188. Janke, M.; Peeters, B.; de Leeuw, O.; Moorman, R.; Arnold, A.*, et al.* Recombinant Newcastle disease virus (NDV) with inserted gene coding for GM-CSF as a new vector for cancer immunogene therapy. *Gene Ther* **2007**, *14*, 1639-1649.
189. Janke, M.; Peeters, B.; Zhao, H.; de Leeuw, O.; Moorman, R.*, et al.* Activation of human T cells by a tumor vaccine infected with recombinant Newcastle disease virus producing IL-2. *Int J Oncol* **2008**, *33*, 823-832.
190. Masters, J.R.; Thomson, J.A.; Daly-Burns, B.; Reid, Y.A.; Dirks, W.G.*, et al.* Short tandem repeat profiling provides an international reference standard for human cell lines. *Proc Natl Acad Sci U S A* **2001**, *98*, 8012-8017.
191. Kuiken, T.; van den Hoogen, B.G.; van Riel, D.A.; Laman, J.D.; van Amerongen, G.*, et al.* Experimental human metapneumovirus infection of cynomolgus macaques (Macaca fascicularis) results in virus replication in ciliated epithelial cells and pneumocytes with associated lesions throughout the respiratory tract. *Am J Pathol* **2004**, *164*, 1893-1900.
192. Reed, L.J.; Muench, H. A simple method of estimating fifty per cent endpoints. *Am J Hyg* **1938**, *27*, 493-497.
193. Jordan, M.; Schallhorn, A.; Wurm, F.M. Transfecting mammalian cells: optimization of critical parameters affecting calcium-phosphate precipitate formation. *Nucleic Acids Res* **1996**, *24*, 596-601.
194. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **2001**, *25*, 402-408.
195. Kumar, R.; Tiwari, A.K.; Chaturvedi, U.; Kumar, G.R.; Sahoo, A.P.*, et al.* Velogenic newcastle disease virus as an oncolytic virotherapeutics: in vitro characterization. *Applied biochemistry and biotechnology* **2012**, *167*, 2005-2022.
196. Cassel, W.A.; Garrett, R.E. Newcastle Disease Virus as an Antineoplastic Agent. *Cancer* **1965**, *18*, 863-868.
197. Walter, R.J.; Attar, B.M.; Rafiq, A.; Tejaswi, S.; Delimata, M. Newcastle disease virus LaSota strain kills human pancreatic cancer cells in vitro with high selectivity. *JOP* **2012**, *13*, 45-53.
198. Walter, R.J.; Attar, B.M.; Rafiq, A.; Delimata, M.; Tejaswi, S. Two avirulent, lentogenic strains of Newcastle disease virus are cytotoxic for some human pancreatic tumor lines in vitro. *JOP* **2012**, *13*, 502-513.
199. Miszczuk-Jamska, B.; Merten, M.; Guy-Crotte, O.; Amouric, M.; Clemente, F.*, et al.* Characterization of trypsinogens 1 and 2 in two human pancreatic adenocarcinoma cell lines; CFPAC-1 and CAPAN-1. *FEBS Lett* **1991**, *294*, 175-178.
200. Ohta, T.; Terada, T.; Nagakawa, T.; Tajima, H.; Itoh, H.*, et al.* Pancreatic trypsinogen and cathepsin B in human pancreatic carcinomas and associated metastatic lesions. *Br J Cancer* **1994**, *69*, 152-156.
201. Murphy, A.M.; Besmer, D.M.; Moerdyk-Schauwecker, M.; Moestl, N.; Ornelles, D.A.*, et al.* Vesicular stomatitis virus as an oncolytic agent against pancreatic ductal adenocarcinoma. *J Virol* **2012**, *86*, 3073-3087.
202. Jost, E.; Roos, W.P.; Kaina, B.; Schmidberger, H. Response of pancreatic cancer cells treated with interferon-alpha or beta and co-exposed to ionising radiation. *International journal of radiation biology* **2010**, *86*, 732-741.
203. Tomimaru, Y.; Eguchi, H.; Wada, H.; Tomokuni, A.; Kobayashi, S.*, et al.* Synergistic antitumor effect of interferon-ss with gemcitabine in interferon-alpha-non-responsive pancreatic cancer cells. *Int J Oncol* **2011**, *38*, 1237-1243.
204. Buijs, P.R.; van Eijck, C.H.; Hofland, L.J.; Fouchier, R.A.; van den Hoogen, B.G. Different responses of human pancreatic adenocarcinoma cell lines to oncolytic Newcastle disease virus infection. *Cancer Gene Ther* **2014**, *21*, 24-30.
205. Kortekaas, J.; Dekker, A.; de Boer, S.M.; Weerdmeester, K.; Vloet, R.P.*, et al.* Intramuscular inoculation of calves with an experimental Newcastle disease virus-based vector vaccine elicits neutralizing antibodies against Rift Valley fever virus. *Vaccine* **2010**, *28*, 2271-2276.
206. Peeters, B.P.; Gruijthuijsen, Y.K.; de Leeuw, O.S.; Gielkens, A.L. Genome replication of Newcastle disease virus: involvement of the rule-of-six. *Arch Virol* **2000**, *145*, 1829-1845.
207. Hirst, G.K. The Quantitative Determination of Influenza Virus and Antibodies by Means of Red Cell Agglutination. *J Exp Med* **1942**, *75*, 49-64.
208. de Wit, E.; Spronken, M.I.; Bestebroer, T.M.; Rimmelzwaan, G.F.; Osterhaus, A.D.*, et al.* Efficient generation and growth of influenza virus A/PR/8/34 from eight cDNA fragments. *Virus Res* **2004**, *103*, 155-161.
209. Herfst, S.; de Graaf, M.; Schickli, J.H.; Tang, R.S.; Kaur, J.*, et al.* Recovery of human metapneumovirus genetic lineages a and B from cloned cDNA. *J Virol* **2004**, *78*, 8264-8270.
210. Spann, K.M.; Tran, K.C.; Chi, B.; Rabin, R.L.; Collins, P.L. Suppression of the induction of alpha, beta, and lambda

interferons by the NS1 and NS2 proteins of human respiratory syncytial virus in human epithelial cells and macrophages [corrected]. ***J Virol*** **2004**, *78*, 4363-4369.
211. Euhus, D.M.; Hudd, C.; LaRegina, M.C.; Johnson, F.E. Tumor measurement in the nude mouse. ***J Surg Oncol*** **1986**, *31*, 229-234.
212. Tomayko, M.M.; Reynolds, C.P. Determination of subcutaneous tumor size in athymic (nude) mice. ***Cancer Chemother Pharmacol*** **1989**, *24*, 148-154.
213. Obuchi, M.; Fernandez, M.; Barber, G.N. Development of recombinant vesicular stomatitis viruses that exploit defects in host defense to augment specific oncolytic activity. ***J Virol*** **2003**, *77*, 8843-8856.
214. Saloura, V.; Wang, L.C.; Fridlender, Z.G.; Sun, J.; Cheng, G.***, et al.*** Evaluation of an attenuated vesicular stomatitis virus vector expressing interferon-beta for use in malignant pleural mesothelioma: heterogeneity in interferon responsiveness defines potential efficacy. ***Hum Gene Ther*** **2010**, *21*, 51-64.
215. Naik, S.; Nace, R.; Barber, G.N.; Russell, S.J. Potent systemic therapy of multiple myeloma utilizing oncolytic vesicular stomatitis virus coding for interferon-beta. ***Cancer Gene Ther*** **2012**, *19*, 443-450.
216. Kirn, D.H.; Wang, Y.; Le Boeuf, F.; Bell, J.; Thorne, S.H. Targeting of interferon-beta to produce a specific, multi-mechanistic oncolytic vaccinia virus. ***PLoS medicine*** **2007**, *4*, e353.
217. Moerdyk-Schauwecker, M.; Shah, N.R.; Murphy, A.M.; Hastie, E.; Mukherjee, P.***, et al.*** Resistance of pancreatic cancer cells to oncolytic vesicular stomatitis virus: role of type I interferon signaling. ***Virology*** **2013**, *436*, 221-234.
218. Biswas, M.; Johnson, J.B.; Kumar, S.R.; Parks, G.D.; Elankumarana, S. Incorporation of host complement regulatory proteins into Newcastle disease virus enhances complement evasion. ***J Virol*** **2012**, *86*, 12708-12716.
219. Munster, V.J.; Baas, C.; Lexmond, P.; Bestebroer, T.M.; Guldemeester, J.***, et al.*** Practical considerations for high-throughput influenza A virus surveillance studies of wild birds by use of molecular diagnostic tests. ***J Clin Microbiol*** **2009**, *47*, 666-673.
220. Xie, L.; Xu, F.; Liu, S.; Ji, Y.; Zhou, Q.***, et al.*** Age- and sex-based hematological and biochemical parameters for Macaca fascicularis. ***PLoS One*** **2013**, *8*, e64892.
221. Wise, M.G.; Suarez, D.L.; Seal, B.S.; Pedersen, J.C.; Senne, D.A.***, et al.*** Development of a real-time reverse-transcription PCR for detection of newcastle disease virus RNA in clinical samples. ***J Clin Microbiol*** **2004**, *42*, 329-338.
222. Rimmelzwaan, G.F.; Baars, M.; van Beek, R.; de Lijster, P.; de Jong, J.C.***, et al.*** Influenza virus subtype cross-reactivities of haemagglutination inhibiting and virus neutralising serum antibodies induced by infection or vaccination with an ISCOM-based vaccine. ***Vaccine*** **1999**, *17*, 2512-2516.
223. Freireich, E.J.; Gehan, E.A.; Rall, D.P.; Schmidt, L.H.; Skipper, H.E. Quantitative comparison of toxicity of anticancer agents in mouse, rat, hamster, dog, monkey, and man. ***Cancer chemotherapy reports. Part 1*** **1966**, *50*, 219-244.
224. Lamb, R.A.; Parks, G.D. Paramyxoviridae. In ***Fields Virology***, Knipe, D.M.; Howley, P.M., Eds. Wolters Kluwer: Philadelphia, 2013; pp 957-995.
225. Capua, I.; Alexander, D.J. Human health implications of avian influenza viruses and paramyxoviruses. ***European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology*** **2004**, *23*, 1-6.
226. Hanson, R.P.; Brandly, C.A. Newcastle disease. ***Ann N Y Acad Sci*** **1958**, *70*, 585-597.
227. Swayne, D.E.; King, D.J. Avian influenza and Newcastle disease. ***J Am Vet Med Assoc*** **2003**, *222*, 1534-1540.
228. Rasmussen, A.F. Avian myxoviruses and man. In ***Newcastle disease virus, an evolving pathogen***, Hanson, R.P., Ed. University of Wisconsin Press: Madison, Wisconsin, 1963; pp 313-325.
229. Dortmans, J.C.; Koch, G.; Rottier, P.J.; Peeters, B.P. Virulence of pigeon paramyxovirus type 1 does not always correlate with the cleavability of its fusion protein. ***J Gen Virol*** **2009**, *90*, 2746-2750.
230. Charan, S.; Mahajan, V.M.; Rai, A.; Balaya, S. Ocular pathogenesis of Newcastle disease virus in rabbits and monkeys. ***J Comp Pathol*** **1984**, *94*, 159-163.
231. Khattar, S.K.; Samal, S.; Devico, A.L.; Collins, P.L.; Samal, S.K. Newcastle disease virus expressing human immunodeficiency virus type 1 envelope glycoprotein induces strong mucosal and serum antibody responses in Guinea pigs. ***J Virol*** **2011**, *85*, 10529-10541.
232. Samuel, A.S.; Subbiah, M.; Shive, H.; Collins, P.L.; Samal, S.K. Experimental infection of hamsters with avian paramyxovirus serotypes 1 to 9. ***Vet Res*** **2011**, *42*, 38.
233. Buijs, P.R.; van Amerongen, G.; van Nieuwkoop, S.; Bestebroer, T.M.; van Run, P.R.***, et al.*** Intravenously injected

Newcastle disease virus in non-human primates is safe to use for oncolytic virotherapy. ***Cancer Gene Ther*** **2014**, *21*, 463-471.
234. Lam, H.Y.; Yeap, S.K.; Rasoli, M.; Omar, A.R.; Yusoff, K.***, et al.*** Safety and clinical usage of newcastle disease virus in cancer therapy. ***Journal of biomedicine & biotechnology*** **2011**, *2011*, 718710.
235. Campadelli-Fiume, G.; De Giovanni, C.; Gatta, V.; Nanni, P.; Lollini, P.L.***, et al.*** Rethinking herpes simplex virus: the way to oncolytic agents. ***Reviews in medical virology*** **2011**, *21*, 213-226.
236. Markert, J.M.; Malick, A.; Coen, D.M.; Martuza, R.L. Reduction and elimination of encephalitis in an experimental glioma therapy model with attenuated herpes simplex mutants that retain susceptibility to acyclovir. ***Neurosurgery*** **1993**, *32*, 597-603.
237. Chou, J.; Kern, E.R.; Whitley, R.J.; Roizman, B. Mapping of herpes simplex virus-1 neurovirulence to gamma 134.5, a gene nonessential for growth in culture. ***Science*** **1990**, *250*, 1262-1266.
238. Brown, S.M.; Harland, J.; MacLean, A.R.; Podlech, J.; Clements, J.B. Cell type and cell state determine differential in vitro growth of non-neurovirulent ICP34.5-negative herpes simplex virus types 1 and 2. ***J Gen Virol*** **1994**, *75 (Pt 9)*, 2367-2377.
239. Mohr, I. Neutralizing innate host defenses to control viral translation in HSV-1 infected cells. ***International reviews of immunology*** **2004**, *23*, 199-220.
240. Shah, A.C.; Parker, J.N.; Gillespie, G.Y.; Lakeman, F.D.; Meleth, S.***, et al.*** Enhanced antiglioma activity of chimeric HCMV/HSV-1 oncolytic viruses. ***Gene Ther*** **2007**, *14*, 1045-1054.
241. Kanai, R.; Zaupa, C.; Sgubin, D.; Antoszczyk, S.J.; Martuza, R.L.***, et al.*** Effect of gamma34.5 deletions on oncolytic herpes simplex virus activity in brain tumors. ***J Virol*** **2012**, *86*, 4420-4431.
242. Kasai, K.; Nakashima, H.; Nguyen, T.; Kaufmann, J.; I., S.***, et al.*** Improvement of safety profile and efficacy of an HSV type 1-based oncolytic virus through replacement of the viral ICP34.5 gene with GADD34. ***8th International Conference on Oncolytic Virus Therapeutics*** **2014**.
243. Mavromara-Nazos, P.; Ackermann, M.; Roizman, B. Construction and properties of a viable herpes simplex virus 1 recombinant lacking coding sequences of the alpha 47 gene. ***J Virol*** **1986**, *60*, 807-812.
244. Todo, T.; Martuza, R.L.; Rabkin, S.D.; Johnson, P.A. Oncolytic herpes simplex virus vector with enhanced MHC class I presentation and tumor cell killing. ***Proc Natl Acad Sci U S A*** **2001**, *98*, 6396-6401.
245. Zhou, G.; Roizman, B. Construction and properties of a herpes simplex virus 1 designed to enter cells solely via the IL-13alpha2 receptor. ***Proc Natl Acad Sci U S A*** **2006**, *103*, 5508-5513.
246. Menotti, L.; Cerretani, A.; Hengel, H.; Campadelli-Fiume, G. Construction of a fully retargeted herpes simplex virus 1 recombinant capable of entering cells solely via human epidermal growth factor receptor 2. ***J Virol*** **2008**, *82*, 10153-10161.
247. Zhou, G.; Ye, G.J.; Debinski, W.; Roizman, B. Engineered herpes simplex virus 1 is dependent on IL13Ralpha 2 receptor for cell entry and independent of glycoprotein D receptor interaction. ***Proc Natl Acad Sci U S A*** **2002**, *99*, 15124-15129.
248. Menotti, L.; Cerretani, A.; Campadelli-Fiume, G. A herpes simplex virus recombinant that exhibits a single-chain antibody to HER2/neu enters cells through the mammary tumor receptor, independently of the gD receptors. ***J Virol*** **2006**, *80*, 5531-5539.
249. Grandi, P.; Fernandez, J.; Szentirmai, O.; Carter, R.; Gianni, D.***, et al.*** Targeting HSV-1 virions for specific binding to epidermal growth factor receptor-vIII-bearing tumor cells. ***Cancer Gene Ther*** **2010**, *17*, 655-663.
250. Kambara, H.; Okano, H.; Chiocca, E.A.; Saeki, Y. An oncolytic HSV-1 mutant expressing ICP34.5 under control of a nestin promoter increases survival of animals even when symptomatic from a brain tumor. ***Cancer Res*** **2005**, *65*, 2832-2839.
251. Pesonen, S.; Kangasniemi, L.; Hemminki, A. Oncolytic adenoviruses for the treatment of human cancer: focus on translational and clinical data. ***Mol Pharm*** **2011**, *8*, 12-28.
252. Bischoff, J.R.; Kirn, D.H.; Williams, A.; Heise, C.; Horn, S.***, et al.*** An adenovirus mutant that replicates selectively in p53-deficient human tumor cells. ***Science*** **1996**, *274*, 373-376.
253. O'Shea, C.C.; Johnson, L.; Bagus, B.; Choi, S.; Nicholas, C.***, et al.*** Late viral RNA export, rather than p53 inactivation, determines ONYX-015 tumor selectivity. ***Cancer Cell*** **2004**, *6*, 611-623.
254. Fueyo, J.; Gomez-Manzano, C.; Alemany, R.; Lee, P.S.; McDonnell, T.J.***, et al.*** A mutant oncolytic adenovirus targeting the Rb pathway produces anti-glioma effect in vivo. ***Oncogene*** **2000**, *19*, 2-12.
255. Hakkarainen, T.; Rajecki, M.; Sarparanta, M.; Tenhunen, M.; Airaksinen, A.J.***, et al.*** Targeted radiotherapy for prostate cancer with an oncolytic adenovirus coding for human sodium iodide symporter. ***Clin Cancer Res*** **2009**, *15*, 5396-5403.

256. Johnson, L.; Shen, A.; Boyle, L.; Kunich, J.; Pandey, K.*, et al.* Selectively replicating adenoviruses targeting deregulated E2F activity are potent, systemic antitumor agents. *Cancer Cell* **2002**, *1*, 325-337.
257. Kim, J.; Cho, J.Y.; Kim, J.H.; Jung, K.C.; Yun, C.O. Evaluation of E1B gene-attenuated replicating adenoviruses for cancer gene therapy. *Cancer Gene Ther* **2002**, *9*, 725-736.
258. Lee, B.; Choi, J.; Kim, J.; Kim, J.H.; Joo, C.H.*, et al.* Oncolysis of human gastric cancers by an E1B 55 kDa-deleted YKL-1 adenovirus. *Cancer Lett* **2002**, *185*, 225-233.
259. Hallden, G. Optimisation of replication-selective oncolytic adenoviral mutants in combination with chemotherapeutics. *J BUON* **2009**, *14 Suppl 1*, S61-67.
260. Cascallo, M.; Capella, G.; Mazo, A.; Alemany, R. Ras-dependent oncolysis with an adenovirus VAI mutant. *Cancer Res* **2003**, *63*, 5544-5550.
261. Mantwill, K.; Naumann, U.; Seznec, J.; Girbinger, V.; Lage, H.*, et al.* YB-1 dependent oncolytic adenovirus efficiently inhibits tumor growth of glioma cancer stem like cells. *J Transl Med* **2013**, *11*, 216.
262. Holm, P.S.; Lage, H.; Bergmann, S.; Jurchott, K.; Glockzin, G.*, et al.* Multidrug-resistant cancer cells facilitate E1-independent adenoviral replication: impact for cancer gene therapy. *Cancer Res* **2004**, *64*, 322-328.
263. Rognoni, E.; Widmaier, M.; Haczek, C.; Mantwill, K.; Holzmuller, R.*, et al.* Adenovirus-based virotherapy enabled by cellular YB-1 expression in vitro and in vivo. *Cancer Gene Ther* **2009**, *16*, 753-763.
264. Holzmuller, R.; Mantwill, K.; Haczek, C.; Rognoni, E.; Anton, M.*, et al.* YB-1 dependent virotherapy in combination with temozolomide as a multimodal therapy approach to eradicate malignant glioma. *Int J Cancer* **2011**, *129*, 1265-1276.
265. Heise, C.; Kirn, D.H. Replication-selective adenoviruses as oncolytic agents. *J Clin Invest* **2000**, *105*, 847-851.
266. Nettelbeck, D.M. Virotherapeutics: conditionally replicative adenoviruses for viral oncolysis. *Anticancer Drugs* **2003**, *14*, 577-584.
267. Li, X.; Liu, Y.; Wen, Z.; Li, C.; Lu, H.*, et al.* Potent anti-tumor effects of a dual specific oncolytic adenovirus expressing apoptin in vitro and in vivo. *Mol Cancer* **2010**, *9*, 10.
268. Gao, Q.; Chen, C.; Ji, T.; Wu, P.; Han, Z.*, et al.* A systematic comparison of the anti-tumoural activity and toxicity of the three Adv-TKs. *PLoS One* **2014**, *9*, e94050.
269. Koski, A.; Kangasniemi, L.; Escutenaire, S.; Pesonen, S.; Cerullo, V.*, et al.* Treatment of cancer patients with a serotype 5/3 chimeric oncolytic adenovirus expressing GMCSF. *Mol Ther* **2010**, *18*, 1874-1884.
270. Burke, J.M.; Lamm, D.L.; Meng, M.V.; Nemunaitis, J.J.; Stephenson, J.J.*, et al.* A first in human phase 1 study of CG0070, a GM-CSF expressing oncolytic adenovirus, for the treatment of nonmuscle invasive bladder cancer. *The Journal of urology* **2012**, *188*, 2391-2397.
271. European Medicines Agency (EMA). Withdrawal assessment report for Advexin. *EMEA/692328/2008* **2008**.
272. European Medicines Agency (EMA). Withdrawal assessment report for Cerepro. *EMEA/203243/2008* **2007**.
273. Kim, M.; Zinn, K.R.; Barnett, B.G.; Sumerel, L.A.; Krasnykh, V.*, et al.* The therapeutic efficacy of adenoviral vectors for cancer gene therapy is limited by a low level of primary adenovirus receptors on tumour cells. *Eur J Cancer* **2002**, *38*, 1917-1926.
274. Tao, N.; Gao, G.P.; Parr, M.; Johnston, J.; Baradet, T.*, et al.* Sequestration of adenoviral vector by Kupffer cells leads to a nonlinear dose response of transduction in liver. *Mol Ther* **2001**, *3*, 28-35.
275. Alemany, R.; Curiel, D.T. CAR-binding ablation does not change biodistribution and toxicity of adenoviral vectors. *Gene Ther* **2001**, *8*, 1347-1353.
276. Smith, T.; Idamakanti, N.; Kylefjord, H.; Rollence, M.; King, L.*, et al.* In vivo hepatic adenoviral gene delivery occurs independently of the coxsackievirus-adenovirus receptor. *Mol Ther* **2002**, *5*, 770-779.
277. Wood, M.; Perrotte, P.; Onishi, E.; Harper, M.E.; Dinney, C.*, et al.* Biodistribution of an adenoviral vector carrying the luciferase reporter gene following intravesical or intravenous administration to a mouse. *Cancer Gene Ther* **1999**, *6*, 367-372.
278. Alba, R.; Bradshaw, A.C.; Coughlan, L.; Denby, L.; McDonald, R.A.*, et al.* Biodistribution and retargeting of FX-binding ablated adenovirus serotype 5 vectors. *Blood* **2010**, *116*, 2656-2664.
279. Short, J.J.; Rivera, A.A.; Wu, H.; Walter, M.R.; Yamamoto, M.*, et al.* Substitution of adenovirus serotype 3 hexon onto a serotype 5 oncolytic adenovirus reduces factor X binding, decreases liver tropism, and improves antitumor efficacy. *Mol Cancer Ther* **2010**, *9*, 2536-2544.
280. Alba, R.; Bradshaw, A.C.; Parker, A.L.; Bhella, D.; Waddington, S.N.*, et al.* Identification of coagulation factor (F)X binding sites on the adenovirus serotype 5 hexon: effect of mutagenesis on FX interactions and gene transfer. *Blood* **2009**, *114*, 965-971.
281. Doronin, K.; Shashkova, E.V.; May, S.M.; Hofherr, S.E.; Barry, M.A. Chemical modification with high molecular

weight polyethylene glycol reduces transduction of hepatocytes and increases efficacy of intravenously delivered oncolytic adenovirus. ***Hum Gene Ther*** **2009**, *20*, 975-988.
282. Fisher, K.D.; Seymour, L.W. HPMA copolymers for masking and retargeting of therapeutic viruses. ***Adv Drug Deliv Rev*** **2010**, *62*, 240-245.
283. Yoshihara, C.; Hamada, K.; Koyama, Y. Preparation of a novel adenovirus formulation with artificial envelope of multilayer polymer-coatings: therapeutic effect on metastatic ovarian cancer. ***Oncol Rep*** **2010**, *23*, 733-738.
284. Kim, J.; Nam, H.Y.; Kim, T.I.; Kim, P.H.; Ryu, J.***, et al.*** Active targeting of RGD-conjugated bioreducible polymer for delivery of oncolytic adenovirus expressing shRNA against IL-8 mRNA. ***Biomaterials*** **2011**, *32*, 5158-5166.
285. Kim, P.H.; Kim, J.; Kim, T.I.; Nam, H.Y.; Yockman, J.W.***, et al.*** Bioreducible polymer-conjugated oncolytic adenovirus for hepatoma-specific therapy via systemic administration. ***Biomaterials*** **2011**, *32*, 9328-9342.
286. Kim, P.H.; Sohn, J.H.; Choi, J.W.; Jung, Y.; Kim, S.W.***, et al.*** Active targeting and safety profile of PEG-modified adenovirus conjugated with herceptin. ***Biomaterials*** **2011**, *32*, 2314-2326.
287. Green, N.K.; Hale, A.; Cawood, R.; Illingworth, S.; Herbert, C.***, et al.*** Tropism ablation and stealthing of oncolytic adenovirus enhances systemic delivery to tumors and improves virotherapy of cancer. ***Nanomedicine (London, England)*** **2012**, *7*, 1683-1695.
288. Carlisle, R.; Choi, J.; Bazan-Peregrino, M.; Laga, R.; Subr, V.***, et al.*** Enhanced tumor uptake and penetration of virotherapy using polymer stealthing and focused ultrasound. ***J Natl Cancer Inst*** **2013**, *105*, 1701-1710.
289. Grunwald, G.K.; Vetter, A.; Klutz, K.; Willhauck, M.J.; Schwenk, N.***, et al.*** EGFR-Targeted Adenovirus Dendrimer Coating for Improved Systemic Delivery of the Theranostic NIS Gene. ***Molecular therapy. Nucleic acids*** **2013**, *2*, e131.
290. Grunwald, G.K.; Vetter, A.; Klutz, K.; Willhauck, M.J.; Schwenk, N.***, et al.*** Systemic image-guided liver cancer radiovirotherapy using dendrimer-coated adenovirus encoding the sodium iodide symporter as theranostic gene. ***Journal of nuclear medicine : official publication, Society of Nuclear Medicine*** **2013**, *54*, 1450-1457.
291. Kim, J.; Li, Y.; Kim, S.W.; Lee, D.S.; Yun, C.O. Therapeutic efficacy of a systemically delivered oncolytic adenovirus - biodegradable polymer complex. ***Biomaterials*** **2013**, *34*, 4622-4631.
292. Kwon, O.J.; Kang, E.; Choi, J.W.; Kim, S.W.; Yun, C.O. Therapeutic targeting of chitosan-PEG-folate-complexed oncolytic adenovirus for active and systemic cancer gene therapy. ***J Control Release*** **2013**, *169*, 257-265.
293. Kim, J.; Nam, H.Y.; Choi, J.W.; Yun, C.O.; Kim, S.W. Efficient lung orthotopic tumor-growth suppression of oncolytic adenovirus complexed with RGD-targeted bioreducible polymer. ***Gene Ther*** **2014**, *21*, 476-483.
294. Lee, C.H.; Kasala, D.; Na, Y.; Lee, M.S.; Kim, S.W.***, et al.*** Enhanced therapeutic efficacy of an adenovirus-PEI-bile-acid complex in tumors with low coxsackie and adenovirus receptor expression. ***Biomaterials*** **2014**, *35*, 5505-5516.
295. Carlisle, R.; Mo, S.; Myers, R.; Graham, S.; Laga, R.***, et al.*** Improving delivery of oncolytic viruses to solid tumors. ***8th International Conference on Oncolytic Virus Therapeutics*** **2014**.
296. Kranzler, J.; Tyler, M.A.; Sonabend, A.M.; Ulasov, I.V.; Lesniak, M.S. Stem cells as delivery vehicles for oncolytic adenoviral virotherapy. ***Current gene therapy*** **2009**, *9*, 389-395.
297. Yong, R.L.; Shinojima, N.; Fueyo, J.; Gumin, J.; Vecil, G.G.***, et al.*** Human bone marrow-derived mesenchymal stem cells for intravascular delivery of oncolytic adenovirus Delta24-RGD to human gliomas. ***Cancer Res*** **2009**, *69*, 8932-8940.
298. Ahmed, A.U.; Rolle, C.E.; Tyler, M.A.; Han, Y.; Sengupta, S.***, et al.*** Bone marrow mesenchymal stem cells loaded with an oncolytic adenovirus suppress the anti-adenoviral immune response in the cotton rat model. ***Mol Ther*** **2010**, *18*, 1846-1856.
299. Alcayaga-Miranda, F.; Cascallo, M.; Rojas, J.J.; Pastor, J.; Alemany, R. Osteosarcoma cells as carriers to allow antitumor activity of canine oncolytic adenovirus in the presence of neutralizing antibodies. ***Cancer Gene Ther*** **2010**, *17*, 792-802.
300. Dembinski, J.L.; Spaeth, E.L.; Fueyo, J.; Gomez-Manzano, C.; Studeny, M.***, et al.*** Reduction of nontarget infection and systemic toxicity by targeted delivery of conditionally replicating viruses transported in mesenchymal stem cells. ***Cancer Gene Ther*** **2010**, *17*, 289-297.
301. Garcia-Castro, J.; Alemany, R.; Cascallo, M.; Martinez-Quintanilla, J.; Arriero Mdel, M.***, et al.*** Treatment of metastatic neuroblastoma with systemic oncolytic virotherapy delivered by autologous mesenchymal stem cells: an exploratory study. ***Cancer Gene Ther*** **2010**, *17*, 476-483.
302. Hamada, K.; Zhang, T.; Desaki, J.; Nakashiro, K.; Itoh, H.***, et al.*** Carrier cell-mediated cell lysis of squamous cell carcinoma cells by squamous cell carcinoma antigen 1 promoter-driven oncolytic adenovirus. ***J Gene Med*** **2010**, *12*, 545-554.

303. Nakashima, H.; Kaur, B.; Chiocca, E.A. Directing systemic oncolytic viral delivery to tumors via carrier cells. *Cytokine Growth Factor Rev* **2010**, *21*, 119-126.
304. Tresilwised, N.; Pithayanukul, P.; Mykhaylyk, O.; Holm, P.S.; Holzmuller, R.*, et al.* Boosting oncolytic adenovirus potency with magnetic nanoparticles and magnetic force. *Mol Pharm* **2010**, *7*, 1069-1089.
305. Yang, L.; Wang, L.; Su, X.Q.; Wang, L.; Chen, X.C.*, et al.* Suppression of ovarian cancer growth via systemic administration with liposome-encapsulated adenovirus-encoding endostatin. *Cancer Gene Ther* **2010**, *17*, 49-57.
306. Ahmed, A.U.; Thaci, B.; Alexiades, N.G.; Han, Y.; Qian, S.*, et al.* Neural stem cell-based cell carriers enhance therapeutic efficacy of an oncolytic adenovirus in an orthotopic mouse model of human glioblastoma. *Mol Ther* **2011**, *19*, 1714-1726.
307. Ahmed, A.U.; Tyler, M.A.; Thaci, B.; Alexiades, N.G.; Han, Y.*, et al.* A comparative study of neural and mesenchymal stem cell-based carriers for oncolytic adenovirus in a model of malignant glioma. *Mol Pharm* **2011**, *8*, 1559-1572.
308. Kwon, O.J.; Kang, E.; Kim, S.; Yun, C.O. Viral genome DNA/lipoplexes elicit in situ oncolytic viral replication and potent antitumor efficacy via systemic delivery. *J Control Release* **2011**, *155*, 317-325.
309. Muthana, M.; Giannoudis, A.; Scott, S.D.; Fang, H.Y.; Coffelt, S.B.*, et al.* Use of macrophages to target therapeutic adenovirus to human prostate tumors. *Cancer Res* **2011**, *71*, 1805-1815.
310. Saito, A.; Morishita, N.; Mitsuoka, C.; Kitajima, S.; Hamada, K.*, et al.* Intravenous injection of irradiated tumor cell vaccine carrying oncolytic adenovirus suppressed the growth of multiple lung tumors in a mouse squamous cell carcinoma model. *J Gene Med* **2011**, *13*, 353-361.
311. Zhang, T.; Hamada, K.; Hyodo, M.; Itoh, H.; Tani, K.*, et al.* Gene therapy for oral squamous cell carcinoma with IAI.3B promoter-driven oncolytic adenovirus-infected carrier cells. *Oncol Rep* **2011**, *25*, 795-802.
312. Ahmed, A.U.; Ulasov, I.V.; Mercer, R.W.; Lesniak, M.S. Maintaining and loading neural stem cells for delivery of oncolytic adenovirus to brain tumors. *Methods Mol Biol* **2012**, *797*, 97-109.
313. Bolontrade, M.F.; Sganga, L.; Piaggio, E.; Viale, D.L.; Sorrentino, M.A.*, et al.* A specific subpopulation of mesenchymal stromal cell carriers overrides melanoma resistance to an oncolytic adenovirus. *Stem cells and development* **2012**, *21*, 2689-2702.
314. Bunuales, M.; Garcia-Aragoncillo, E.; Casado, R.; Quetglas, J.I.; Hervas-Stubbs, S.*, et al.* Evaluation of monocytes as carriers for armed oncolytic adenoviruses in murine and Syrian hamster models of cancer. *Hum Gene Ther* **2012**, *23*, 1258-1268.
315. Hai, C.; Jin, Y.M.; Jin, W.B.; Han, Z.Z.; Cui, M.N.*, et al.* Application of mesenchymal stem cells as a vehicle to deliver replication-competent adenovirus for treating malignant glioma. *Chinese journal of cancer* **2012**, *31*, 233-240.
316. Hsiao, W.C.; Sung, S.Y.; Liao, C.H.; Wu, H.C.; Hsieh, C.L. Vitamin D3-inducible mesenchymal stem cell-based delivery of conditionally replicating adenoviruses effectively targets renal cell carcinoma and inhibits tumor growth. *Mol Pharm* **2012**, *9*, 1396-1408.
317. Iguchi, K.; Sakurai, F.; Tomita, K.; Katayama, K.; Yamaguchi, T.*, et al.* Efficient antitumor effects of carrier cells loaded with a fiber-substituted conditionally replicating adenovirus on CAR-negative tumor cells. *Cancer Gene Ther* **2012**, *19*, 118-125.
318. Thaci, B.; Ahmed, A.U.; Ulasov, I.V.; Tobias, A.L.; Han, Y.*, et al.* Pharmacokinetic study of neural stem cell-based cell carrier for oncolytic virotherapy: targeted delivery of the therapeutic payload in an orthotopic brain tumor model. *Cancer Gene Ther* **2012**, *19*, 431-442.
319. Tresilwised, N.; Pithayanukul, P.; Holm, P.S.; Schillinger, U.; Plank, C.*, et al.* Effects of nanoparticle coatings on the activity of oncolytic adenovirus-magnetic nanoparticle complexes. *Biomaterials* **2012**, *33*, 256-269.
320. Ahmed, A.U.; Thaci, B.; Tobias, A.L.; Auffinger, B.; Zhang, L.*, et al.* A preclinical evaluation of neural stem cell-based cell carrier for targeted antiglioma oncolytic virotherapy. *J Natl Cancer Inst* **2013**, *105*, 968-977.
321. Kim, C.K.; Ahmed, A.U.; Auffinger, B.; Ulasov, I.V.; Tobias, A.L.*, et al.* N-acetylcysteine amide augments the therapeutic effect of neural stem cell-based antiglioma oncolytic virotherapy. *Mol Ther* **2013**, *21*, 2063-2073.
322. Shinojima, N.; Hossain, A.; Takezaki, T.; Fueyo, J.; Gumin, J.*, et al.* TGF-beta mediates homing of bone marrow-derived human mesenchymal stem cells to glioma stem cells. *Cancer Res* **2013**, *73*, 2333-2344.
323. Tobias, A.L.; Thaci, B.; Auffinger, B.; Rincon, E.; Balyasnikova, I.V.*, et al.* The timing of neural stem cell-based virotherapy is critical for optimal therapeutic efficacy when applied with radiation and chemotherapy for the treatment of glioblastoma. *Stem cells translational medicine* **2013**, *2*, 655-666.
324. Coughlan, L.; Alba, R.; Parker, A.L.; Bradshaw, A.C.; McNeish, I.A.*, et al.* Tropism-modification strategies for

targeted gene delivery using adenoviral vectors. ***Viruses*** **2010**, ***2***, 2290-2355.
325. Hemminki, O.; Diaconu, I.; Cerullo, V.; Pesonen, S.K.; Kanerva, A.***, et al.*** Ad3-hTERT-E1A, a fully serotype 3 oncolytic adenovirus, in patients with chemotherapy refractory cancer. ***Mol Ther*** **2012**, ***20***, 1821-1830.
326. Belousova, N.; Mikheeva, G.; Xiong, C.; Soghomonian, S.; Young, D.***, et al.*** Development of a targeted gene vector platform based on simian adenovirus serotype 24. ***J Virol*** **2010**, ***84***, 10087-10101.
327. Sharma, A.; Bangari, D.S.; Tandon, M.; Pandey, A.; HogenEsch, H.***, et al.*** Comparative analysis of vector biodistribution, persistence and gene expression following intravenous delivery of bovine, porcine and human adenoviral vectors in a mouse model. ***Virology*** **2009**, ***386***, 44-54.
328. Strauss, R.; Sova, P.; Liu, Y.; Li, Z.Y.; Tuve, S.***, et al.*** Epithelial phenotype confers resistance of ovarian cancer cells to oncolytic adenoviruses. ***Cancer Res*** **2009**, ***69***, 5115-5125.
329. Hemminki, O.; Bauerschmitz, G.; Hemmi, S.; Lavilla-Alonso, S.; Diaconu, I.***, et al.*** Oncolytic adenovirus based on serotype 3. ***Cancer Gene Ther*** **2011**, ***18***, 288-296.
330. Shashkova, E.V.; May, S.M.; Barry, M.A. Characterization of human adenovirus serotypes 5, 6, 11, and 35 as anticancer agents. ***Virology*** **2009**, ***394***, 311-320.
331. Senac, J.S.; Doronin, K.; Russell, S.J.; Jelinek, D.F.; Greipp, P.R.***, et al.*** Infection and killing of multiple myeloma by adenoviruses. ***Hum Gene Ther*** **2010**, ***21***, 179-190.
332. van Zeeburg, H.J.; Huizenga, A.; Brink, A.; van den Doel, P.B.; Zhu, Z.B.***, et al.*** Comparison of oncolytic adenoviruses for selective eradication of oral cancer and pre-cancerous lesions. ***Gene Ther*** **2010**, ***17***, 1517-1524.
333. Chen, C.Y.; Senac, J.S.; Weaver, E.A.; May, S.M.; Jelinek, D.F.***, et al.*** Species D adenoviruses as oncolytics against B-cell cancers. ***Clin Cancer Res*** **2011**, ***17***, 6712-6722.
334. Chen, C.Y.; Weaver, E.A.; Khare, R.; May, S.M.; Barry, M.A. Mining the adenovirus virome for oncolytics against multiple solid tumor types. ***Cancer Gene Ther*** **2011**, ***18***, 744-750.
335. Weaver, E.A.; Chen, C.Y.; May, S.M.; Barry, M.E.; Barry, M.A. Comparison of adenoviruses as oncolytics and cancer vaccines in an immunocompetent B cell lymphoma model. ***Hum Gene Ther*** **2011**, ***22***, 1095-1100.
336. Barry, M.A.; Weaver, E.A.; Chen, C.Y. Mining the adenovirus "virome" for systemic oncolytics. ***Current pharmaceutical biotechnology*** **2012**, ***13***, 1804-1808.
337. Wong, H.H.; Jiang, G.; Gangeswaran, R.; Wang, P.; Wang, J.***, et al.*** Modification of the early gene enhancer-promoter improves the oncolytic potency of adenovirus 11. ***Mol Ther*** **2012**, ***20***, 306-316.
338. Zhang, Z.; Zhang, X.; Newman, K.; Liu, X.; Seth, P. MicroRNA regulation of oncolytic adenovirus 6 for selective treatment of castration-resistant prostate cancer. ***Mol Cancer Ther*** **2012**, ***11***, 2410-2418.
339. Richter, M.; Yumul, R.; Lu, Z.; Wang, H.; Fender, P.***, et al.*** Species B adenovirus spread in solid tumros. ***8th International Conference on Oncolytic Virus Therapeutics*** **2014**.
340. Kuhn, I.; Harden, P.; Bauzon, M.; Chartier, C.; Nye, J.***, et al.*** Directed evolution generates a novel oncolytic virus for the treatment of colon cancer. ***PLoS One*** **2008**, ***3***, e2409.
341. Miura, Y.; Yamasaki, S.; Davydova, J.; Brown, E.; Aoki, K.***, et al.*** Infectivity-selective oncolytic adenovirus developed by high-throughput screening of adenovirus-formatted library. ***Mol Ther*** **2013**, ***21***, 139-148.
342. Uil, T.G.; Vellinga, J.; de Vrij, J.; van den Hengel, S.K.; Rabelink, M.J.***, et al.*** Directed adenovirus evolution using engineered mutator viral polymerases. ***Nucleic Acids Res*** **2011**, ***39***, e30.
343. Bauzon, M.; Jin, F.; Kretschmer, P.; Hermiston, T. In vitro analysis of cidofovir and genetically engineered TK expression as potential approaches for the intervention of ColoAd1-based treatment of cancer. ***Gene Ther*** **2009**, ***16***, 1169-1174.
344. Reid, T.; Galanis, E.; Abbruzzese, J.; Sze, D.; Wein, L.M.***, et al.*** Hepatic arterial infusion of a replication-selective oncolytic adenovirus (dl1520): phase II viral, immunologic, and clinical endpoints. ***Cancer Res*** **2002**, ***62***, 6070-6079.
345. Kimball, K.J.; Preuss, M.A.; Barnes, M.N.; Wang, M.; Siegal, G.P.***, et al.*** A phase I study of a tropism-modified conditionally replicative adenovirus for recurrent malignant gynecologic diseases. ***Clin Cancer Res*** **2010**, ***16***, 5277-5287.
346. Pesonen, S.; Diaconu, I.; Cerullo, V.; Escutenaire, S.; Raki, M.***, et al.*** Integrin targeted oncolytic adenoviruses Ad5-D24-RGD and Ad5-RGD-D24-GMCSF for treatment of patients with advanced chemotherapy refractory solid tumors. ***Int J Cancer*** **2012**, ***130***, 1937-1947.
347. Pesonen, S.; Nokisalmi, P.; Escutenaire, S.; Sarkioja, M.; Raki, M.***, et al.*** Prolonged systemic circulation of chimeric oncolytic adenovirus Ad5/3-Cox2L-D24 in patients with metastatic and refractory solid tumors. ***Gene Ther*** **2010**, ***17***, 892-904.

348. Raki, M.; Sarkioja, M.; Escutenaire, S.; Kangasniemi, L.; Haavisto, E.*, et al.* Switching the fiber knob of oncolytic adenoviruses to avoid neutralizing antibodies in human cancer patients. *J Gene Med* **2011**, *13*, 253-261.
349. Bramante, S.; Koski, A.; Kipar, A.; Diaconu, I.; Liikanen, I.*, et al.* Serotype chimeric oncolytic adenovirus coding for GM-CSF for treatment of sarcoma in rodents and humans. *Int J Cancer* **2014**, *135*, 720-730.
350. Kim, K.H.; Dmitriev, I.P.; Saddekni, S.; Kashentseva, E.A.; Harris, R.D.*, et al.* A phase I clinical trial of Ad5/3-Delta24, a novel serotype-chimeric, infectivity-enhanced, conditionally-replicative adenovirus (CRAd), in patients with recurrent ovarian cancer. *Gynecologic oncology* **2013**, *130*, 518-524.
351. Blanc, C.; Calvo, E.; Martin, M.G.; Machiels, J.P.; Rottey, S.*, et al.* Review of the current status of phase I clinical studies of Enadenotucirev (ColoAd1), an Ad11/Ad3 chimeric group B adenovirus, in patients with metastatic epithelial solid tumors. *8th International Conference on Oncolytic Virus Therapeutics* **2014**.
352. Nemunaitis, J.; Tong, A.W.; Nemunaitis, M.; Senzer, N.; Phadke, A.P.*, et al.* A phase I study of telomerase-specific replication competent oncolytic adenovirus (telomelysin) for various solid tumors. *Mol Ther* **2010**, *18*, 429-434.
353. Gimenez-Alejandre, M.; Rodriguez-Garcia, A.; Moreno-Olie, R.; Condom i Mundó, E.; Nadal, M.*, et al.* Chemosensitization to gemcitabine by VCN-01, a tumor-targeted oncolytic adenovirus armed with hyaluronidase: from bench to bedside. *8th International Conference on Oncolytic Virus Therapeutics* **2014**.
354. Chang, J.; Zhao, X.; Wu, X.; Guo, Y.; Guo, H.*, et al.* A Phase I study of KH901, a conditionally replicating granulocyte-macrophage colony-stimulating factor: armed oncolytic adenovirus for the treatment of head and neck cancers. *Cancer Biol Ther* **2009**, *8*, 676-682.
355. Cerullo, V.; Pesonen, S.; Diaconu, I.; Escutenaire, S.; Arstila, P.T.*, et al.* Oncolytic adenovirus coding for granulocyte macrophage colony-stimulating factor induces antitumoral immunity in cancer patients. *Cancer Res* **2010**, *70*, 4297-4309.
356. Pesonen, S.; Diaconu, I.; Kangasniemi, L.; Ranki, T.; Kanerva, A.*, et al.* Oncolytic immunotherapy of advanced solid tumors with a CD40L-expressing replicating adenovirus: assessment of safety and immunologic responses in patients. *Cancer Res* **2012**, *72*, 1621-1631.
357. Kanerva, A.; Nokisalmi, P.; Diaconu, I.; Koski, A.; Cerullo, V.*, et al.* Antiviral and antitumor T-cell immunity in patients treated with GM-CSF-coding oncolytic adenovirus. *Clin Cancer Res* **2013**, *19*, 2734-2744.
358. Msaouel, P.; Iankov, I.D.; Dispenzieri, A.; Galanis, E. Attenuated oncolytic measles virus strains as cancer therapeutics. *Current pharmaceutical biotechnology* **2012**, *13*, 1732-1741.
359. Msaouel, P.; Dispenzieri, A.; Galanis, E. Clinical testing of engineered oncolytic measles virus strains in the treatment of cancer: an overview. *Curr Opin Mol Ther* **2009**, *11*, 43-53.
360. Blechacz, B.; Russell, S.J. Measles virus as an oncolytic vector platform. *Current gene therapy* **2008**, *8*, 162-175.
361. Peng, K.W.; Facteau, S.; Wegman, T.; O'Kane, D.; Russell, S.J. Non-invasive in vivo monitoring of trackable viruses expressing soluble marker peptides. *Nat Med* **2002**, *8*, 527-531.
362. Hadac, E.M.; Peng, K.W.; Nakamura, T.; Russell, S.J. Reengineering paramyxovirus tropism. *Virology* **2004**, *329*, 217-225.
363. Nakamura, T.; Peng, K.W.; Harvey, M.; Greiner, S.; Lorimer, I.A.*, et al.* Rescue and propagation of fully retargeted oncolytic measles viruses. *Nat Biotechnol* **2005**, *23*, 209-214.
364. Billeter, M.A.; Naim, H.Y.; Udem, S.A. Reverse genetics of measles virus and resulting multivalent recombinant vaccines: applications of recombinant measles viruses. *Current topics in microbiology and immunology* **2009**, *329*, 129-162.
365. Galanis, E.; Hartmann, L.C.; Cliby, W.A.; Long, H.J.; Peethambaram, P.P.*, et al.* Phase I trial of intraperitoneal administration of an oncolytic measles virus strain engineered to express carcinoembryonic antigen for recurrent ovarian cancer. *Cancer Res* **2010**, *70*, 875-882.
366. Russell, S.J.; Federspiel, M.J.; Peng, K.W.; Tong, C.; Dingli, D.*, et al.* Remission of disseminated cancer after systemic oncolytic virotherapy. *Mayo Clinic proceedings* **2014**, *89*, 926-933.
367. Hastie, E.; Grdzelishvili, V.Z. Vesicular stomatitis virus as a flexible platform for oncolytic virotherapy against cancer. *J Gen Virol* **2012**, *93*, 2529-2545.
368. Quiroz, E.; Moreno, N.; Peralta, P.H.; Tesh, R.B. A human case of encephalitis associated with vesicular stomatitis virus (Indiana serotype) infection. *Am J Trop Med Hyg* **1988**, *39*, 312-314.
369. Stojdl, D.F.; Lichty, B.; Knowles, S.; Marius, R.; Atkins, H.*, et al.* Exploiting tumor-specific defects in the interferon pathway with a previously unknown oncolytic virus. *Nat Med* **2000**, *6*, 821-825.
370. Stojdl, D.F.; Lichty, B.D.; tenOever, B.R.; Paterson, J.M.; Power, A.T.*, et al.* VSV strains with defects in their ability to shutdown innate immunity are potent systemic anti-cancer agents. *Cancer Cell* **2003**, *4*, 263-275.

371. Barber, G.N. Vesicular stomatitis virus as an oncolytic vector. ***Viral immunology*** **2004**, *17*, 516-527.
372. Lichty, B.D.; Power, A.T.; Stojdl, D.F.; Bell, J.C. Vesicular stomatitis virus: re-inventing the bullet. ***Trends in molecular medicine*** **2004**, *10*, 210-216.
373. Barber, G.N. VSV-tumor selective replication and protein translation. ***Oncogene*** **2005**, *24*, 7710-7719.
374. Oliere, S.; Arguello, M.; Mesplede, T.; Tumilasci, V.; Nakhaei, P.***, et al.*** Vesicular stomatitis virus oncolysis of T lymphocytes requires cell cycle entry and translation initiation. ***J Virol*** **2008**, *82*, 5735-5749.
375. Balachandran, S.; Barber, G.N. PKR in innate immunity, cancer, and viral oncolysis. ***Methods Mol Biol*** **2007**, *383*, 277-301.
376. Harashima, A.; Guettouche, T.; Barber, G.N. Phosphorylation of the NFAR proteins by the dsRNA-dependent protein kinase PKR constitutes a novel mechanism of translational regulation and cellular defense. ***Genes Dev*** **2010**, *24*, 2640-2653.
377. Kottke, T.; Diaz, R.M.; Kaluza, K.; Pulido, J.; Galivo, F.***, et al.*** Use of biological therapy to enhance both virotherapy and adoptive T-cell therapy for cancer. ***Mol Ther*** **2008**, *16*, 1910-1918.
378. Qiao, J.; Wang, H.; Kottke, T.; Diaz, R.M.; Willmon, C.***, et al.*** Loading of oncolytic vesicular stomatitis virus onto antigen-specific T cells enhances the efficacy of adoptive T-cell therapy of tumors. ***Gene Ther*** **2008**, *15*, 604-616.
379. Labib, M.; Zamay, A.S.; Muharemagic, D.; Chechik, A.; Bell, J.C.***, et al.*** Electrochemical sensing of aptamer-facilitated virus immunoshielding. ***Anal Chem*** **2012**, *84*, 1677-1686.
380. Tesfay, M.Z.; Kirk, A.C.; Hadac, E.M.; Griesmann, G.E.; Federspiel, M.J.***, et al.*** PEGylation of vesicular stomatitis virus extends virus persistence in blood circulation of passively immunized mice. ***J Virol*** **2013**, *87*, 3752-3759.
381. Shafren, D.R.; Dorahy, D.J.; Ingham, R.A.; Burns, G.F.; Barry, R.D. Coxsackievirus A21 binds to decay-accelerating factor but requires intercellular adhesion molecule 1 for cell entry. ***J Virol*** **1997**, *71*, 4736-4743.
382. Shafren, D.R.; Au, G.G.; Nguyen, T.; Newcombe, N.G.; Haley, E.S.***, et al.*** Systemic therapy of malignant human melanoma tumors by a common cold-producing enterovirus, coxsackievirus a21. ***Clin Cancer Res*** **2004**, *10*, 53-60.
383. Andtbacka, R.H.I.; Au, G.G.; Weisberg, J.I.; Post, L.; Shafren, D.R. CAVATAK-mediated oncolytic immunotherapy in advanced melanoma patients. ***8th International Conference on Oncolytic Virus Therapeutics*** **2014**.
384. Merrill, M.K.; Bernhardt, G.; Sampson, J.H.; Wikstrand, C.J.; Bigner, D.D.***, et al.*** Poliovirus receptor CD155-targeted oncolysis of glioma. ***Neuro-oncology*** **2004**, *6*, 208-217.
385. Goetz, C.; Everson, R.G.; Zhang, L.C.; Gromeier, M. MAPK signal-integrating kinase controls cap-independent translation and cell type-specific cytotoxicity of an oncolytic poliovirus. ***Mol Ther*** **2010**, *18*, 1937-1946.
386. Gromeier, M.; Bossert, B.; Arita, M.; Nomoto, A.; Wimmer, E. Dual stem loops within the poliovirus internal ribosomal entry site control neurovirulence. ***J Virol*** **1999**, *73*, 958-964.
387. Dobrikova, E.Y.; Broadt, T.; Poiley-Nelson, J.; Yang, X.; Soman, G.***, et al.*** Recombinant oncolytic poliovirus eliminates glioma in vivo without genetic adaptation to a pathogenic phenotype. ***Mol Ther*** **2008**, *16*, 1865-1872.
388. Gromeier, M.; Dobrikova, E.; Dobrikov, M.; Brown, M.; Desjardins, A.***, et al.*** Oncolytic poliovirus immunotherapy of glioblastoma. ***8th International Conference on Oncolytic Virus Therapeutics*** **2014**.
389. Reddy, P.S.; Burroughs, K.D.; Hales, L.M.; Ganesh, S.; Jones, B.H.***, et al.*** Seneca Valley virus, a systemically deliverable oncolytic picornavirus, and the treatment of neuroendocrine cancers. ***J Natl Cancer Inst*** **2007**, *99*, 1623-1633.
390. Poirier, J.T.; Dobromilskaya, I.; Moriarty, W.F.; Peacock, C.D.; Hann, C.L.***, et al.*** Selective tropism of Seneca Valley virus for variant subtype small cell lung cancer. ***J Natl Cancer Inst*** **2013**, *105*, 1059-1065.
391. Morton, C.L.; Houghton, P.J.; Kolb, E.A.; Gorlick, R.; Reynolds, C.P.***, et al.*** Initial testing of the replication competent Seneca Valley virus (NTX-010) by the pediatric preclinical testing program. ***Pediatric blood & cancer*** **2010**, *55*, 295-303.
392. Yu, L.; Baxter, P.A.; Zhao, X.; Liu, Z.; Wadhwa, L.***, et al.*** A single intravenous injection of oncolytic picornavirus SVV-001 eliminates medulloblastomas in primary tumor-based orthotopic xenograft mouse models. ***Neuro-oncology*** **2011**, *13*, 14-27.
393. Liu, Z.; Zhao, X.; Mao, H.; Baxter, P.A.; Huang, Y.***, et al.*** Intravenous injection of oncolytic picornavirus SVV-001 prolongs animal survival in a panel of primary tumor-based orthotopic xenograft mouse models of pediatric glioma. ***Neuro-oncology*** **2013**, *15*, 1173-1185.
394. Wadhwa, L.; Hurwitz, M.Y.; Chevez-Barrios, P.; Hurwitz, R.L. Treatment of invasive retinoblastoma in a murine model using an oncolytic picornavirus. ***Cancer Res*** **2007**, *67*, 10653-10656.

395. Rudin, C.M.; Poirier, J.T.; Senzer, N.N.; Stephenson, J., Jr.; Loesch, D.*, et al.* Phase I clinical study of Seneca Valley Virus (SVV-001), a replication-competent picornavirus, in advanced solid tumors with neuroendocrine features. *Clin Cancer Res* **2011**, *17*, 888-895.
396. Burke, M.J.; Ahern, C.; Weigel, B.J.; Poirier, J.T.; Rudin, C.M.*, et al.* Phase I trial of Seneca Valley Virus (NTX-010) in children with relapsed/refractory solid tumors: a report of the Children's Oncology Group. *Pediatric blood & cancer* **2015**, *62*, 743-750.
397. Miller, J.D.; van der Most, R.G.; Akondy, R.S.; Glidewell, J.T.; Albott, S.*, et al.* Human effector and memory CD8+ T cell responses to smallpox and yellow fever vaccines. *Immunity* **2008**, *28*, 710-722.
398. Putz, M.M.; Midgley, C.M.; Law, M.; Smith, G.L. Quantification of antibody responses against multiple antigens of the two infectious forms of Vaccinia virus provides a benchmark for smallpox vaccination. *Nat Med* **2006**, *12*, 1310-1315.
399. Wein, L.M.; Wu, J.T.; Kirn, D.H. Validation and analysis of a mathematical model of a replication-competent oncolytic virus for cancer treatment: implications for virus design and delivery. *Cancer Res* **2003**, *63*, 1317-1324.
400. Thorne, S.H.; Hwang, T.H.; O'Gorman, W.E.; Bartlett, D.L.; Sei, S.*, et al.* Rational strain selection and engineering creates a broad-spectrum, systemically effective oncolytic poxvirus, JX-963. *J Clin Invest* **2007**, *117*, 3350-3358.
401. McCart, J.A.; Ward, J.M.; Lee, J.; Hu, Y.; Alexander, H.R.*, et al.* Systemic cancer therapy with a tumor-selective vaccinia virus mutant lacking thymidine kinase and vaccinia growth factor genes. *Cancer Res* **2001**, *61*, 8751-8757.
402. Vanderplasschen, A.; Mathew, E.; Hollinshead, M.; Sim, R.B.; Smith, G.L. Extracellular enveloped vaccinia virus is resistant to complement because of incorporation of host complement control proteins into its envelope. *Proc Natl Acad Sci U S A* **1998**, *95*, 7544-7549.
403. Bell, E.; Shamim, M.; Whitbeck, J.C.; Sfyroera, G.; Lambris, J.D.*, et al.* Antibodies against the extracellular enveloped virus B5R protein are mainly responsible for the EEV neutralizing capacity of vaccinia immune globulin. *Virology* **2004**, *325*, 425-431.
404. Kirn, D.H.; Wang, Y.; Liang, W.; Contag, C.H.; Thorne, S.H. Enhancing poxvirus oncolytic effects through increased spread and immune evasion. *Cancer Res* **2008**, *68*, 2071-2075.
405. Buller, R.M.; Chakrabarti, S.; Moss, B.; Fredrickson, T. Cell proliferative response to vaccinia virus is mediated by VGF. *Virology* **1988**, *164*, 182-192.
406. Tzahar, E.; Moyer, J.D.; Waterman, H.; Barbacci, E.G.; Bao, J.*, et al.* Pathogenic poxviruses reveal viral strategies to exploit the ErbB signaling network. *EMBO J* **1998**, *17*, 5948-5963.
407. de Magalhaes, J.C.; Andrade, A.A.; Silva, P.N.; Sousa, L.P.; Ropert, C.*, et al.* A mitogenic signal triggered at an early stage of vaccinia virus infection: implication of MEK/ERK and protein kinase A in virus multiplication. *J Biol Chem* **2001**, *276*, 38353-38360.
408. Katsafanas, G.C.; Moss, B. Vaccinia virus intermediate stage transcription is complemented by Ras-GTPase-activating protein SH3 domain-binding protein (G3BP) and cytoplasmic activation/proliferation-associated protein (p137) individually or as a heterodimer. *J Biol Chem* **2004**, *279*, 52210-52217.
409. Puhlmann, M.; Brown, C.K.; Gnant, M.; Huang, J.; Libutti, S.K.*, et al.* Vaccinia as a vector for tumor-directed gene therapy: biodistribution of a thymidine kinase-deleted mutant. *Cancer Gene Ther* **2000**, *7*, 66-73.
410. Hengstschlager, M.; Knofler, M.; Mullner, E.W.; Ogris, E.; Wintersberger, E.*, et al.* Different regulation of thymidine kinase during the cell cycle of normal versus DNA tumor virus-transformed cells. *J Biol Chem* **1994**, *269*, 13836-13842.
411. Buller, R.M.; Smith, G.L.; Cremer, K.; Notkins, A.L.; Moss, B. Decreased virulence of recombinant vaccinia virus expression vectors is associated with a thymidine kinase-negative phenotype. *Nature* **1985**, *317*, 813-815.
412. Gnant, M.F.; Noll, L.A.; Irvine, K.R.; Puhlmann, M.; Terrill, R.E.*, et al.* Tumor-specific gene delivery using recombinant vaccinia virus in a rabbit model of liver metastases. *J Natl Cancer Inst* **1999**, *91*, 1744-1750.
413. Colamonici, O.R.; Domanski, P.; Sweitzer, S.M.; Larner, A.; Buller, R.M. Vaccinia virus B18R gene encodes a type I interferon-binding protein that blocks interferon alpha transmembrane signaling. *J Biol Chem* **1995**, *270*, 15974-15978.
414. Symons, J.A.; Alcami, A.; Smith, G.L. Vaccinia virus encodes a soluble type I interferon receptor of novel structure and broad species specificity. *Cell* **1995**, *81*, 551-560.
415. Shida, H.; Hinuma, Y.; Hatanaka, M.; Morita, M.; Kidokoro, M.*, et al.* Effects and virulences of recombinant vaccinia viruses derived from attenuated strains that express the human T-cell leukemia virus type I envelope

gene. *J Virol* **1988**, *62*, 4474-4480.
416. Roenigk, H.H., Jr.; Deodhar, S.; St Jacques, R.; Burdick, K. Immunotherapy of malignant melanoma with vaccinia virus. *Archives of dermatology* **1974**, *109*, 668-673.
417. Lee, S.S.; Eisenlohr, L.C.; McCue, P.A.; Mastrangelo, M.J.; Lattime, E.C. Intravesical gene therapy: in vivo gene transfer using recombinant vaccinia virus vectors. *Cancer Res* **1994**, *54*, 3325-3328.
418. Hunter-Craig, I.; Newton, K.A.; Westbury, G.; Lacey, B.W. Use of vaccinia virus in the treatment of metastatic malignant melanoma. *British medical journal* **1970**, *2*, 512-515.
419. Lusky, M.; Erbs, P.; Foloppe, J.; Acres, R.B. Oncolytic vaccinia virus: a silver bullet? *Expert Rev Vaccines* **2010**, *9*, 1353-1356.
420. Kim, J.H.; Oh, J.Y.; Park, B.H.; Lee, D.E.; Kim, J.S.*, et al.* Systemic armed oncolytic and immunologic therapy for cancer with JX-594, a targeted poxvirus expressing GM-CSF. *Mol Ther* **2006**, *14*, 361-370.
421. Parato, K.A.; Breitbach, C.J.; Le Boeuf, F.; Wang, J.; Storbeck, C.*, et al.* The oncolytic poxvirus JX-594 selectively replicates in and destroys cancer cells driven by genetic pathways commonly activated in cancers. *Mol Ther* **2012**, *20*, 749-758.
422. Mastrangelo, M.J.; Maguire, H.C., Jr.; Eisenlohr, L.C.; Laughlin, C.E.*, et al.* Intratumoral recombinant GM-CSF-encoding virus as gene therapy in patients with cutaneous melanoma. *Cancer Gene Ther* **1999**, *6*, 409-422.
423. Hwang, T.H.; Moon, A.; Burke, J.; Ribas, A.; Stephenson, J.*, et al.* A mechanistic proof-of-concept clinical trial with JX-594, a targeted multi-mechanistic oncolytic poxvirus, in patients with metastatic melanoma. *Mol Ther* **2011**, *19*, 1913-1922.
424. Liu, T.C.; Hwang, T.; Park, B.H.; Bell, J.; Kirn, D.H. The targeted oncolytic poxvirus JX-594 demonstrates antitumoral, antivascular, and anti-HBV activities in patients with hepatocellular carcinoma. *Mol Ther* **2008**, *16*, 1637-1642.
425. Park, B.H.; Hwang, T.; Liu, T.C.; Sze, D.Y.; Kim, J.S.*, et al.* Use of a targeted oncolytic poxvirus, JX-594, in patients with refractory primary or metastatic liver cancer: a phase I trial. *The lancet oncology* **2008**, *9*, 533-542.
426. Heo, J.; Breitbach, C.J.; Moon, A.; Kim, C.W.; Patt, R.*, et al.* Sequential therapy with JX-594, a targeted oncolytic poxvirus, followed by sorafenib in hepatocellular carcinoma: preclinical and clinical demonstration of combination efficacy. *Mol Ther* **2011**, *19*, 1170-1179.
427. Heo, J.; Reid, T.; Ruo, L.; Breitbach, C.J.; Rose, S.*, et al.* Randomized dose-finding clinical trial of oncolytic immunotherapeutic vaccinia JX-594 in liver cancer. *Nat Med* **2013**, *19*, 329-336.
428. Breitbach, C.J.; Burke, J.; Jonker, D.; Stephenson, J.; Haas, A.R.*, et al.* Intravenous delivery of a multi-mechanistic cancer-targeted oncolytic poxvirus in humans. *Nature* **2011**, *477*, 99-102.
429. Lauer, U.M.; Beil, J.; Berchtold, S.; Zimmermann, M.; Koppenhöfer, U.*, et al.* Tracking of tumor cell colonization, in-patient replication, and oncolysis by GL-ONC1 employed in a phase I/II virotherapy study on patients with peritoneal carcinomatosis. *8th International Conference on Oncolytic Virus Therapeutics* **2014**.
430. Reinboth, J.; Ralph, C.; Sciagalla, P.; Yu, Y.A.; Agular, J.*, et al.* Oncolytic vaccinia virus GL-ONC1 treatment of colorectal cancer patients. *8th International Conference on Oncolytic Virus Therapeutics* **2014**.
431. Shmulevitz, M.; Marcato, P.; Lee, P.W. Unshackling the links between reovirus oncolysis, Ras signaling, translational control and cancer. *Oncogene* **2005**, *24*, 7720-7728.
432. Lemay, G.; Tumilasci, V.; Hiscott, J. Uncoating reo: uncovering the steps critical for oncolysis. *Mol Ther* **2007**, *15*, 1406-1407.
433. Hashiro, G.; Loh, P.C.; Yau, J.T. The preferential cytotoxicity of reovirus for certain transformed cell lines. *Arch Virol* **1977**, *54*, 307-315.
434. Strong, J.E.; Lee, P.W. The v-erbB oncogene confers enhanced cellular susceptibility to reovirus infection. *J Virol* **1996**, *70*, 612-616.
435. Min, H.J.; Koh, S.S.; Cho, I.R.; Srisuttee, R.; Park, E.H.*, et al.* Inhibition of GSK-3beta enhances reovirus-induced apoptosis in colon cancer cells. *Int J Oncol* **2009**, *35*, 617-624.
436. Wilcox, M.E.; Yang, W.; Senger, D.; Rewcastle, N.B.; Morris, D.G.*, et al.* Reovirus as an oncolytic agent against experimental human malignant gliomas. *J Natl Cancer Inst* **2001**, *93*, 903-912.
437. Coffey, M.C.; Strong, J.E.; Forsyth, P.A.; Lee, P.W. Reovirus therapy of tumors with activated Ras pathway. *Science* **1998**, *282*, 1332-1334.
438. Strong, J.E.; Coffey, M.C.; Tang, D.; Sabinin, P.; Lee, P.W. The molecular basis of viral oncolysis: usurpation of the Ras signaling pathway by reovirus. *EMBO J* **1998**, *17*, 3351-3362.
439. Park, E.H.; Park, E.H.; Cho, I.R.; Srisuttee, R.; Min, H.J.*, et al.* CUG2, a novel oncogene confers reoviral replication

through Ras and p38 signaling pathway. ***Cancer Gene Ther*** **2010**, *17*, 307-314.
440. Shmulevitz, M.; Marcato, P.; Lee, P.W. Activated Ras signaling significantly enhances reovirus replication and spread. ***Cancer Gene Ther*** **2010**, *17*, 69-70.
441. Song, L.; Ohnuma, T.; Gelman, I.H.; Holland, J.F. Reovirus infection of cancer cells is not due to activated Ras pathway. ***Cancer Gene Ther*** **2009**, *16*, 382.
442. Smakman, N.; van den Wollenberg, D.J.; Borel Rinkes, I.H.; Hoeben, R.C.; Kranenburg, O. Sensitization to apoptosis underlies KrasD12-dependent oncolysis of murine C26 colorectal carcinoma cells by reovirus T3D. ***J Virol*** **2005**, *79*, 14981-14985.
443. Smakman, N.; van den Wollenberg, D.J.; Elias, S.G.; Sasazuki, T.; Shirasawa, S.***, et al.*** KRAS(D13) Promotes apoptosis of human colorectal tumor cells by ReovirusT3D and oxaliplatin but not by tumor necrosis factor-related apoptosis-inducing ligand. ***Cancer Res*** **2006**, *66*, 5403-5408.
444. Alain, T.; Kim, T.S.; Lun, X.; Liacini, A.; Schiff, L.A.***, et al.*** Proteolytic disassembly is a critical determinant for reovirus oncolysis. ***Mol Ther*** **2007**, *15*, 1512-1521.
445. Kim, M.; Egan, C.; Alain, T.; Urbanski, S.J.; Lee, P.W.***, et al.*** Acquired resistance to reoviral oncolysis in Ras-transformed fibrosarcoma cells. ***Oncogene*** **2007**, *26*, 4124-4134.
446. Marcato, P.; Shmulevitz, M.; Pan, D.; Stoltz, D.; Lee, P.W. Ras transformation mediates reovirus oncolysis by enhancing virus uncoating, particle infectivity, and apoptosis-dependent release. ***Mol Ther*** **2007**, *15*, 1522-1530.
447. Sheng, J.; Organ, E.L.; Hao, C.; Wells, K.S.; Ruley, H.E.***, et al.*** Mutations in the IGF-II pathway that confer resistance to lytic reovirus infection. ***BMC cell biology*** **2004**, *5*, 32.
448. Norman, K.L.; Hirasawa, K.; Yang, A.D.; Shields, M.A.; Lee, P.W. Reovirus oncolysis: the Ras/RalGEF/p38 pathway dictates host cell permissiveness to reovirus infection. ***Proc Natl Acad Sci U S A*** **2004**, *101*, 11099-11104.
449. Yang, W.Q.; Lun, X.; Palmer, C.A.; Wilcox, M.E.; Muzik, H.***, et al.*** Efficacy and safety evaluation of human reovirus type 3 in immunocompetent animals: racine and nonhuman primates. ***Clin Cancer Res*** **2004**, *10*, 8561-8576.
450. Alkassar, M.; Gartner, B.; Roemer, K.; Graesser, F.; Rommelaere, J.***, et al.*** The combined effects of oncolytic reovirus plus Newcastle disease virus and reovirus plus parvovirus on U87 and U373 cells in vitro and in vivo. ***J Neurooncol*** **2011**, *104*, 715-727.
451. van den Hengel, S.K.; Balvers, R.K.; Dautzenberg, I.J.; van den Wollenberg, D.J.; Kloezeman, J.J.***, et al.*** Heterogeneous reovirus susceptibility in human glioblastoma stem-like cell cultures. ***Cancer Gene Ther*** **2013**, *20*, 507-513.
452. Norman, K.L.; Coffey, M.C.; Hirasawa, K.; Demetrick, D.J.; Nishikawa, S.G.***, et al.*** Reovirus oncolysis of human breast cancer. ***Hum Gene Ther*** **2002**, *13*, 641-652.
453. Hata, Y.; Etoh, T.; Inomata, M.; Shiraishi, N.; Nishizono, A.***, et al.*** Efficacy of oncolytic reovirus against human breast cancer cells. ***Oncol Rep*** **2008**, *19*, 1395-1398.
454. Marcato, P.; Dean, C.A.; Giacomantonio, C.A.; Lee, P.W.K. Oncolytic reovirus effectively targets breast cancer stem cells. ***Mol Ther*** **2009**, *17*, 972-979.
455. Alain, T.; Wong, J.F.; Endersby, R.; Urbanski, S.J.; Lee, P.W.***, et al.*** Reovirus decreases azoxymethane-induced aberrant crypt foci and colon cancer in a rodent model. ***Cancer Gene Ther*** **2007**, *14*, 867-872.
456. van Houdt, W.J.; Smakman, N.; van den Wollenberg, D.J.; Emmink, B.L.; Veenendaal, L.M.***, et al.*** Transient infection of freshly isolated human colorectal tumor cells by reovirus T3D intermediate subviral particles. ***Cancer Gene Ther*** **2008**, *15*, 284-292.
457. Adair, R.A.; Scott, K.J.; Fraser, S.; Errington-Mais, F.; Pandha, H.***, et al.*** Cytotoxic and immune-mediated killing of human colorectal cancer by reovirus-loaded blood and liver mononuclear cells. ***Int J Cancer*** **2013**, *132*, 2327-2338.
458. Maitra, R.; Seetharam, R.; Tesfa, L.; Augustine, T.A.; Klampfer, L.***, et al.*** Oncolytic reovirus preferentially induces apoptosis in KRAS mutant colorectal cancer cells, and synergizes with irinotecan. ***Oncotarget*** **2014**, *5*, 2807-2819.
459. Errington, F.; White, C.L.; Twigger, K.R.; Rose, A.; Scott, K.***, et al.*** Inflammatory tumour cell killing by oncolytic reovirus for the treatment of melanoma. ***Gene Ther*** **2008**, *15*, 1257-1270.
460. Prestwich, R.J.; Errington, F.; Ilett, E.J.; Morgan, R.S.; Scott, K.J.***, et al.*** Tumor infection by oncolytic reovirus primes adaptive antitumor immunity. ***Clin Cancer Res*** **2008**, *14*, 7358-7366.
461. Pandha, H.S.; Heinemann, L.; Simpson, G.R.; Melcher, A.; Prestwich, R.***, et al.*** Synergistic effects of oncolytic reovirus and cisplatin chemotherapy in murine malignant melanoma. ***Clin Cancer Res*** **2009**, *15*, 6158-6166.
462. Heinemann, L.; Simpson, G.R.; Annels, N.E.; Vile, R.; Melcher, A.***, et al.*** The effect of cell cycle synchronization

on tumor sensitivity to reovirus oncolysis. ***Mol Ther*** **2010**, *18*, 2085-2093.
463. Kottke, T.; Chester, J.; Ilett, E.; Thompson, J.; Diaz, R.***, et al.*** Precise scheduling of chemotherapy primes VEGF-producing tumors for successful systemic oncolytic virotherapy. ***Mol Ther*** **2011**, *19*, 1802-1812.
464. Steele, L.; Errington, F.; Prestwich, R.; Ilett, E.; Harrington, K.***, et al.*** Pro-inflammatory cytokine/chemokine production by reovirus treated melanoma cells is PKR/NF-kappaB mediated and supports innate and adaptive anti-tumour immune priming. ***Mol Cancer*** **2011**, *10*, 20.
465. Hirano, S.; Etoh, T.; Okunaga, R.; Shibata, K.; Ohta, M.***, et al.*** Reovirus inhibits the peritoneal dissemination of pancreatic cancer cells in an immunocompetent animal model. ***Oncol Rep*** **2009**, *21*, 1381-1384.
466. Carew, J.S.; Espitia, C.M.; Zhao, W.; Kelly, K.R.; Coffey, M.***, et al.*** Reolysin is a novel reovirus-based agent that induces endoplasmic reticular stress-mediated apoptosis in pancreatic cancer. ***Cell death & disease*** **2013**, *4*, e728.
467. Alain, T.; Muzik, H.; Otsuka, S.; Chan, S.; Magliocco, T.***, et al.*** Susceptibility of mantle cell lymphomas to reovirus oncolysis. ***Leukemia research*** **2010**, *34*, 100-108.
468. Cho, I.R.; Koh, S.S.; Min, H.J.; Park, E.H.; Srisuttee, R.***, et al.*** Reovirus infection induces apoptosis of TRAIL-resistant gastric cancer cells by down-regulation of Akt activation. ***Int J Oncol*** **2010**, *36*, 1023-1030.
469. Kawaguchi, K.; Etoh, T.; Suzuki, K.; Mitui, M.T.; Nishizono, A.***, et al.*** Efficacy of oncolytic reovirus against human gastric cancer with peritoneal metastasis in experimental animal model. ***Int J Oncol*** **2010**, *37*, 1433-1438.
470. Thirukkumaran, C.M.; Nodwell, M.J.; Hirasawa, K.; Shi, Z.Q.; Diaz, R.***, et al.*** Oncolytic viral therapy for prostate cancer: efficacy of reovirus as a biological therapeutic. ***Cancer Res*** **2010**, *70*, 2435-2444.
471. Gujar, S.A.; Pan, D.A.; Marcato, P.; Garant, K.A.; Lee, P.W. Oncolytic virus-initiated protective immunity against prostate cancer. ***Mol Ther*** **2011**, *19*, 797-804.
472. Heinemann, L.; Simpson, G.R.; Boxall, A.; Kottke, T.; Relph, K.L.***, et al.*** Synergistic effects of oncolytic reovirus and docetaxel chemotherapy in prostate cancer. ***BMC Cancer*** **2011**, *11*, 221.
473. Gupta-Saraf, P.; Miller, C.L. HIF-1alpha downregulation and apoptosis in hypoxic prostate tumor cells infected with oncolytic mammalian orthoreovirus. ***Oncotarget*** **2014**, *5*, 561-574.
474. Hingorani, P.; Zhang, W.; Lin, J.; Liu, L.; Guha, C.***, et al.*** Systemic administration of reovirus (Reolysin) inhibits growth of human sarcoma xenografts. ***Cancer*** **2011**, *117*, 1764-1774.
475. Hall, K.; Scott, K.J.; Rose, A.; Desborough, M.; Harrington, K.***, et al.*** Reovirus-mediated cytotoxicity and enhancement of innate immune responses against acute myeloid leukemia. ***BioResearch open access*** **2012**, *1*, 3-15.
476. Kelly, K.R.; Espitia, C.M.; Mahalingam, D.; Oyajobi, B.O.; Coffey, M.***, et al.*** Reovirus therapy stimulates endoplasmic reticular stress, NOXA induction, and augments bortezomib-mediated apoptosis in multiple myeloma. ***Oncogene*** **2012**, *31*, 3023-3038.
477. Thirukkumaran, C.M.; Shi, Z.Q.; Luider, J.; Kopciuk, K.; Gao, H.***, et al.*** Reovirus as a viable therapeutic option for the treatment of multiple myeloma. ***Clin Cancer Res*** **2012**, *18*, 4962-4972.
478. Thirukkumaran, C.M.; Shi, Z.Q.; Luider, J.; Kopciuk, K.; Gao, H.***, et al.*** Reovirus modulates autophagy during oncolysis of multiple myeloma. ***Autophagy*** **2013**, *9*, 413-414.
479. Thirukkumaran, C.M.; Shi, Z.Q.; Luider, J.; Kopciuk, K.; Bahlis, N.***, et al.*** Reovirus as a successful ex vivo purging modality for multiple myeloma. ***Bone marrow transplantation*** **2014**, *49*, 80-86.
480. Twigger, K.; Roulstone, V.; Kyula, J.; Karapanagiotou, E.M.; Syrigos, K.N.***, et al.*** Reovirus exerts potent oncolytic effects in head and neck cancer cell lines that are independent of signalling in the EGFR pathway. ***BMC Cancer*** **2012**, *12*, 368.
481. Roulstone, V.; Twigger, K.; Zaidi, S.; Pencavel, T.; Kyula, J.N.***, et al.*** Synergistic cytotoxicity of oncolytic reovirus in combination with cisplatin-paclitaxel doublet chemotherapy. ***Gene Ther*** **2013**, *20*, 521-528.
482. Jebar, A.; Bentham, M.; Errington, F.; Scott, K.; Peckham-Cooper, A.***, et al.*** Combined anti-viral and anti-tumor therapy for virus associated liver cancer. ***8th International Conference on Oncolytic Virus Therapeutics*** **2014**.
483. Gujar, S.; Dielschneider, R.; Clements, D.; Helson, E.; Shmulevitz, M.***, et al.*** Multifaceted therapeutic targeting of ovarian peritoneal carcinomatosis through virus-induced immunomodulation. ***Mol Ther*** **2013**, *21*, 338-347.
484. Maitra, R.; Ghalib, M.H.; Goel, S. Reovirus: a targeted therapeutic--progress and potential. ***Mol Cancer Res*** **2012**, *10*, 1514-1525.
485. Prestwich, R.J.; Errington, F.; Steele, L.P.; Ilett, E.J.; Morgan, R.S.***, et al.*** Reciprocal human dendritic cell-natural killer cell interactions induce antitumor activity following tumor cell infection by oncolytic reovirus. ***J Immunol*** **2009**, *183*, 4312-4321.
486. Prestwich, R.J.; Ilett, E.J.; Errington, F.; Diaz, R.M.; Steele, L.P.***, et al.*** Immune-mediated antitumor activity of

reovirus is required for therapy and is independent of direct viral oncolysis and replication. ***Clin Cancer Res*** **2009**, *15*, 4374-4381.
487. Gujar, S.A.; Marcato, P.; Pan, D.; Lee, P.W. Reovirus virotherapy overrides tumor antigen presentation evasion and promotes protective antitumor immunity. ***Mol Cancer Ther*** **2010**, *9*, 2924-2933.
488. van den Wollenberg, D.J.; van den Hengel, S.K.; Dautzenberg, I.J.; Cramer, S.J.; Kranenburg, O.***, et al.*** A strategy for genetic modification of the spike-encoding segment of human reovirus T3D for reovirus targeting. ***Gene Ther*** **2008**, *15*, 1567-1578.
489. van den Wollenberg, D.J.; Dautzenberg, I.J.; van den Hengel, S.K.; Cramer, S.J.; de Groot, R.J.***, et al.*** Isolation of reovirus T3D mutants capable of infecting human tumor cells independent of junction adhesion molecule-A. ***PLoS One*** **2012**, *7*, e48064.
490. Bergeron, J.; Mabrouk, T.; Garzon, S.; Lemay, G. Characterization of the thermosensitive ts453 reovirus mutant: increased dsRNA binding of sigma 3 protein correlates with interferon resistance. ***Virology*** **1998**, *246*, 199-210.
491. Chappell, J.D.; Gunn, V.L.; Wetzel, J.D.; Baer, G.S.; Dermody, T.S. Mutations in type 3 reovirus that determine binding to sialic acid are contained in the fibrous tail domain of viral attachment protein sigma1. ***J Virol*** **1997**, *71*, 1834-1841.
492. Rudd, P.; Lemay, G. Correlation between interferon sensitivity of reovirus isolates and ability to discriminate between normal and Ras-transformed cells. ***J Gen Virol*** **2005**, *86*, 1489-1497.
493. Shmulevitz, M.; Gujar, S.A.; Ahn, D.G.; Mohamed, A.; Lee, P.W. Reovirus variants with mutations in genome segments S1 and L2 exhibit enhanced virion infectivity and superior oncolysis. ***J Virol*** **2012**, *86*, 7403-7413.
494. Van den Wollenberg, D.J.; Dautzenberg, I.J.; Van den Hengel, S.K.; Ros, W.; Nadif, S.***, et al.*** Development of replication-competent, expanded-tropism oncolytic reovirus carrying a heterologous transgene. ***8th International Conference on Oncolytic Virus Therapeutics*** **2014**.
495. Harrington, K.J.; Vile, R.G.; Melcher, A.; Chester, J.; Pandha, H.S. Clinical trials with oncolytic reovirus: moving beyond phase I into combinations with standard therapeutics. ***Cytokine Growth Factor Rev*** **2010**, *21*, 91-98.
496. Solly, S.K.; Trajcevski, S.; Frisen, C.; Holzer, G.W.; Nelson, E.***, et al.*** Replicative retroviral vectors for cancer gene therapy. ***Cancer Gene Ther*** **2003**, *10*, 30-39.
497. Logg, C.R.; Logg, A.; Tai, C.K.; Cannon, P.M.; Kasahara, N. Genomic stability of murine leukemia viruses containing insertions at the Env-3′ untranslated region boundary. ***J Virol*** **2001**, *75*, 6989-6998.
498. Jespersen, T.; Duch, M.; Carrasco, M.L.; Warming, S.; Pedersen, F.S. Expression of heterologous genes from an IRES translational cassette in replication competent murine leukemia virus vectors. ***Gene*** **1999**, *239*, 227-235.
499. Hlavaty, J.; Jandl, G.; Liszt, M.; Petznek, H.; Konig-Schuster, M.***, et al.*** Comparative evaluation of preclinical in vivo models for the assessment of replicating retroviral vectors for the treatment of glioblastoma. ***J Neurooncol*** **2011**, *102*, 59-69.
500. Perez, O.D.; Logg, C.R.; Hiraoka, K.; Diago, O.; Burnett, R.***, et al.*** Design and selection of Toca 511 for clinical use: modified retroviral replicating vector with improved stability and gene expression. ***Mol Ther*** **2012**, *20*, 1689-1698.
501. Kawasaki, Y.; Tamamoto, A.; Takagi-Kimura, M.; Maeyama, Y.; Yamaoka, N.***, et al.*** Replication-competent retrovirus vector-mediated prodrug activator gene therapy in experimental models of human malignant mesothelioma. ***Cancer Gene Ther*** **2011**, *18*, 571-578.
502. Ostertag, D.; Amundson, K.K.; Lopez Espinoza, F.; Martin, B.; Buckley, T.***, et al.*** Brain tumor eradication and prolonged survival from intratumoral conversion of 5-fluorocytosine to 5-fluorouracil using a nonlytic retroviral replicating vector. ***Neuro-oncology*** **2012**, *14*, 145-159.
503. Huang, T.T.; Hlavaty, J.; Ostertag, D.; Espinoza, F.L.; Martin, B.***, et al.*** Toca 511 gene transfer and 5-fluorocytosine in combination with temozolomide demonstrates synergistic therapeutic efficacy in a temozolomide-sensitive glioblastoma model. ***Cancer Gene Ther*** **2013**, *20*, 544-551.
504. Yin, D.; Zhai, Y.; Gruber, H.E.; Ibanez, C.E.; Robbins, J.M.***, et al.*** Convection-enhanced delivery improves distribution and efficacy of tumor-selective retroviral replicating vectors in a rodent brain tumor model. ***Cancer Gene Ther*** **2013**, *20*, 336-341.
505. Jolly, D.J.; Ibanez, C.E.; Ostertag, D.; Robbins, J.M.; Kasahara, N.***, et al.*** Toca 511: update on the use of Retroviral Replicating Vector as an anti-tumor agent in preclinical and clinical trials. ***8th International Conference on Oncolytic Virus Therapeutics*** **2014**.
506. Buijs, P.; van Nieuwkoop, S.; Van Eijck, C.; Fouchier, R.; Van den Hoogen, B. Recombinant oncolytic Newcastle disease virus for treatment of pancreatic adenocarcinoma. ***8th International Conference on Oncolytic Virus***

Therapeutics **2014**.
507. Varghese, S.; Newsome, J.T.; Rabkin, S.D.; McGeagh, K.; Mahoney, D.***, et al.*** Preclinical safety evaluation of G207, a replication-competent herpes simplex virus type 1, inoculated intraprostatically in mice and nonhuman primates. ***Hum Gene Ther*** **2001**, *12*, 999-1010.
508. Wolfe, D.; Niranjan, A.; Trichel, A.; Wiley, C.; Ozuer, A.***, et al.*** Safety and biodistribution studies of an HSV multigene vector following intracranial delivery to non-human primates. ***Gene Ther*** **2004**, *11*, 1675-1684.
509. Markert, J.M.; Medlock, M.D.; Rabkin, S.D.; Gillespie, G.Y.; Todo, T.***, et al.*** Conditionally replicating herpes simplex virus mutant, G207 for the treatment of malignant glioma: results of a phase I trial. ***Gene Ther*** **2000**, *7*, 867-874.
510. Markert, J.M.; Liechty, P.G.; Wang, W.; Gaston, S.; Braz, E.***, et al.*** Phase Ib trial of mutant herpes simplex virus G207 inoculated pre-and post-tumor resection for recurrent GBM. ***Mol Ther*** **2009**, *17*, 199-207.
511. Hu, J.C.; Coffin, R.S.; Davis, C.J.; Graham, N.J.; Groves, N.***, et al.*** A phase I study of OncoVEXGM-CSF, a second-generation oncolytic herpes simplex virus expressing granulocyte macrophage colony-stimulating factor. ***Clin Cancer Res*** **2006**, *12*, 6737-6747.
512. Senzer, N.N.; Kaufman, H.L.; Amatruda, T.; Nemunaitis, M.; Reid, T.***, et al.*** Phase II clinical trial of a granulocyte-macrophage colony-stimulating factor-encoding, second-generation oncolytic herpesvirus in patients with unresectable metastatic melanoma. ***J Clin Oncol*** **2009**, *27*, 5763-5771.
513. Harrington, K.J.; Hingorani, M.; Tanay, M.A.; Hickey, J.; Bhide, S.A.***, et al.*** Phase I/II study of oncolytic HSV GM-CSF in combination with radiotherapy and cisplatin in untreated stage III/IV squamous cell cancer of the head and neck. ***Clin Cancer Res*** **2010**, *16*, 4005-4015.
514. Geevarghese, S.K.; Geller, D.A.; de Haan, H.A.; Horer, M.; Knoll, A.E.***, et al.*** Phase I/II study of oncolytic herpes simplex virus NV1020 in patients with extensively pretreated refractory colorectal cancer metastatic to the liver. ***Hum Gene Ther*** **2010**, *21*, 1119-1128.
515. Umene, K. Mechanism and application of genetic recombination in herpesviruses. ***Reviews in medical virology*** **1999**, *9*, 171-182.
516. Cassady, K.A.; Gross, M.; Roizman, B. The second-site mutation in the herpes simplex virus recombinants lacking the gamma134.5 genes precludes shutoff of protein synthesis by blocking the phosphorylation of eIF-2alpha. ***J Virol*** **1998**, *72*, 7005-7011.
517. Chiocca, E.A.; Smith, K.M.; McKinney, B.; Palmer, C.A.; Rosenfeld, S.***, et al.*** A phase I trial of Ad.hIFN-beta gene therapy for glioma. ***Mol Ther*** **2008**, *16*, 618-626.
518. Keedy, V.; Wang, W.; Schiller, J.; Chada, S.; Slovis, B.***, et al.*** Phase I study of adenovirus p53 administered by bronchoalveolar lavage in patients with bronchioloalveolar cell lung carcinoma: ECOG 6597. ***J Clin Oncol*** **2008**, *26*, 4166-4171.
519. Tian, G.; Liu, J.; Zhou, J.S.; Chen, W. Multiple hepatic arterial injections of recombinant adenovirus p53 and 5-fluorouracil after transcatheter arterial chemoembolization for unresectable hepatocellular carcinoma: a pilot phase II trial. ***Anticancer Drugs*** **2009**, *20*, 389-395.
520. Xu, F.; Li, S.; Li, X.L.; Guo, Y.; Zou, B.Y.***, et al.*** Phase I and biodistribution study of recombinant adenovirus vector-mediated herpes simplex virus thymidine kinase gene and ganciclovir administration in patients with head and neck cancer and other malignant tumors. ***Cancer Gene Ther*** **2009**, *16*, 723-730.
521. Page, J.G.; Tian, B.; Schweikart, K.; Tomaszewski, J.; Harris, R.***, et al.*** Identifying the safety profile of a novel infectivity-enhanced conditionally replicative adenovirus, Ad5-delta24-RGD, in anticipation of a phase I trial for recurrent ovarian cancer. ***American journal of obstetrics and gynecology*** **2007**, *196*, 389 e381-389; discussion 389 e389-310.
522. Griscelli, F.; Opolon, P.; Saulnier, P.; Mami-Chouaib, F.; Gautier, E.***, et al.*** Recombinant adenovirus shedding after intratumoral gene transfer in lung cancer patients. ***Gene Ther*** **2003**, *10*, 386-395.
523. Schenk-Braat, E.A.; van Mierlo, M.M.; Wagemaker, G.; Bangma, C.H.; Kaptein, L.C. An inventory of shedding data from clinical gene therapy trials. ***J Gene Med*** **2007**, *9*, 910-921.
524. Robinson, C.M.; Rajaiya, J.; Walsh, M.P.; Seto, D.; Dyer, D.W.***, et al.*** Computational analysis of human adenovirus type 22 provides evidence for recombination among species D human adenoviruses in the penton base gene. ***J Virol*** **2009**, *83*, 8980-8985.
525. Walsh, M.P.; Chintakuntlawar, A.; Robinson, C.M.; Madisch, I.; Harrach, B.***, et al.*** Evidence of molecular evolution driven by recombination events influencing tropism in a novel human adenovirus that causes epidemic keratoconjunctivitis. ***PLoS One*** **2009**, *4*, e5635.
526. Singh, G.; Robinson, C.M.; Dehghan, S.; Jones, M.S.; Dyer, D.W.***, et al.*** Homologous recombination in E3 genes

of human adenovirus species D. ***J Virol*** **2013**, ***87***, 12481-12488.
527. Griffin, D.E.; Pan, C.H. Measles: old vaccines, new vaccines. ***Current topics in microbiology and immunology*** **2009**, ***330***, 191-212.
528. LeBlanc, A.K.; Naik, S.; Galyon, G.D.; Jenks, N.; Steele, M.***, et al.*** Safety studies on intravenous administration of oncolytic recombinant vesicular stomatitis virus in purpose-bred beagle dogs. ***Human gene therapy. Clinical development*** **2013**, ***24***, 174-181.
529. Jenks, N.; Myers, R.; Greiner, S.M.; Thompson, J.; Mader, E.K.***, et al.*** Safety studies on intrahepatic or intratumoral injection of oncolytic vesicular stomatitis virus expressing interferon-beta in rodents and nonhuman primates. ***Hum Gene Ther*** **2010**, ***21***, 451-462.
530. Naik, S.; LeBlanc, A.K.; Galyon, G.D.; Frazier, S.; Jenks, N.***, et al.*** Early findings from a comparative oncology study evaluating systemic VSV therapy in client owned dogs with spontaneous hematologic malignancies. ***8th International Conference on Oncolytic Virus Therapeutics*** **2014**.
531. Stanifer, M.L.; Cureton, D.K.; Whelan, S.P. A recombinant vesicular stomatitis virus bearing a lethal mutation in the glycoprotein gene uncovers a second site suppressor that restores fusion. ***J Virol*** **2011**, ***85***, 8105-8115.
532. Harouaka, D.; Wertz, G.W. Second-site mutations selected in transcriptional regulatory sequences compensate for engineered mutations in the vesicular stomatitis virus nucleocapsid protein. ***J Virol*** **2012**, ***86***, 11266-11275.
533. Quinones-Kochs, M.I.; Schnell, M.J.; Buonocore, L.; Rose, J.K. Mechanisms of loss of foreign gene expression in recombinant vesicular stomatitis viruses. ***Virology*** **2001**, ***287***, 427-435.
534. Gao, Y.; Whitaker-Dowling, P.; Watkins, S.C.; Griffin, J.A.; Bergman, I. Rapid adaptation of a recombinant vesicular stomatitis virus to a targeted cell line. ***J Virol*** **2006**, ***80***, 8603-8612.
535. Dinh, P.X.; Panda, D.; Das, P.B.; Das, S.C.; Das, A.***, et al.*** A single amino acid change resulting in loss of fluorescence of eGFP in a viral fusion protein confers fitness and growth advantage to the recombinant vesicular stomatitis virus. ***Virology*** **2012**, ***432***, 460-469.
536. Dobrikova, E.Y.; Goetz, C.; Walters, R.W.; Lawson, S.K.; Peggins, J.O.***, et al.*** Attenuation of neurovirulence, biodistribution, and shedding of a poliovirus:rhinovirus chimera after intrathalamic inoculation in Macaca fascicularis. ***J Virol*** **2012**, ***86***, 2750-2759.
537. Jahan, N.; Wimmer, E.; Mueller, S. A host-specific, temperature-sensitive translation defect determines the attenuation phenotype of a human rhinovirus/poliovirus chimera, PV1(RIPO). ***J Virol*** **2011**, ***85***, 7225-7235.
538. Fedorko, D.P.; Preuss, J.C.; Fahle, G.A.; Li, L.; Fischer, S.H.***, et al.*** Comparison of methods for detection of vaccinia virus in patient specimens. ***J Clin Microbiol*** **2005**, ***43***, 4602-4606.
539. Cono, J.; Casey, C.G.; Bell, D.M.; Centers for Disease, C.; Prevention. Smallpox vaccination and adverse reactions. Guidance for clinicians. ***MMWR. Recommendations and reports : Morbidity and mortality weekly report. Recommendations and reports / Centers for Disease Control*** **2003**, ***52***, 1-28.
540. Qin, L.; Evans, D.H. Genome scale patterns of recombination between coinfecting vaccinia viruses. ***J Virol*** **2014**, ***88***, 5277-5286.
541. Chakrabarty, R.; Tran, H.; Fortin, Y.; Yu, Z.; Shen, S.H.***, et al.*** Evaluation of homogeneity and genetic stability of REOLYSIN (pelareorep) by complete genome sequencing of reovirus after large scale production. ***Appl Microbiol Biotechnol*** **2014**, ***98***, 1763-1770.

chapter 6

Appendices

Dankwoord
About the Author
List of Publications
Erasmus MC PhD Portfolio

Dankwoord

Tot zover het wetenschappelijke deel. Nu is het tijd om iedereen te bedanken zonder wie dit proefschrift nooit tot stand was gekomen. Zoals vaker gezegd en geschreven: het meest gelezen onderdeel van een boekje. Mocht U ondanks aandachtig lezen Uw eigen naam missen, vergeef me, dat is dan mijn tekortkoming. Ook U ben ik uiteraard dankbaar!

Mijn heelkundige promotor, professor van Eijck. Ik had nooit gedacht dat een gemeenschappelijke kennis zou leiden tot het boekje wat hier voor U ligt. Vanaf het eerste kennismakings-/sollicitatiegesprek bent U altijd de stille motor geweest achter mijn promotietraject. Leidend met een losse hand, maar altijd op de hoogte en doortastend wanneer nodig. Dank voor Uw vertrouwen en stimulatie om dit project tot een goed einde te brengen. Ik hoop U in de toekomst als heelkundige collega nog vaak te kunnen spreken.

Mijn virologische promotor, professor Fouchier, beste Ron. Dank voor alle mogelijkheden die je me hebt gegeven op de afdeling Viroscience. Ondanks drukte met de zoveelste (vogel)griepepidemie of dodelijke coronavirussen was er altijd genoeg tijd en ruimte om mijn project aandacht te geven. Je hebt een fantastische groep mensen om je heen verzameld, wat mij betreft het kenmerk van een echte leider.

Mijn copromotor, Dr. van den Hoogen, beste Bernadette. Vanaf begin tot eind: jij was er voor me als ik hulp nodig had. En of het nu ging om basale wetenschap, het schrijven van lappen tekst voor manuscripten en beursaanvragen, of het begeleiden van samenwerkingen, jij kunt het allemaal. Liefst in de kleine uurtjes van de dag of in het weekend, want ook thuis is genoeg te doen. Zonder jou was dit project nooit zover gekomen, en daarvoor ben ik je ontzettend dankbaar. Ik hoop dat het gaat lukken om verder te kunnen gaan met 'ons kindje' om er een volwassen onderzoekslijn van te maken. Wanneer is er weer een lab-diner bij jullie thuis…?

Leden van de leescommissie, prof.dr. C.M.F. Dirven, prof.dr. L.J. Hofland en prof.dr. R.C. Hoeben, dank voor het beoordelen van mijn proefschrift.

Beste (oud)collega's van de afdeling Viroscience, bedankt voor jullie hulp en gezelligheid over de jaren. Wie denkt dat wetenschappers saai zijn, heeft nog nooit een SinterKerst feest of de bijna maandelijkse borrels meegemaakt op de 17e verdieping. Marina, Jonneke en Stefan v N., bedankt voor het begeleiden, helpen en onderwijzen van deze simpele jongen op het gebied van laboratoriumtechnieken. Kamergenoten Martin, Sander, Kim, Marine en Sasha: ik had geen betere buren kunnen wensen. Overige collega's van de Flu-groep, bedankt voor jullie hulp door de jaren heen. Iedere vrijdagmiddag frituurlunch was altijd haute cuisine! Robert, jij bent waarschijnlijk de meest onderschatte collega van de afdeling, altijd (vroeg) paraat om alles in goede banen te leiden, dank voor je hulp en ondersteuning bij duizend en één dingen. Met name de Goody-Goody box en Surinaamse broodjes waren altijd goed voor de arbeidsproductiviteit. Geert, bedankt voor je hulp met de proeven in het EDC; je verhalen over vroege trektochten door de Duitse velden werkten altijd inspirerend.
Dames van het secretariaat, Simone, Loubna, Anouk, Maria, bedankt voor jullie ondersteuning door de jaren heen. Zonder jullie hulp en adviezen was ik waarschijnlijk niet ver gekomen.

Lieve (oud)collega's van de afdeling Heelkunde, bedankt voor de gezelligheid van de afgelopen jaren. Ik vond het altijd een leuk moment om op dinsdag even de week te breken met onze onderzoekerslunch. Stephanie, bedankt voor het ondersteunen van de 17e verdieping met jullie expertise van de celletjes. Mensen in de kelder, houd vol, er is licht aan het einde van de tunnel! Mensen in de Z-flat, geen idee wat jullie daar de hele dag doen, maar ga zo door! Ik zal altijd met trots refereren aan mijn periode in Rotterdam, met name de jaarlijkse ski-reis en maandelijkse regioborrel zijn uniek te noemen.
Dames van het secretariaat Heelkunde, bedankt voor jullie ondersteuning. Conny, bedankt voor je luisterende en ontnuchterende oor, de snoepjes gingen er altijd goed in. Carola, bedankt voor het begeleiden van de laatste loodjes richting het einde.

Timo en Gerben, en alle promovendi en medewerkers van LECO. Bedankt dat jullie mij op weg hebben geholpen in de chirurgische oncologie.

Heren en dames van het EDC, dank dat jullie mij hebben geholpen door alles altijd tot in de puntjes voorbereid te hebben. Vincent, er is geen betere prikkert dan jij. Dennis & Dennis, dank voor jullie hulp met het onderhouden van mijn proeven, met name in de weekenden.

People from UMD, professor Perez, dear Daniel. Thank you for enabling me to perform some cool experiments in your lab. Preparation was key (read hell), but we managed in the end anyway. Ade(bimpe), Troy and Matthew, thank you for teaching and escorting me around the lab and beyond. I am still on the lookout for a Chipotle and Sardi's in Rotterdam.

People from Georgetown, professor Wellstein, thank you for enabling me to use your expertise regarding tumor models. Eveline en Marianne, bedankt voor jullie hulp bij het regelen van ons zeer speciale transport.

IJsvogels, ondanks dat ik inmiddels het nest verlaten heb, zal ik mijzelf altijd blijven beschouwen als een Capellenaar. Dr. Dawson, dank voor Uw begeleiding gedurende mijn tijd in het IJsselland, ik heb het gevoel dat ik U ook nog voor iets anders moet bedanken. Michiel, bedankt dat je voor mij een awesome kaft hebt ontworpen.

Nieuwe collega's uit het Elisabeth-TweeSteden ziekenhuis, bedankt voor jullie warme Tilburgse welkom. Opleiders dr. Vriens en dr. Ibelings, bedankt voor het in mij gestelde vertrouwen. Ik verheug mij op de toekomst met jullie allemaal.

Roy en Manja met kids, Ward en Marloes met kid en Bastiaan en Anniek met kids. Alsof er niet genoeg clichés in het voorgaande stuk zitten: we zien elkaar te weinig. Ik hoop dan ook dat dat vanaf nu, ondanks de afstand tot sommige van jullie, gaat toenemen om alle ervaringen op het gebied van poepluiers en snottebellen te kunnen uitwisselen. Peek, ik hoop dat je in de toekomst nog veel bios-afspraken niet vergeet.

Lieve schoonfamilie, Geert, Goof, Christianne, Gerard, Cockie en Kees. Jullie hebben de koude kant toch een warm gevoel gegeven. Bedankt voor de nimmer aflatende gastvrijheid en gezelligheid door de jaren heen.

Paranimf Stefan, nooit te beroerd om mee te gaan lunchen op elke dag behalve dinsdag. Leuk dat je vandaag naast me staat. Ik hoop dat we nog vaak kunnen ontspannen in de bios of op de tribune van ons cluppie.

Paranimf Manon, maar uiteraard ook lieve zus. Met bewondering heb ik vaak een voorbeeld aan jou proberen te nemen. Vanzelfsprekend sta jij vandaag ook naast mij, nadat eerder de rollen omgedraaid waren. Ik wil je bedanken voor de ontelbare dingen die je voor mij gedaan hebt, in binnen- en buitenland. Je bent de beste zus ever!

Lieve papa en mama, mijn Limburgse voorouders. Zonder jullie liefde en steun was ik nooit kunnen worden wie ik nu ben. Bedankt voor jullie zorg, vertrouwen, raad en daad. Ik hoop dat jullie trots op mij zijn.

Lieve Cox, project van mijn leven. Als geen ander begrijp jij mij. In vijf jaar tijd hebben we samen een fantastisch leven opgebouwd. Binnenkort begint ons grootste avontuur tot nu toe en ik weet zeker dat het heel leuk gaat worden. Ik hou van jou!

About the author

Pascal Buijs was born on Sunday June 17th, 1984 in Landgraaf, the Netherlands. In Kerkrade he attended the Catholic Gymnasium Rolduc, from which he graduated 'with honors' in 2002. From 2002 to 2008 he completed his medical training 'with honors' at the Maastricht University, of which he spent his last year senior internship at the department of Surgery in the Atrium Medical Centre in Heerlen (supervisor: dr. Jan Siebenga). After his graduation, he worked as a surgical non-training resident (ANIOS) in the TweeSteden Hospital in Tilburg (supervisor: dr. Maaike Ibelings). In 2009, he started as a PhD-student at the Erasmus Medical Centre's departments of Surgery (promotor: prof.dr. Casper van Eijck) and Viroscience (promotor: prof.dr. Ron Fouchier and co-promotor dr. Bernadette van den Hoogen) in Rotterdam, performing the research that is presented in this thesis on oncolytic NDV as treatment for pancreatic adenocarcinoma. As part of his PhD-training, he spent a 3 months' period at the department of Veterinary Medicine at the University of Maryland, USA (supervisor: prof.dr. Daniel Perez) supported by a personal grant of the Lisa Waller Hayes foundation. Following his PhD training, he worked as a surgical ANIOS in the IJsselland Hospital in Capelle aan den IJssel (supervisor: dr. Imro Dawson). July 2015 he started his training as a surgical resident (AIOS) at the Elisabeth-TweeSteden Hospital in Tilburg (supervisor: dr. Patrick Vriens), which will be continued in the Radboud University Hospital in Nijmegen in 2018 (supervisor: dr. Bas Verhoeven). Pascal currently resides in Rotterdam with his girlfriend Cox van de Weg and their two cats Tommie and James, with a new addition to the family in the making.

List of Publications

- **Buijs P**, van Eijck C, Hofland L, Fouchier R, van den Hoogen B. Different responses of human pancreatic adenocarcinoma cell lines to oncolytic Newcastle disease virus infection.
  Cancer Gene Therapy. 2014 Jan;21(1):24-30
- **Buijs P**, van Amerongen G, van Nieuwkoop S, Bestebroer T, van Run P, Kuiken T, Fouchier R, van Eijck C, van den Hoogen B. Intravenously injected Newcastle disease virus in non-human primates is safe to use for oncolytic virotherapy.
  Cancer Gene Therapy. 2014 Nov;21(11):463-71
- **Buijs P**, van Nieuwkoop S, Vaes V, Fouchier R, van Eijck C, van den Hoogen B. Recombinant immunomodulating lentogenic or mesogenic oncolytic Newcastle disease virus for treatment of pancreatic adenocarcinoma.
  Viruses. 2015 Jun;7(6):2980-2998
- **Buijs P***, Kuiken T*, van Run P, van Amerongen G, Svraka S, Breitbart M, Koopmans M, van den Hoogen B. Pigeon paramyxovirus type 1 from a fatal human case induces pneumonia in cynomolgus macaques *(Macaca Fascicularis).*
  *In preparation, * shared first author*
- **Buijs P**, Verhagen J, van Eijck C, van den Hoogen B. Oncolytic viruses: from bench to bedside with a focus on safety. (review)
  Human Vaccines and Immunotherapy. 2015 Jul 3;11(7):1573-84
- Verhagen J, **Buijs P**, van den Hoogen B, van Eijck C. COGEM rapport: Inventarisatie preklinische gentherapiestudies.
  In press
- **Buijs P**, Maring J. Snapshots in Surgery: intussusception of the appendix due to mucocele.
  British Journal of Surgery 2009; 96: 1415.

Erasmus MC PhD Portfolio

Name PhD student: Pascal Buijs Erasmus MC Department: Surgery Research School: Molecular Medicine	PhD period: 2010-2015 Promotor(s): Prof.dr. C.H.J. van Eijck & Prof.dr. R.A.M. Fouchier Supervisor: Dr. B.G. van den Hoogen

1. PhD training		
	Year	**Workload (ECTS)**
General courses		
Basic and translational oncology (MolMed)	2010	2
Course in Virology (MolMed)	2010	2
Research Integrity	2010	0.5
Laboratory animal science	2010	3
Indesign CS5 (MolMed)	2013	0.15
Photoshop & Illustrator CS5 (MolMed)	2013	0.3
Seminars and workshops		
Daniel den Hoed day (2x)	2012-2013	0.3
Science in Transition	2014	0.15
Presentations		
Labmeetings department of Viroscience	2010-2015	2
Labmeetings LECO	2010-2012	1
University of Maryland/Georgetown research project	2014	1
(Inter)national conferences		
Dutch Annual Virology Symposium	2011	1
Symposium Experimenteel Onderzoek Heelkundige Specialismen	2012	1
MolMed Day (3x)	2010-2014	3
Oncolytic virus meeting (3x)	2010-2014	3
Chirurgendagen (3x)	2010-2014	3
Other		
Stafdag Heelkunde (2x)	2012-2013	1
Wetenschapsmiddag AAV	2013	0.15

2. Teaching		
	Year	**Workload (ECTS)**
Lecturing		
Minor MDL	2013	0.5
Supervising practicals and excursions, Tutoring		
Supervising HLO student	2012	2
Other		
Resuscitation exams Faculty of Medicine, Erasmus MC	2010-2014	1

Stellingen behorende bij het proefschrift

Oncolytisch Newcastle Disease Virus als Behandeling voor Pancreascarcinoom

1. Hoog virulent Newcastle disease virus heeft de meeste potentie als oncolytisch virus voor de behandeling van pancreascarcinoom. *(dit proefschrift)*

2. Defecten in aangeboren immuniteit van pancreascarcinoom cellen bepalen niet alleen de gevoeligheid voor oncolytisch Newcastle disease virus. *(dit proefschrift)*

3. Het intraveneus toedienen van hoge doses oncolytisch Newcastle disease virus is veilig voor niet-humane primaten voor wat betreft toxiciteit en uitscheiding. *(dit proefschrift)*

4. Omdat een hoog virulent oncolytisch Newcastle disease virus een risico kan vormen voor het milieu, dient beperking van het gastheerbereik of immunomodulatie verder onderzocht te worden. *(dit proefschrift)*

5. Het heterogene karakter van pancreastumoren vraagt om een 'personalized medicine' aanpak, wat goed mogelijk is met verschillende oncolytische virussen. *(dit proefschrift)*

6. De grootste uitdaging voor het onderzoeksveld van oncolytische virotherapie is niet het genereren van verbeterde virussen, maar de vraag welke te selecteren voor klinische onderzoeken. *(Stephen Russell et al, Nat Biotechnol. 2012;30(7):658-70)*

7. De recente successen behaald in kanker immunotherapie zullen versterkt worden wanneer deze gecombineerd worden met oncolytische virotherapie.

8. Oncolytische virotherapie zal in de nabije toekomst een plaats krijgen binnen de bestaande kanker behandelingen.

9. Ondanks investeringen in nieuwe en betere behandelmethoden zijn er helaas maar weinig tot geen patiënten met ductaal type pancreascarcinoom die genezen kunnen worden. *(Monika Carpelan-Holmström, Gut, 2005;54:385-387)*

10. Eet als je kan, slaap als je kan, en sol niet met het pancreas.
(Miranda Bailey, Grey's Anatomy - S07E08 - "Something's Gotta Give")

11. Het is belangrijker om een arts te leren pipetteren, dan een giraf te leren touwtje springen.

Pascal Buijs, 30 september 2015

Propositions accompanying the doctoral thesis

Oncolytic Newcastle Disease Virus as Treatment for Pancreatic Cancer

1. High virulent Newcastle disease virus is the most potent oncolytic virus as treatment for pancreatic carcinoma. *(this thesis)*

2. Defects in innate immunity of pancreatic carcinoma cell lines do not solely dictate the susceptibility for oncolytic Newcastle disease virus. *(this thesis)*

3. The intravenous administration of high dose oncolytic Newcastle disease virus is safe for non-human primates with regards to toxicity and shedding. *(this thesis)*

4. Because a high virulent oncolytic Newcastle disease virus can pose a risk for the environment, further research should focus on restriction of the host range or immunomodulation. *(this thesis)*

5. The heterogeneity of pancreatic tumors demands a 'personalized medicine' approach, which can be acheived by using different oncolytic viruses. *(this thesis)*

6. The biggest overall challenges facing the field [of oncolytic virotherapy] now have less to do with the development of technology solutions to enhance virus delivery and spread than with how to get new viruses clinically tested.
(Stephen Russell et al, Nat Biotechnol. 2012;30(7):658-70)

7. Recent successes in cancer immunotherapy will be enhanced when combined with oncolytic virotherapy.

8. Oncolytic virotherapy will get its place among regular cancer treatments in the near future.

9. Despite investments in new and better treatment methods there are few to no patients with ductal pancreatic carcinoma that can be cured.
(Monika Carpelan-Holmström, Gut, 2005;54:385-387)

10. Eat when you can, sleep when you can, and don't screw with the pancreas.
(Miranda Bailey, Grey's Anatomy - S07E08 - "Something's Gotta Give")

11. It is more important to teach a medical doctor how to pipet, than to teach a giraffe how to jump rope.

Pascal Buijs, 30 September 2015