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In-vitro characzerization of a novel recombinant oncolytic virus vector for improved therapy of hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC), the most common type of primary liver cancer, is a major health concern worldwide. Unfortunately, therapeutic options for HCC are very limited, and the overall outcome is still poor. Over the past few decades, oncolytic viruses have arisen as novel therapeutic approaches for the treatment of various types of cancer, including HCC. Amongst the most investigated vectors are Vesicular stomatitis virus (VSV) and Newcastle disease virus (NDV). VSV has previously been demonstrated to be an effective oncolytic agent for the treatment of HCC. However, intratumoral spread of the virus was limited, and high doses of VSV resulted in neuro- and hepatotoxicity. NDV, on the other hand, has been shown to be both safe and effective when applied in humans. Especially rNDV/F3aa(L289), a recombinant NDV (rNDV) bearing a hyperfusogenic fusion (F) protein mutation, has demonstrated great results in preclinical studies. Nevertheless, NDV is known to be a severe pathogen in its avian hosts, thus posing a great environmental threat as well as bearing economic risks for the poultry industry.

Considering the positive and negative features of both VSV and NDV, we engineered a chimeric vector named rVSV-NDV. In this hybrid construct, VSV's glycoprotein G, to which VSV's cytotoxic side effects are often attributed, was deleted and replaced with rNDV's glycoproteins, the hemagglutinin-neuraminidase (HN) attachment protein and the hyperfusogenic fusion protein F3aa(L289). In proof-of-concept *in-vitro* studies, we compared rVSV-NDV to its parental vectors with regard to its safety and efficacy profile.

Our results suggest that the engineering strategy resulted in a significantly reduced off-target toxicity of the new vector in comparison with VSV, and that efficient cell killing abilities are mediated by its new glycoproteins. Having established a production protocol in this study, we were also able to produce high titered stocks of the recombinant vector, which allowed for subsequently conducted *in-vivo* studies, the results of which are consonant with the findings of this study (Abdullahi et al., 2018).

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Abbreviations

°C	Degree Celcius
APS	Ammonium persulphate
ATCC	American Type Culture Collection
BCLC	Barcelona Clinic Liver Cancer
BHK	Baby Hamster Kidney Cells
BSA	Bovine Serum Albumin
CPE	Cytopathic Effect
CO_2	Carbon dioxide
DAMP	Damage-associated molecular patterns
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
dH_2O	Distilled water
DMEM	Dulbecco's Modified Eagle´s Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECOG	Eastern Cooperative Oncology Group
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
FDA	Food and Drug Administration
G	Glycoprotein
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein
GM-CSF	granulocyte macrophage colony-stimulating factor
GMEM	Glasgow Minimal Essential Medium
h	Hour(s)
HBV	Hepatitis B Virus
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C Virus
HEK	Human Embryonic Kidney
HSV	Herpes simplex virus
HTCR	Human Tissue and Cell Research
ICD	Immunogenic cell death
ICI	Immuno checkpoint inhibitor
IF	Immunofluorescence
IFN	Interferon

IU	International unit
l	Liter
L	Large polymerase protein
LDH	Lactate Dehydrogenase
LDLR	Low-density lipoprotein receptor
LiCl	Lithium Chloride
M	Molar
M	Matrix protein
ml	Milliliter
MOI	Multiplicity of Infection
MTD	Maximum tolerated dose
MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carbooxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)
N	Nucleoprotein
ND	Newcastle disease
NDV	Newcastle disease virus
NEAA	Non Essential Amino Acids
NH_4Cl	Ammonium Chloride
OD	Optical density
OV	Oncolytic Virus
OVT	Oncolytic Virotherapy
P	Phosphoprotein
P/S	P/S
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PBS-T	PBS Tween-20
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PFU	Plaque forming units
q-RT-PCR	Quantitative reverse transcription PCR
RCF	Relative centrifugal force
RFA	Radiofrequency Ablation
RNA	Ribonucleic acid
rNDV	Recombinant Newcastle Disease Virus
RNP	Ribonucleoprotein
rpm	Revolutions per minute
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction

rVSV	Recombinant Vesicular stomatitis virus
rVSV-NDV	Recombinant Vesicular stomatitis/ Newcastle disease hybrid virus
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SPF	Specific pathogen free
TAA	Tumor-associated antigen
TACE	Transarterial Chemoembolization
TAE	Tris-acetate-EDTA
$TCID_{50}$	50% Tissue culture Infective Dose
TPB	Tryptose Phosphate Broth
TBS	Tris-buffered saline
TBS/T	Tris-buffered saline with Tween20
TEMED	N,N,N',N'-Tetramethylethylenediamine
V	Volt
VSV	Vesicular Stomatitis Virus
WB	Western Blot
wt	Wild type

1 Introduction

1.1 Hepatocellular Carcinoma

Hepatocellular Carcinoma (HCC) is the most common type of primary liver cancer and a major health problem worldwide (Forner et al., 2018). Recent data analyzed by the International Agency for Research on Cancer (IARC), revealed continuously rising incidence and mortality rates, with 905 677 new cases of primary liver cancer and 830 180 deaths caused in 2020, making HCC the sixth most common type of cancer in the world and the third most common cause of cancer-associated death (*International Agency for Research on Cancer (IARC). GLOBOCAN*, 2020). HCC usually develops in patients suffering from chronic liver disease, most often (in 80-90%) on the basis of a cirrhotic liver (Daoudaki & Fouzas, 2014; Fattovich et al., 2004). Important risk factors for developing such cirrhosis are chronic viral infections with Hepatitis B virus (HBV) or Hepatitis C virus (HCV), alcoholic liver disease, nonalcoholic steatohepatitis (NASH), hemochromatosis, exposure to aflatoxin B1 or autoimmune hepatitis (Daoudaki & Fouzas, 2014; Forner et al., 2018; Galle et al., 2018). Approximately 80% of the reported cases of HCC occur in developing countries, primarily in eastern Asia and Sub-Saharan Africa (*International Agency for Research on Cancer (IARC). GLOBOCAN*, 2020), where its high incidence is attributed mainly to high infection rates with HBV as well as to the exposure to aflatoxin B1 (El-Serag, 2012; Fitzmaurice et al., 2017; Forner et al., 2018). But also in developed regions such as Europe or the United States of America (USA), the incidence of HCC is rising, especially due to an increased prevalence of HCV infection, an increased alcohol consumption and a rising incidence of obesity and the metabolic syndrome (e.g. type 2 diabetes) (Bosetti et al., 2014; Daoudaki & Fouzas, 2014; El-Serag et al., 2004; El-Serag & Kanwal, 2014; Younossi et al., 2014). In the USA, for instance, age-adjusted incidence rates for HCC have tripled between 1975 and 2005 (Altekruse et al., 2009) and HCC mortality rates seem to have increased by 40% from 1990 to 2004, while overall cancer-associated death rates decreased by about 18% during the same time span (Galle et al., 2018; Jemal et al., 2010).

Today, the most widely accepted staging and treatment strategy is the Barcelona Clinic Liver Cancer (BCLC) classification (Galle et al., 2018). It was first published in 1999 (Llovet et al., 1999) and has regularly been updated ever since. Figure 1 shows the most recently updated version (Forner et al., 2018).

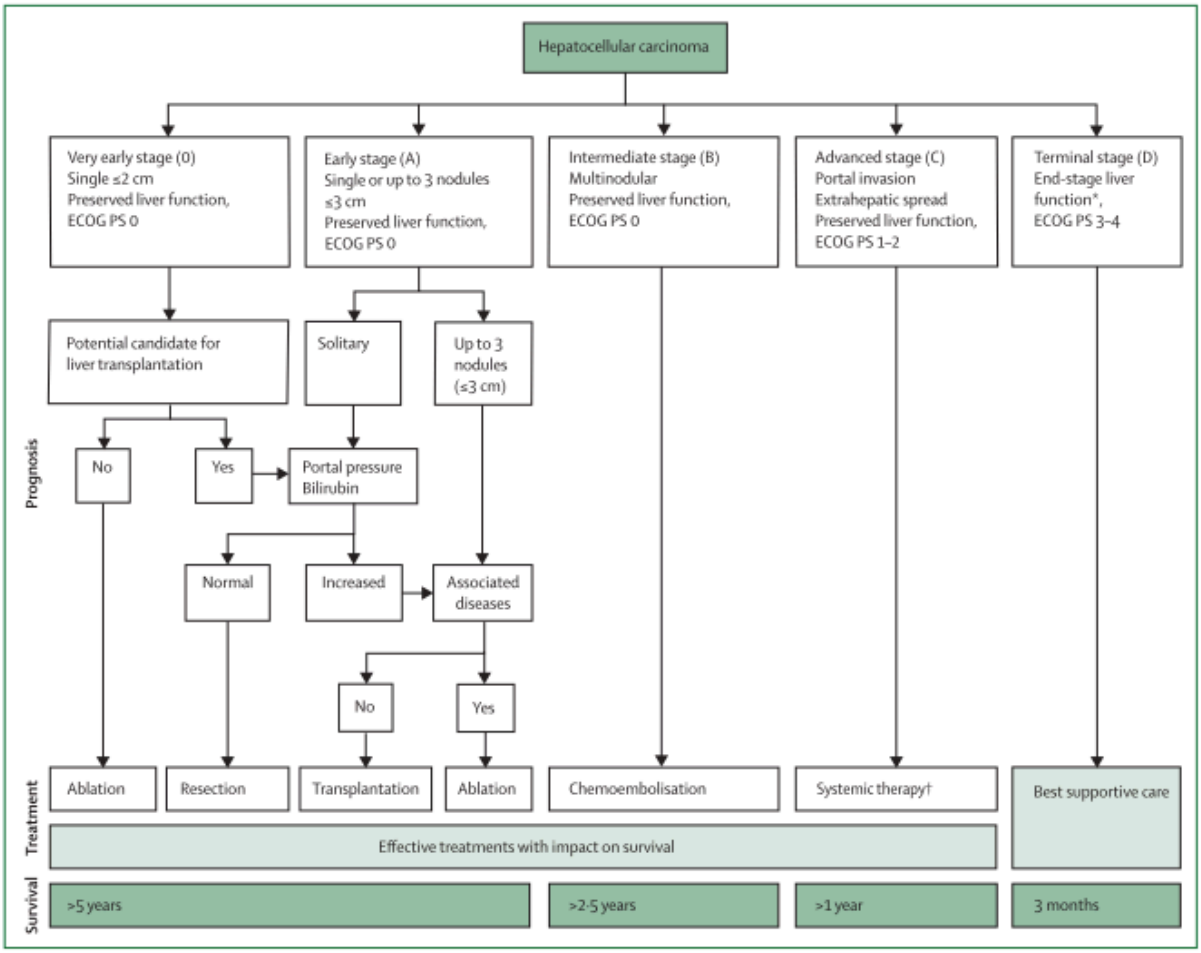

Figure 1 Barcelona Clinic Liver Cancer (BCLC) staging and treatment strategy

The BCLC classification assigns patients diagnosed with HCC to five categories (tumor stage 0, A, B, C, D), based on their tumor burden (size and number of nodules, vascular invasion, metastases), their liver function (as determined by the Child–Pugh Score) and their performance status (PS) (classified using the Eastern Cooperative Oncology Group (ECOG) scale. Subsequently, first-line treatment recommendations and survival expectations (expressed as median survival) are given for each tumor stage. Systemic therapy †: The standard first line therapy systemic therapy is sorafenib, Lenvatinib has been shown to be non- inferior to sorafenib, so it is also recommended as first line therapy. Regorafenib is recommended as second-line therapy after sorafenib. (from (Forner et al., 2018)).

Surgical resection is the treatment of choice for patients diagnosed at an (very) early stage of HCC with no underlying cirrhosis or with only small, solitary tumors, no portal hypertension and normal bilirubin. These criteria are met by only 5-10% of the patients, and those treated with resection have a recurrence risk of 70% within five years and a five-year survival of only 50-70% (Daoudaki & Fouzas, 2014; Forner et al., 2018).

Liver transplantation, theoretically the best treatment option as it not only provides cure of the tumor but also of the underlying chronic liver disease, can be offered to patients meeting the so-called Milan criteria (one single HCC tumor of ≤ 5cm in diameter or up to three nodules measuring less than 3cm, respectively). For these patients, the five-year survival is expected to be higher than 70%, and recurrence rates are low (<10–15%). However, an extreme

shortage of liver donors is unfortunately a great limitation in the feasibility of liver transplantations (Forner et al., 2018; Mazzaferro et al., 1996)

An alternative for patients not eligible for resection or transplantation with BCLC 0 or A tumors is local ablation. Here, necrosis of tumor tissue is induced by either injection of chemicals (e.g. ethanol) or by temperature modification (e.g. radiofrequency, microwave, laser, or cryoablation) (Forner et al., 2018). Radiofrequency ablation (RFA)) is the ablation technique of choice (Breen & Lencioni, 2015) and has an excellent efficacy of almost 100% in patients with BCLC 0, so that lately it has been regarded as first line therapy for patients with very early-stage hepatocellular carcinoma if liver transplantation is not feasible (Forner et al., 2018; Galle et al., 2018) However, again, as it is not a curative treatment option, recurrence rates are as high as 70%, and five-year survival is only 50-70% (Daoudaki & Fouzas, 2014; Forner et al., 2012).

Patients diagnosed at an intermediate stage of tumor progression (BCLC B) can be treated with transarterial chemoembolization (TACE). This combines the injection of a chemotherapeutic agent with the embolization of the tumor-feeding arterial blood supply, with the aim of inducing a strong cytotoxic, as well as ischemic, response. If patients are well selected and treatment is delivered correctly the median survival can exceed 30–40 months (Forner et al., 2018). Otherwise, the overall median survival is thought to be around 20 months, which is about four months longer than in patients treated conservatively (Llovet et al., 2012).

At advanced stages (BCLC C), the only promising treatment option currently is the administration of oral multi-kinase inhibitors, drugs with anti-proliferative and anti-angiogenic properties that block the actions of several protein kinases such as Rapidly Accelerated Fibrosarcoma (Raf-) kinases, vascular endothelial growth factor receptors, platelet-derived growth factor receptors or fibroblast growth factor receptors (Forner et al., 2018). So far, three agents have shown survival benefits: sorafenib and lenvatinib in first-line treatment and regorafenib in second-line after sorafenib (Galle et al., 2018). Despite this relevant improvement for the treatment of advanced HCC since the approval of sorafenib in 2007, the overall survival prolongation of these drugs is still marginal: The median overall survival was reported to be 10.7 months versus 7.9 months for sorafenib versus placebo (Llovet et al., 2008), and 10.6 months versus 7.8 months for regorafenib versus placebo (Bruix et al., 2017). Levatinib was shown to be non-inferior to sorafenib (median overall survival 13.6 months for lenvatinib vs. 12.3 months for sorafenib (Kudo et al., 2018). Also, all these treatment options are only eligible for patients with compensated liver disease (Child-Pugh A) (Galle et al., 2018)

Patients with terminal stage HCC (BLCL D) are left with best supportive care.

Taken together, despite a continuously increasing incidence of HCC, therapeutic options are still very limited. Curative treatment options are restricted to patients diagnosed at early stages of HCC. This, however, is the minority only (about 30% (Pons et al., 2005)), as a lack of efficient surveillance and diagnostic tools, especially in less-developed countries, and difficulties in detecting the very small nodules at early stages, even in well-developed regions, often impede early diagnosis (Llovet et al., 2012). As a result, only few patients meet the strict criteria for curative treatments and, in addition, the feasibility of performing liver transplantations is extremely limited due to scarcity of donated livers. The majority of the patients is left with palliative treatment options and still faces a relatively poor prognosis. Therefore, the continuation of research in this field and the development of new therapeutic agents are strongly needed.

1.2 Oncolytic Virotherapy (OVT)

To address the limited availability of effective treatment options for HCC, as well as for many other types of cancer, many novel therapeutic approaches are being investigated. Among those options under development are oncolytic viruses (OVs). OVs are viruses that preferentially infect, replicate in and kill tumor cells, while leaving healthy cells unharmed (Kirn et al., 2001; S. J. Russell & Peng, 2007).

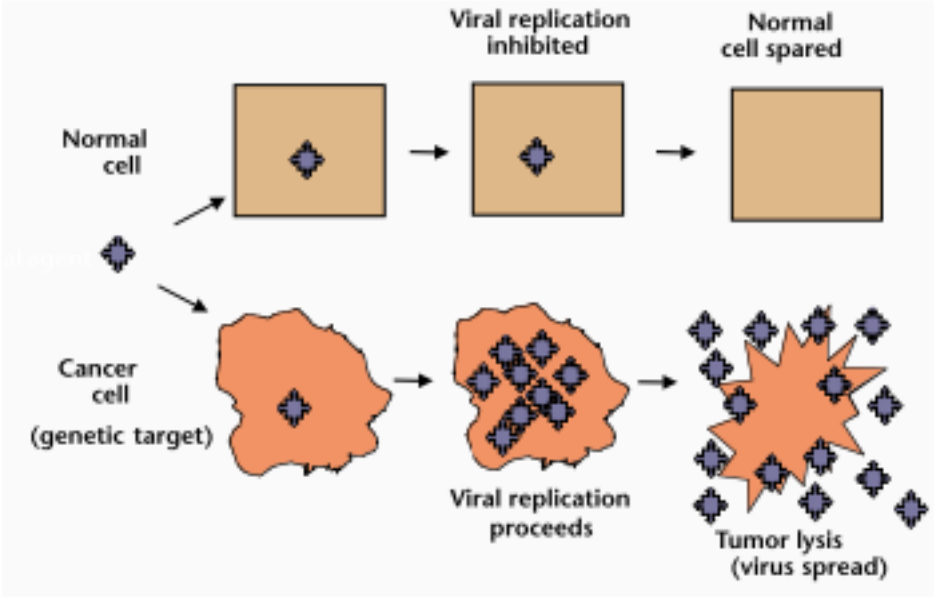

Figure 2 Schematic representation of tumor selective viral replication and oncolysis

Oncolytic viruses preferentially replicate in and cause cell lysis of cancer cells, while replication in normal cells is inhibited (from (Kirn et al., 2001)

Oncolytic virotherapy (OVT) has been studied for the last century. First reports on oncolytic activity of certain viruses were based on temporary tumor regression observed in patients undergoing naturally acquired infectious diseases. Those first findings were published at the early beginning of the 20th century: In 1904, George Dock described the case of a female patient suffering from myelogenous leukemia, that went into remission after contracting an influenza infection in 1896 (Dock, 1904). Bierman et al. reported on the regression of the lymphatic leukemia of a four-year old boy following an infection with varicella virus (Bierman et al., 1953). In the following years, several other reports emphasized the coincidences of viral infections and tumor regression (Bluming & Ziegler, 1971; Pasquinucci, 1971; Pelner et al., 1958; Zygiert, 1971). Herewith, the idea of viruses being able to destroy tumors was born, and first clinical trials were on their way (Asada, 1974; Georgiades et al., 1959; Hoster et al., 1949; E. Kelly & Russell, 2007; Southam & Moore, 1952). Despite rapid progress in the field in the 1950s and 1960s, research on oncolytic virotherapy stagnated soon. This was presumably due to the fact that research was initially limited to finding naturally occurring oncolytic viruses, which additionally often led to controversial results only, as well as to the rapid and successful development of alternative treatment options for cancer such as chemo- and radiotherapy. In the early 1990s however, a better understanding of the viral biology and carcinogenesis, as well as the introduction of recombinant technologies, brought OVT back into the focus of research. This innovational approach allowed researchers to directly engineer viral genomes in order to improve the viruses' tumor specificity and antitumor activity (E. Kelly & Russell, 2007). As a result, OVT was investigated intensively throughout the past 25 years, leading to several advanced clinical trials (Cook & Chauhan, 2020; Macedo et al., 2020) and the first oncolytic viruses to be approved globally: In 2004, Rigvir, a native enteric cytopathic human orphan (ECHO)-7 derived virus was approved in Latvia and later on in Georgia, Armenia and Uzbekistan for the treatment of melanoma (Alberts et al., 2018). In 2005, H101, a genetically modified adenovirus was approved in China for the treatment of head and neck cancer (P.-I. Huang et al., 2009). Finally, in 2015 Talimogene laherparepvec (T-Vec), an attenuated herpes simplex virus type 1 (HSV-1) encoding the immunostimulant granulocyte macrophage colony-stimulating factor (GM-CSF) was approved by the Food and Drug Administration (FDA) and is registered for the treatment of advanced, non-resectable metastatic multiple melanoma in the US, Europe, Australia and Israel (Andtbacka et al., 2015; Rehman et al., 2016).

The mechanisms used by OVs to attack and destroy tumorous tissues are diverse: On the one hand, virus-infected cells are killed directly by oncolysis. On the other hand, uninfected cells are tackled indirectly, by either induction of anti-angiogenic and anti-vascular factors or

by stimulation of the innate and adaptive immune system, resulting in an increased antitumor immunity directed against neighboring or metastatic cancer cells (Bartlett et al., 2013; Breitbach et al., 2007; Tong et al., 2012). By genetic engineering, some of these oncolytic properties can be newly integrated into the viral genome or, if present already, enhanced even further.

Replication competent adenovirus, herpes simplex virus, measles virus, Newcastle disease virus, reovirus, vaccinia virus and vesicular stomatitis virus are amongst the most investigated viruses as oncolytic agents (S. J. Russell et al., 2012). They can be roughly classified into two groups: naturally tumor selective viruses, such as vesicular stomatitis virus (VSV) and Newcastle disease virus (NDV), and viruses that had to be genetically engineered in order to become so, such as adeno- or herpesviridae. The reasons for natural tumor selectivity are multiple, and not all of them are fully understood yet. It is known though, that cancer cells often show deficiencies in effective anti-viral defense mechanisms (Wang et al., 2011), a fact that is often exploited by oncolytic viruses: Normal cells respond to viral infections by activating the innate immune system (notably by activation of the Interferon (IFN) signaling pathway), by down-regulating their metabolism or by undergoing apoptosis in order to prevent a viral invasion. Cancer cells, however, often display mutated cellular signaling pathways that developed during the process of carcinogenesis. These altered pathways may help them to escape the immune system, providing them growth and survival benefits, but they are therefore also a lot more susceptible to viral infections, allowing OVs to replicate efficiently (Katsoulidis et al., 2010; Naik & Russell, 2009; S. J. Russell et al., 2012).

1.3 Vesicular stomatitis virus

Vesicular stomatitis virus (VSV) is an enveloped, non-segmented, single-stranded, negative sense RNA-virus that belongs to the family of the *Rhabdoviridae.* Its natural hosts include cattle, horses, pigs as well as a range of other mammals and their insect vectors. In these hosts, an infection leads to symptoms almost identical to those induced by the foot and mouth disease virus. In humans however, infections are very rare and mostly asymptomatic although mild flu-like symptoms have been observed (Rodríguez, 2002; Wagner & Rose, 1996). Vesicular stomatitis virions are typically bullet-shaped and measure about 75nm x 185nm.

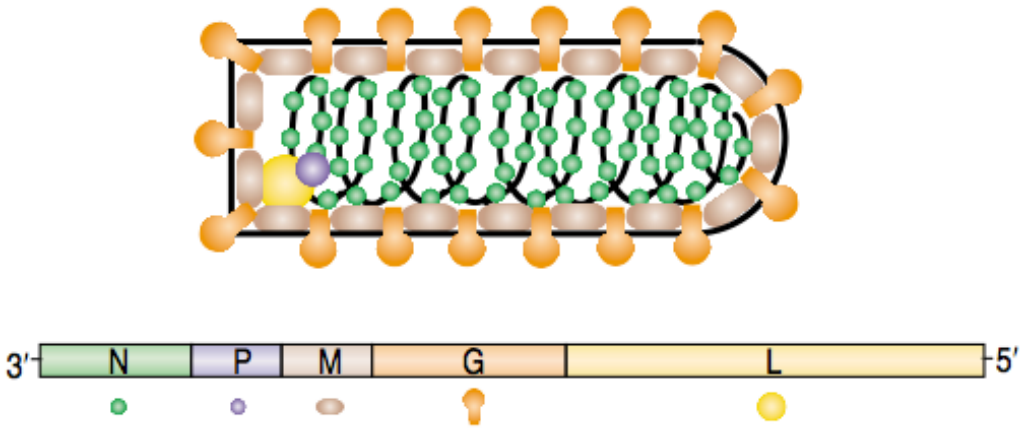

Figure 3 Structure and genome of VSV

Schematic representation of VSV's bullet shaped virion: Its genome consists of five genes encoding the five major viral proteins: the nucleoprotein (N), the phosphoprotein (P), the matrix protein (M), the glycoprotein (G) and the large polymerase (L) (from (Lichty et al., 2004)*).*

VSV's simply composed 11-kilobase (kb) genome holds five genes which encode for its five major proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and large polymerase (L) (Ge et al., 2010; Lichty et al., 2004). N, P and L form the RNA-polymerase complex, which is responsible for viral transcription and replication. The multifunctional M protein is important for virus assembly and budding and responsible for cell apoptosis and interruption of the host-cell innate-immunity system. VSV-G mediates receptor binding and cell entry (Barr et al., 2002; Lichty et al., 2004). Only recently, the low-density lipoprotein receptor (LDLR) and its family members have been identified as the main cell surface receptors used by VSV-G for cell attachment. Considering their almost ubiquitous expression, one can understand the broad tropism of VSV (Finkelshtein et al., 2013). After cell binding, the virus enters the cell by endocytosis, the viral RNA-polymerase complex is released into the cytoplasm and transcription is initiated. Transcription of viral genes starts at the 3' end and proceeds to the 5' end. At each intergenic region however, the polymerase stops, and as re-initiation is incomplete, viral mRNA levels of the proteins decrease steadily (N>P>M>G>L) (Ball et al., 1999; Hastie & Grdzelishvili, 2012).

For several reasons, VSV is considered a promising oncolytic vector: It has a short replication cycle, which allows it to replicate efficiently before being cleared by the immune system. Its replication cycle is strictly limited to the cytoplasm, which prevents integration of the vector into the hosts genome and, therefore, potentially dangerous host-cell mutations (Lichty et al., 2004; Roberts et al., 1999). Furthermore, as infections in humans are very rare, there is almost no pre-existing immunity and therefore almost no pre-existing antibodies in the general human population, which would lead to a premature clearing of the virus (Hastie & Grdzelishvili, 2012). Another important beneficial characteristic is VSV's extraordinary

sensitivity to type I Interferon (IFN). IFNs are a group of signaling proteins that belong to the class of cytokines and that play an important role in human anti-viral defense mechanisms. They can be categorized into three groups: Type I (IFN-α, IFN-β, IFN-ε, IFN-κ and IFN-ω), type II (IFN-γ) and type III Interferon (IFN-λ). IFNs are produced and released by host cells upon viral infection, causing IFN-susceptible cells to raise their anti-viral defenses. By activating immune cells or mediating apoptosis, they prevent an effective replication of the invading virus and therefore limit the spread of the infection (Haller et al., 2006; Muller et al., 1994; Stark et al., 1998). As previously mentioned, cancer cells often present mutated IFN-signaling pathways in order to escape the immune system and the IFN-mediated growth-inhibiting and apoptotic effects (Wang et al., 2011). Specific genes known to be related to the type I IFN responses were shown to be down-regulated or inactivated in some types of cancer (Balachandran & Barber, 2004; Marozin et al., 2008; Zhang et al., 2010), providing tumor cells growth and survival benefits, but at the same time, rendering them a lot more vulnerable to viral infections. The exquisite sensitivity of VSV to type I IFN is believed to be the key mechanism of VSV's tumor selectivity, allowing it to replicate efficiently in tumor cells while sparing the surrounding 'normal', meaning IFN-susceptible cells (Balachandran & Barber, 2000; Barber, 2004; Lichty et al., 2004; Stojdl et al., 2000).

VSV was shown to be an effective oncolytic agent in several *in vitro* as well as *in vivo* studies (Balachandran et al., 2001; Balachandran & Barber, 2000; Stojdl et al., 2003), particularly for the treatment of hepatocellular carcinoma (Jennifer Altomonte et al., 2013; Jennifer Altomonte, Braren, et al., 2008; Jennifer Altomonte, Wu, et al., 2008; Ebert et al., 2003; Katsunori Shinozaki et al., 2004). However, it has also been reported that the administration of wild type (wt)VSV causes severe neurotoxicity (Johnson et al., 2007; Plakhov et al., 1995; Quiroz et al., 1988; K. Shinozaki et al., 2005; van den Pol et al., 2002). This unfavorable property has greatly limited the clinical applicability of VSV as an oncolytic vector. As a result, several recombinant viruses were engineered over the past few years in order to address this major safety concern, using the established reverse genetics system (Hastie & Grdzelishvili, 2012; Lawson et al., 1995; Whelan et al., 1995). Amongst these approaches are strategies of using mutated M proteins, especially a recombinant haboring the ΔM51 mutation (VSV-ΔM51), which lacks the ability to inhibit anti-viral responses, leading to increased protective IFN levels and a significant reduction of systemic side effects (Ahmed et al., 2003; Coulon et al., 1990; Ebert et al., 2005; Stojdl et al., 2003). Other approaches use a VSV-recombinant encoding INFβ (VSV-INFβ), which stimulates the innate immune responses in IFN-competent, non-malignant cells (Jenks et al., 2010; Obuchi et al., 2003). This modification was shown to lead to an improved safety profile with greatly diminished neurotoxicity (Jenks et al., 2010) and resulted in several phase I clinical trials taking place in the USA (see ClinicalTrials.gov: NCT02923466, NCT03120624 and NCT03017820). While

these two concepts are based on exploiting defective anti-viral defense mechanisms of tumor cells, a different approach is to address VSV's very broad tropism, which is attributed to the almost ubiquitous prevalence of its binding molecules, the LDLR-family (Finkelshtein et al., 2013). This broad tropism can be advantageous in terms of allowing VSV to infect a wide range of tumor types (Felt & Grdzelishvili, 2017). However, the cause of VSV's toxicity could be exactly this unselective targeting. As VSV-G is the responsible structure for receptor binding and cell entry (Roche et al., 2008), VSVs neurotropism has often been attributed to this protein (Boritz et al., 1999; Hastie et al., 2013; A. Muik et al., 2011; Tani et al., 2007). Therefore, a promising approach to detarget VSV from healthy tissue could be to try and alter VSV's tropism by replacing its G protein by the one of another oncolytic vector, a process that is called pseudotyping.

In summary, VSV is an interesting and promising oncolytic vector due to a lot of benefits, including its well-studied biology, its rapid and strictly cytoplasmatic replication cycle, its exquisite sensitivity to type I IFN, the possibility to engineer recombinants via reverse genetics, the lack of pre-existing immunity in the general population, and the possibility to produce very high virus yields in a broad range of cell lines (Hastie & Grdzelishvili, 2012). However, its inherent neurotoxicity poses an obstacle that to date still needs to be overcome.

1.4 Newcastle disease virus

Another promising oncolytic vector is the avian Newcastle disease virus (NDV). NDV is the causative agent of Newcastle disease (ND), a highly infectious and economically very important disease in poultry and other avian species (Lancaster, 1976). It was named after the site of the original outbreak among chicken at a farm near Newcastle-upon-Tyne in England in 1926 (Doyle, 1927; Suarez et al., 2019). ND is widely spread and endemic in many countries, bearing the risk of large economic losses in the poultry industry (Ganar et al., 2014; Lancaster, 1976). However, despite its strong impact on avian species, NDV is not a pathogen in human, causing at most mild fever or conjunctivitis (Fournier & Schirrmacher, 2013).

As VSV, NDV is a naturally tumor selective virus. Four mechanisms seem to contribute to this selectivity (Zamarin & Palese, 2012):

I) Defects in activation of antiviral signaling pathways (Fiola et al., 2006)
II) Defects in type I IFN signaling pathway (Krishnamurthy et al., 2006)
III) Defects in apoptotic pathways (Lazar et al., 2010; Mansour et al., 2011)
IV) Activation of Ras signaling and expression of Rac1 protein (Puhlmann et al., 2010)

As a member of the *Paramyxoviridae* family, NDV is an enveloped, non-segmented, single-stranded negative sense RNA virus. It is spherically shaped and about 100-300 nm in diameter. Its 15,186 nucleotide genome contains six genes encoding six structural proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase protein (HN), and large polymerase protein (L) (Lamb & Parks, 2007).

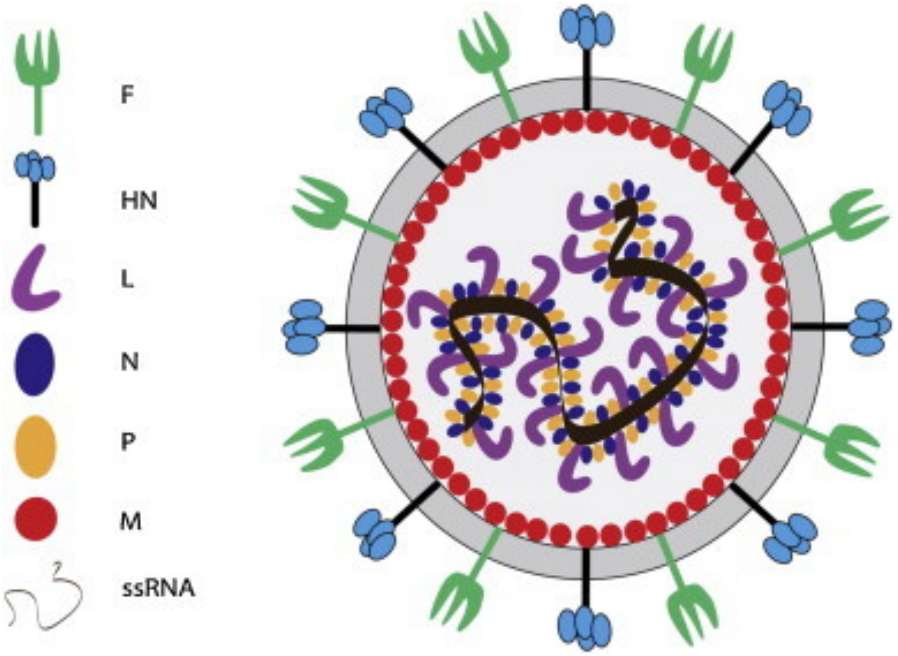

Figure 4 Structure and genome of NDV

Schematic representation of the spherically shaped NDV-virion: Its genome consists of six genes encoding the six structural proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase protein (HN), and large polymerase protein (L) (from (Ganar et al., 2014)*).*

N, P and L form the nucleocapsid, and together with the genomic RNA they build the ribonucleoprotein complex, which serves as template for RNA synthesis (Lamb & Parks, 2007). The nucleocapsid is surrounded by a bilayer lipid envelope: The inner layer is lined by the matrix protein which is necessary for virus assembly and budding (Pantua et al., 2006; Shnyrova et al., 2007); the outer layer is spiked with the two glycosylated surface proteins HN and F, which are responsible for viral entry (Villar & Barroso, 2006). To this end, the virus' attachment protein HN binds to what have been identified as sialic acid molecules, present at the cell surface of a wide range of cells (Herrler et al., 1995). It then interacts with the F protein, which hereupon induces membrane fusion between the viral and the host cell membranes. Subsequently, the nucleocapsid is released into the cytoplasm where replication takes place. Transcription of the viral RNA starts at the 3' end of the genome, and again, as already described for VSV, re-initiation of transcription in between the genes is incomplete, resulting in a decrease of expression of the viral proteins from the 3' to the 5' end (N>P>M>F>HN>L) (Lamb & Parks, 2007). Two additional non-structural proteins, V and W,

are released upon RNA editing of the phosphoprotein (Steward et al., 1993). The V protein antagonizes the antiviral actions of IFNs (Park, Shaw, et al., 2003); however this activity was found to be limited to avian species, suggesting that V is an important determinant of host cell restriction (Park, García-Sastre, et al., 2003).

Based on the severity of the disease NDV causes in birds, it can be categorized into three distinct pathotypes: lentogenic (non-/ low-virulent), mesogenic (intermediate) and velogenic (virulent) (Suarez et al., 2019). Lentogenic strains usually do not cause disease in adult birds and if, only mild or asymptomatic forms with negligible mortality occur. As intermediate classified viruses, mesogenic strains provoke respiratory disease with moderate mortality (<10%). Velogenic NDV strains are highly virulent with mortality rates up to 100% and can be further subcategorized into viscerotropic, producing hemorrhagic gastrointestinal lesions or neurotropic, causing respiratory and neurological problems (Dortmans et al., 2011; Fournier & Schirrmacher, 2013; Suarez et al., 2019). The F protein is known to be an important determinant of NDV's virulence (Morrison, 2003; Nagai et al., 1976; Panda et al., 2004; Peeters et al., 1999; Toyoda et al., 1987): It is synthesized as an inactive precursor F_0 that needs to be cleaved into its functional subunits F_1 and F_2 in order to mediate fusion of the viral and the host cell membranes. Lentogenic strains have F_0 proteins with monobasic amino acid cleavage sites, which can only be cleaved by exogenous trypsin-like proteases found in the respiratory and intestinal tracts of birds. Meso- and velogenic strains however have a polybasic amino acid motif at the F_0 cleavage site that can be cleaved by ubiquitous, intracellular furin-like proteases. This polybasic cleavage site allows virulent strains to infect a broader range of tissues, increasing NDV's virulence immensely (Garten et al., 1980; Toyoda et al., 1987).

The oncolytic qualities of NDV were first described in 1965, when Cassel et al. reported on a patient treated with NDV for advanced cervical cancer (Cassel & Garrett, 1965). Since this initial report, several *in-vitro* and *in-vivo* studies as well as clinical trials have indicated that NDV is a safe and effective anti-cancer therapeutic agent (Csatary et al., 2004; Freeman et al., 2006; Hotte et al., 2007; Laurie et al., 2006; Lorence et al., 2007; Nemunaitis, 2002; Pecora et al., 2002; Reichard et al., 1992; Zamarin & Palese, 2012). Additionally, with the help of reverse genetics (Lawson et al., 1995; Whelan et al., 1995), several recombinant versions of NDV have been engineered in order to enhance its therapeutic efficacy even more (Janke et al., 2007; Pühler et al., 2008; Zamarin et al., 2009; H. Zhao et al., 2008; L. Zhao & Liu, 2012). Amongst these is a recombinant from the non-pathogenic NDV strain Hitchner B1, whose virulence level was raised from lentogenic to mesogenic by introducing a polybasic cleavage site to the F protein (rNDV/F3aa). This modified fusion protein had previously been reported to be highly fusogenic and to effectively kill tumor cells by mediating syncytia formation (large multinucleated cells produced by cell-to-cell fusion) (A. R.

Bateman et al., 2002). In accordance with these findings, encouraging results were achieved by the recombinant rNDV/F3aa in comparison with rNDV/B1, a recombinant not bearing the modification, when evaluated in an immune competent colon carcinoma tumor model (Vigil et al., 2007). Furthermore, it was shown that a single amino acid change in the F protein from alanine to leucine at position 289 (L289A) results in even greater syncytia formation (Li et al., 2005; Sergel et al., 2000). A recombinant NDV harboring both the polybasic cleavage site F3aa and the amino acid mutation L289A (rNDV/F3aa(L289A)) presented an improved oncolytic profile, with increased tumor-specific syncytia formation and necrosis in the absence of local or systemic toxicity when tested in an orthotopic immunocompetent rat model for hepatocellular carcinoma (Jennifer Altomonte et al., 2010).

As briefly mentioned, syncytia are large, multinucleated cells. They are formed by the fusion of infected cells with uninfected neighboring cells. The underlying mechanism is that cells, once infected, express the viral fusion protein at their cell surface, which allows them to make contact and fuse with the plasma membrane of adjacent, until then uninfected cells. By this means, the virus can easily spread from cell to cell, thereby rapidly extending the syncytium. This provides a very effective mechanism of viral spread, as a single virion can possibly infect and kill a large number of cells. Additionally, as it is an intracellular way of spread and cell killing, the exposure of the virus to potentially neutralizing antibodies is limited (Higuchi et al., 2000; Matveeva et al., 2015).

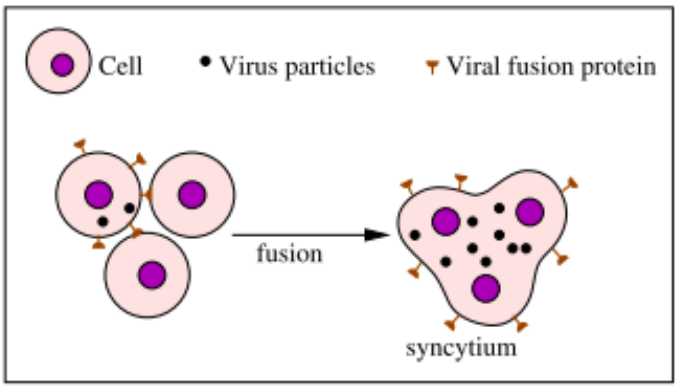

Figure 5 Mechanism of syncytia formation

Syncytia are large multinucleated cells formed by the fusion of Infected cells with uninfected, neighboring cells. To this end, the infected cells express the viral fusion protein at their cell surface, allowing them to make contact and fuse with the plasma membrane of adjacent cells. This mechanism allows for an efficient mode of viral spread and, as the virus remains intracellularly, minimizes the exposure to neutralizing antibodies. (from (Alzahrani et al., 2020)*).*

Furthermore, syncytia-induced cell death has been identified as a cause of immunogenic cell death (ICD). This type of cell death goes along with the release or the expression of damage-associated molecular patterns (DAMPs) or pathogen-associated molecular patterns (PAMPs) by virus-infected cells, such as the membrane localization of calreticulin (ecto-CRT) or the release of heat-shock proteins (Hsp70, Hsp90), high mobility group box 1(HMGB1) protein, ATP and uric acids. These danger signals activate antigen-presenting cells, which results in increased adaptive immune responses known to trigger an increased antitumor immunity and therefore potentially long-lasting tumor regression (Cuadrado-Castano et al., 2015; Guo et al., 2014; Koks et al., 2015; Kroemer et al., 2013; Matveeva et al., 2015; Zelenay & Reis e Sousa, 2013).

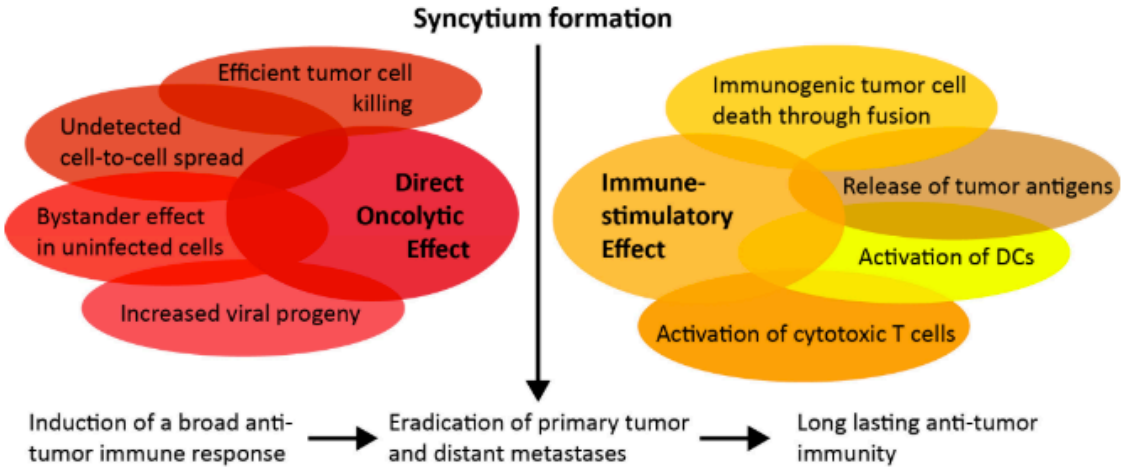

Figure 6 Benefits of syncytia formation

Syncytia formation enhances both the direct oncolytic effect by providing an efficient mode of intratumoral viral spread with limited contact to neutralizing antibodies and efficient cell killing of uninfected neighboring cells, as well as the "indirect" oncolytic effect by a potent stimulation of the adaptive immune system, triggered by its immunogenic cell death. (from (Krabbe & Altomonte, 2018)*).*

In conclusion, the ability to induce syncytia formation seems to be a crucial weapon for NDV as an oncolytic agent, being efficient in direct cell killing as well as in activating the adaptive immune system and therefore inducing a long-lasting anti-tumor immunity. Fusogenicity even appears to be such a beneficial feature, that it should be considered when designing an optimal virus vector platform (Krabbe & Altomonte, 2018).

Taken together, NDV offers multiple positive features: As it is an avian virus, there is almost no preexisting immunity or pathogenicity in humans. Side-effects when administered to humans are usually limited to flu-like symptoms or mild conjunctivitis (Nemunaitis, 2002; Suarez et al., 2019). Secondly, as discussed, it demonstrates tumor-selective replication. Moreover, its genome is susceptible to manipulation, and with its replication being limited to

the cytoplasm, no integration of foreign genes into the host genome has to be feared. It can mediate syncytia-formation and a powerful anti-tumor immunity by effectively activating the immune system (Fournier & Schirrmacher, 2013; Jarahian et al., 2009; Washburn & Schirrmacher, 2002). Lastly, owing to the ubiquitous prevalence of its receptor, namely sialic acid molecules, it can potentially tackle a broad range of cancer types (Zamarin & Palese, 2012).

However, despite all these positive features and the promising results in both preclinical and clinical studies, NDV remains a severe pathogen in birds. While being rather safe in humans, it poses a great environmental and economical risk to the poultry industry (Ganar et al., 2014; Lancaster, 1976). Its mesogenic and velogenic strains, which are generally the most efficient as oncolytic agents, were classified as select agents by the U.S. Department of Agriculture, meaning that they "have the potential to pose a severe threat to public health and safety, to animal or plant health, or to animal or plant products" (www.selectagents.gov). This unfavorable property of NDV is sadly greatly limiting its clinical applicability as a therapeutic agent, creating the need to develop alternative oncolytic vectors that are both safe and effective.

1.5 Aim of this thesis

New therapeutic approaches for the treatment of cancer are urgently needed. Oncolytic viruses have arisen as promising candidates, as they preferentially target tumor cells. Two of the most investigated vectors are vesicular stomatitis virus (VSV) and Newcastle disease virus (NDV), which have both been studied as cancer therapeutics, amongst others for the treatment of hepatocellular carcinoma (HCC). Unfortunately, their clinical applicability is limited, especially due to major safety concerns. Considering the positive and negative features of both VSV and NDV as oncolytic vector platforms, we engineered a chimeric vector named rVSV-NDV, in which VSV's endogenous glycoprotein VSV-G was deleted and replaced with NDV's envelope glycoproteins HN and F. The aim of this thesis was to establish a high-concentration virus stock production protocol for the newly engineered vector, as well as conduct an *in-vitro* characterization of the recombinant vector regarding its safety and efficacy profile for the treatment of HCC, relative to its parental vectors.

2 Materials and Methods

2.1 Materials

2.1.1 Equipment

Table 1: Equipment

Product	Company
AxioCam ICm1	Carl Zeiss, Germany
Axio Imager	Carl Zeiss, Germany
Balance (AGB, FTB)	Kern, Germany
Centrifuge (5415R, 5415D, 5702R)	Eppendorf, Germany
Electrophoresis system Mini PROTEAN	Bio-Rad, Germany
Film processor Hyperprocessor	GE Healthcare Life Science, Germany
Freezer -20°C	Siemens, Germany
Freezer -80°C (“Hera freeze”)	Heraeus, Germany
Fridge 4°C	Dixell, Italy
Glass plates Mini PROTEAN	Bio-Rad, Germany
Hypercassette (Western Blot)	Amersham Bioscience, UK
Hood (“Hera safe”)	Heraeus, Germany
Incubator (“Hera Cell 240”)	Heraeus, Germany
Microplate reader	TECAN, Switzerland
Microscope (“Optech”)	Exacta Optech, Germany
Microwave	Siemens, Germany
Movie analyzer “JuLI Br”	NanoEN TEK, USA
Multichannel pipette 100	Eppendorf, Germany
Multipipette M4	Eppendorf, Germany
Neubauer counting chamber	Brand, Germany
Pipette (“Research”, 10µl, 20µl, 200µl)	Eppendorf, Germany
Pipette (“Research Plus”, 1000µl)	Eppendorf, Germany
Pipet Controller (“Stripettor Ultra”)	Corning, USA
Pierce Fast Semi-Dry Blotter	Thermo Fisher Scientific, USA
Polycarbonate bottle with Cap Assembly (26.3 mL)	Beckman Coulter, USA
Power Supply Power Pack Basic	Bio-Rad, Germany
Rotor for Ultracentrifugation, 70Ti	Beckman Coulter, Germany

Shaker Duomax 1080	Heidolph, Germany
Spectrophotometer Nanodrop Lite	Thermo Fisher Scientific, USA
Spectrophotometer SmartSpec Plus	Bio-Rad, Germany
Thermomixer compact	Eppendorf, Germany
Tube rotator (“MACSmix”)	Miltenyl Biotec, Germany
Ultracentrifuge OptimaTM XL- 100K	Beckman Coulter, Germany
Vortex Mixer (“Reax top”)	Heidolph, Germany
Waterbath	GFL, Germany

2.1.2 Consumables

Table 2: Consumables

Product	**Company**
Amicon Ultra-15 Centrifugal Filter Units	Merck Millipore, USA
Aspirating Pipette (2ml)	BD Falcon, USA
Blot filter paper	Bio-Rad, Germany
Cell scraper	TPP, Switzerland
Centrifuge tubes (15ml, 50ml)	Greiner Bio One, Germany
Chamber culture slides (8-chambers)	BD Falcon, USA
CL-XPosure Film	Thermo Fisher Scientific, USA
Combitips advanced(1ml,5ml,10ml)	Eppendorf, Germany
Cryogenic tubes	Thermo Fisher Scientific, USA
Filter tips (TipOne, 10µl, 20µl, 200µl, 1000µl)	StarLab, Germany
Needle (20 G)	Braun, Germany
Nitrocellulose membrane (0,45µM)	Bio-Rad, Germany
Parafilm	Bemis, USA
Safe-lock microcentrifuge tubes (1,5ml, 2ml)	Sarstedt, Germany
Serological Pipettes (5ml, 10ml, 25ml, 50ml)	Greiner Bio One, Germany
Syringe filter (0,22µm)	TPP, Switzerland
Syringe sterile (3ml)	BD Falcon, USA
Tissue culture dish 150	TPP, Switzerland
Tissue culture flask (75 cm^2)	TPP, Switzerland
Tissue culture test plates (6-, 24- and 96-well)	TPP, Switzerland

2.1.3 Reagents, buffers and solutions

Table 3: Purchased reagents, buffers and solutions

Product	Company
4',6-diamidino-2-phenylindole (DAPI)	Thermo Fisher Scientific, USA
30% Acrylamide/Bis Solution 37.5:1	Bio-Rad, Germany
Agarose	Biozym, Germany
Ammonium Chloride (NH_4Cl)	Sigma-Aldrich, Germany
Ammonium persulphate (APS)	Bio-Rad, Germany
β-Mercaptoethanol	Bio-Rad, Germany
B-27 Supplement	Thermo Fisher Scientific, USA
Cell lysis buffer	New England Biolabs, Germany
Complete Protease Inhibitor Cocktail,	Roche, Germany
Distilled water (dH2O)	SAV Liquid Production, Germany
Dulbecco's Modified Eagle´s Medium (D-MEM)	ATCC, USA
Di-methyl sulfoxide (DMSO)	Sigma-Aldrich, Germany
Fetal bovine serum (FBS)	Biochrom, Germany
Glasgow Minimal Essential Medium (G-MEM)	Thermo Fisher Scientific, USA
Goat serum 5%	Vector Labs, USA
HepatoZYME-SFM	Thermo Fisher Scientific, USA
Universal Type I Interferon	R&D Systems, USA
L-Glutamine (200mM)	PAN Biotech, Germany
Laemmli Buffer (2x)	Bio-Rad, Germany
Methanol	Roth, Germany
Neurobasal medium	Thermo Fisher Scientific, USA
Non-essential amino acids (NEAA)	PAA, Austria
OptiPRO SFM	Thermo Fisher Scientific, USA
Paraformaldehyde (PFA)	Sigma-Aldrich, Germany
Phosphate buffered saline (PBS) (with and without Ca^{2+} and Mg^{2+})	PAN Biotech, Germany
Protein size standard	Bio Rad, Germany
P/S	PAN Biotech, Germany
Resolving gel buffer pH 8,8	Bio-Rad, Germany
Sodium dodecyl sulfate (SDS) 10%	Bio-Rad, Germany

Skim milk powder	Sigma-Aldrich, Germany
Sodium pyruvate	GE Healthcare, USA
Stacking gel buffer pH 6,8	Bio-Rad, Germany
Stripping buffer	Thermo Fisher Scientific, USA
Tris-acetate-EDTA (TAE) (50x)	Bio-Rad, Germany
Tris-buffered saline (TBS)	Bio-Rad, Germany
Transfer Buffer 1-Step	Thermo Fisher Scientific, USA
Trypan Blue	Sigma-Aldrich, Germany
TEMED (N,N,N',N'-Tetramethylethylenediamine)	Bio-Rad, Germany
Trypsin/EDTA (Ethylenediaminetetraacetic acid)	PAN Biotech, Germany
Tryptose Phosphate Broth (TPB)	Sigma-Aldrich, Germany
Triton-X	Roth, Germany
Tween20 10%	Bio-Rad, Germany
Vecta-Shield Mouting Medium	Vector Labs, USA

2.1.4 Prepared buffers and gels used for western blots

Table 4: Prepared buffers and gels

Buffer or gel	Components
Resolving gel 7,5%	5ml Acrylamide solution 5ml Resolving gel buffer (4X, pH 8,8) 200µl SDS 9,7ml dH_2O 100µl APS 10µl TEMED
Stacking gel 4%	660ml Acrylamide solution 1,26ml Stacking gel buffer (4X, pH 6,8) 50µl SDS 3ml dH_2O 25µl APS 5µl TEMED
Lysis Buffer	100µl Cell lysis buffer 40µl 860µl dH_2O

Running buffer	1,8l dH_2O 200ml Tris-buffered saline (TBS)
PBS/T	1x PBS with 0,1% Tween 20
TBS/T	1x TBS with 0,1% Tween 20

2.1.5 Kits

Table 5: Kits

Product	Company
Amersham ECL Prime Western Blot Detection Kit	GE Healthcare, USA
CellTiter 96 AQ_{ueous} Non-Radioactive Cell Proliferation Assay	Promega, USA
CytoTox 96 Non-Radioactive Cytotoxicity Assay	Promega, USA
Pierce BCA Protein Assay	Thermo Fisher Scientific, USA

2.1.6 Antibodies

Table 6: Primary antibodies

Antibody	Host	Dilution	Application	Company
α-Actin	Mouse	1:2000	Western Blot (WB)	Sigma-Aldrich, USA
α-NDV-HN	Mouse	1:1000	Immunofluorescence (IF)	Bio-Rad, Germany
α-NDV-HN	Mouse	1:500, 1:1000, 1:3000	WB	Bio-Rad, Germany
α-NDV-HN	Mouse		WB	Novus Biologicals, USA
α-VSV-G	Rabbit	1:300	IF, WB	Rockland, USA
α-VSV-M	Mouse	1:500	IF, WB	Alpha Diagnostic, USA

Table 7: Secondary antibodies

Antibody	Host	Dilution	Application	Conjugate	Company
α-Mouse	Mouse	1:250	IF	Cy3	Alpha Diagnostic, USA
α-Mouse	Goat	1:20,000	WB	HRP	Jackson Imnmunoresearch, USA

α-Rabbit	Goat	1:250	IF	FITC	Jackson Immunoresearch, USA
α-Rabbit	Goat	1:20,000	WB	HRP	Jackson Immunoresearch, USA

2.1.7 Cell lines, embryonated chicken eggs and primary cells

2.1.7.1 Cell lines

The following cell lines were purchased as frozen cultures from the American Type Culture Collection (ATCC), USA:

Table 8: Purchased cell lines

Cell line	ATCC No.
A549	CCL-185
BHK-21	CCL-10
HEK-293	CRL-1573
McA-RH7777	CRL-1601

Table 9: Provided cell lines

Cell line	Provided by
DF1	**PD Dr. Roger Vogelmann (formerly Klinikum Rechts der Isar, Munich, Germany)**
Huh7	Prof. Dr. Ulrich Lauer (University Hospital Tübingen, Germany)
HepG2	Prof. Dr. Ulrich Lauer (University Hospital Tübingen, Germany)

2.1.7.2 Specific pathogen free (SPF) embryonated chicken eggs

SPF-embryonated chicken eggs were obtained from the Charles River Laboratories, Sulzfeld, Germany.

2.1.7.3 Primary cell lines

Primary human hepatocytes (PHH) were kindly provided by Maresa Demmel and other members of the non-profit foundation Human Tissue and Cell Research (HTCR). They were isolated from patients who had undergone surgical resection of liver tumors by a two-step collagenase perfusion procedure, as previously described (Lee et al., 2013). The patients

were tested negative for hepatitis B and C virus as well as for human immunodeficiency virus and the resection was performed in accordance with the guidelines of the HTCR (Thasler et al., 2003).

Primary cortical neurons were isolated from mouse embryos (E16.5 C57/Bl6) as previously described (Kuhn et al., 2010). They were kindly provided by Dr. Alessio-Vittoro Colombo and Prof. Dr. Stefan Lichtenthaler, Deutsches Zentrum für Neurodegenerative Erkrankungen (DZNE).

2.1.8 Software

Table 10: Software

Product	Company
Mendeley Ltd.	Elsevier, Netherlands
Microsoft Office 2011	Microsoft, USA
GraphPad Prism 9.0	GraphPad Software, USA

2.2 Methods

2.2.1 Tissue culture

BHK-21 cells were maintained in Glasgow Minimal Essential Medium (G-MEM) supplemented with 10% fetal bovine serum (FBS), 1% Tryptose Phosphate Broth (TPB) and 1% penicillin/streptomycin (P/S).

Huh7, HepG2 and A549 cells were maintained in Dulbecco's Modified Eagle´s Medium (D-MEM) with high glucose supplemented with 10% FBS, 1% L-glutamine, 1% sodium pyruvate, 1% P/S and 1% non-essential amino acids (NEAA).

Morris Cells (McA-RH7777), as well as DF1, cells were cultivated in D-MEM supplemented with 10% FBS and 1% P/S.

Primary human hepatocytes (PHH) were maintained in HepatoZYME-SFM medium supplemented with 1% L-glutamine and 1% P/S.

Neurons were cultured in neurobasal medium supplemented with 2% B-27, 0.5mM L-glutamine, and 1% P/S.

DF1 cells were cultivated in a 39°C incubator with 5% carbon dioxide (CO_2). All other cell lines were cultivated in a 37°C incubator with 5% CO_2.

Primary cells (PHH and neurons) were isolated and plated shortly before use. All other cell lines were cultured in 75cm^2 flasks and passaged on a regularly basis (two to three times per week). To this end, the cell medium was removed, the cells were washed with PBS, and 2-3ml of Trypsin/EDTA were added to detach them. Once detached, the cells were resuspended in culture medium and adequate aliquots were added to new culture flasks. The cells were cultured in a total volume of 10ml per flask.

To freeze cells for later use, they were washed, detached and resuspended as described above. They were then centrifuged at 500 g for 5 minutes. Cell pellets were dissolved in culture medium supplemented with 10% DMSO and transferred to 2ml cryogenic tubes. Consequently, they were stored at -80°C in a cryogenic freezing container holding isopropyl alcohol for 24 to 72 hours. For long time storage they were subsequently moved to the liquid nitrogen tank.

To thaw cells, they were warmed up in a 37°C waterbath and, once melted, quickly resuspended in their respective culture medium. Before use, they were passaged four to five times to establish predictable growth behavior.

Cell numbers were determined using a Neubauer counting chamber. To help recognize viable cells they were dyed with Trypan Blue (1:1 dilution of culture medium and Trypan

Blue). The mean number of cells per square was calculated based on the cells counted in four adjacent squares. The cell concentration was then calculated using the following formula:

Concentration (cells/ml) = mean number of cells per square x diluting factor (2) x 10^4.

2.2.2 Viruses

Recombinant VSV (rVSV) expressing the green fluorescent protein (GFP) reporter and recombinant NDV (rNDV) carrying the modified F3aa(L289A) as well as the GFP reporter gene were engineered, rescued and produced as previously described (Jennifer Altomonte et al., 2010; Jennifer Altomonte, Braren, et al., 2008; T. G. Huang et al., 2003). They were kindly provided by Prof. Dr. Ebert and PD Dr. Altomonte.

Recombinant VSV-NDV (rVSV-NDV), a pseudotyped VSV vector in which VSV's endogenous glycoprotein had been deleted and replaced with rNDV's envelope glycoproteins HN and F3aa(L289A), was constructed and rescued by PD Dr. Altomonte and kindly provided for this study (Abdullahi et al., 2018). Briefly, NDV's modified fusion protein F3aa(L289A) was integrated into a full-length VSV plasmid (pVSV-XN2) by introducing the cDNA encoding for F3aa(L289A) as a supplementary transcription unit between VSV's glycoprotein (G) and large polymerase protein (L) genes, as previously described (Ebert et al., 2004). Then, VSV's G gene was deleted by digestion with the restriction enzymes MluI and XhoI, followed by blunting and self-ligation of the new construct. Then, a short oligonucleotide linker was introduced at the unique NheI restriction site located after the F-gene, hereby generating a multiple cloning site for the insertion of NDV's HN gene. To this end, the HN gene was PCR-amplified from a full-length NDV plasmid, and PacI and PmeI restriction sites were added at the 5' and 3' ends of the PCR product. The product was then introduced into the new plasmid construct at the above-mentioned site to preserve the natural gene order of F and HN genes as they occur in (wt)NDV. Ultimately, the newly engineered plasmid of rVSV-NDV was subjected to sequence analysis and finally rescued with the help of the established reverse genetics system for the rescue of negative-strand RNA viruses (Lawson et al., 1995; Whelan et al., 1995), though for this rescue, a plasmid encoding the VSV-G protein was co-transfected along with the standard rescue plasmids encoding N, P and L.

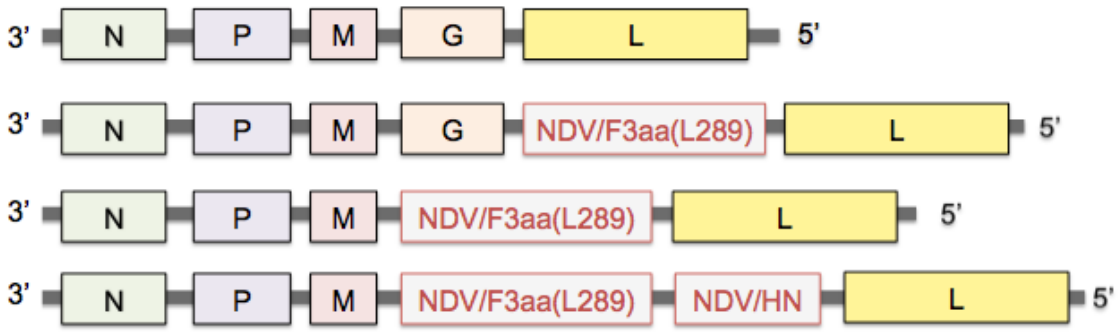

Figure 7 Schematic representation of the construction of rVSV-NDV

A plasmid encoding for the full-length VSV genome was modified in order to express NDV's envelope proteins, comprising the modified fusion protein F3aa(L289A) and the attachment protein HN, instead of VSV's endogenous glycoprotein G. To this end, the F3aa(L289A)-sequence was inserted as a supplementary transcription unit between VSV's G and L genes. Subsequently, the G gene was deleted and the HN gene was introduced behind the F gene, thereby preserving the same gene order in which the inserted envelope proteins naturally occur in NDV's genome.

2.2.3 Determination of viral titers

Viral titers were quantified using the 50% Tissue culture Infective Dose ($TCID_{50}$) assay. This endpoint dilution assay determines the amount of virus needed to cause a cytopathic effect (CPE) in 50% of the infected cells.

BHK-21 cells were seeded in 96-well plates at low confluence. Serial dilutions of the virus-containing samples were prepared with OptiPRO. The dilution range was chosen with regard to the expected viral titers, but usually six 10-fold serial dilutions were prepared. The wells were then infected in quadruplicate, with 100µl of the respective dilution added to each well. After 48 to 72 hours of incubation at 37°C, the percentage of wells containing CPE was determined for each dilution. Viral titers were then calculated by the Reed-Muench-method (Reed & Muench, 1938).

2.2.4 Establishment of a high-concentration virus stock production protocol

2.2.4.1 rVSV-NDV in BHK-21 cells

BHK-21 cells were plated in 24-well dishes at a density of 2,5 $x10^5$ cells per well and infected the following morning with rVSV-NDV at a multiplicity of infection (MOI) of either 0.1, 0.01 or 0.001. At 24, 36, 48 and 60 hours post-infection supernatants were harvested and the cells were collected by scraping in 150µl of PBS per well. The cells were then lysed by three cycles of freezing and thawing and viral titers were determined by $TCID_{50}$ analysis.

2.2.4.2 rVSV-NDV in SPF-embryonated chicken eggs

10-day-old SPF-embryonated chicken eggs were inoculated with either 10, 100 or 1000 $TCID_{50}$ of rVSV-NDV. The infectious solutions were diluted with PBS and a total volume of 100µl was injected into the allantoic cavity. The eggs were incubated at 37,8°C and 60% humidity, and after 24, 48 or 72 hours of incubation the allantoic fluid was collected, centrifuged at 1800 rpm for 5 minutes and viral titers were determined by $TCID_{50}$ analysis.

2.2.4.3 Comparison of virus propagation in different cell lines

BHK-21, DF1, A549 and HEK293 cells were seeded in 6-well plates at a density of 10^6 cells per well and infected with rVSV-NDV at an MOI of 0.001. When CPE was observed in most areas (48 hours in BHK-21 cells, 72 hours in all other cell lines), supernatants were collected and cell lysates were obtained by scraping in 150µl of PBS followed by three cycles of freezing and thawing. Viral titers were determined by $TCID_{50}$ analysis.

2.2.4.4 Comparison of different methods to produce cell lysates

BHK-21 cells were plated in 6-well dishes at a density of 10^6 cells per well and infected with rVSV-NDV at an MOI of 0.001. After 48 hours of incubation, the cells were either lysed by three cycles of freezing and thawing or by adding the detergent Triton-X to the wells. Different concentrations of Triton-X (1%, 0.1%, 0.01%, 0.001% and 0.0001%, prepared in a total volume of 600µl with OptiPRO and different incubation times (10, 30 or 60 minutes) were tested. Cell lysates were subsequently subjected to $TCID_{50}$ analysis for viral titer determination.

As a further development of the above-mentioned experiment, BHK-21 cells were plated and infected as described above. After 48 hours of incubation, cells lysates were produced by either scraping in 600µl of PBS followed by three cycles of freezing and thawing, or by a 60-minutes incubation in 600µl of 0.01% Triton-X. Subsequently, viral titers were either measured directly from the obtained cell lysates or the samples were primarily subjected to three times 30 seconds of sonication. Viral titers were determined by $TCID_{50}$ analysis.

2.2.4.5 Large-scale virus production

For large-scale virus production, three different methods were tested:

(1) Lysing cells by cycles of freezing and thawing and purifying the stock by sucrose gradient.
(2) Lysing cells by cycles of freezing and thawing, concentrating the viral stock by using Amicon Ultra-15 Centrifugal Filter Units and subsequently purifying it by sucrose gradient.

(3) Lysing cells via a combination of Triton-X and sonication, followed by sucrose-gradient-purification.

BHK-21 cells were seeded in 20 (methods (1) and (3)) or 40 (method (2)) 15cm^2 dishes. The cells were cultured until they were approximately 90% confluent. Then the cell medium was discarded and the cells were infected with rVSV-NDV at an MOI of 0.001 in 20 ml of OptiPRO. They were incubated at 37°C until CPE was observed in almost all areas, approximately after 48 hours. After incubation, the cells were collected by scraping in the conditioned medium and separated from supernatants by centrifugation at 1800 rpm for 5 minutes. From here on the methods differ:

(1) and (2): The cells were lysed by three cycles of freezing and thawing and centrifuged one more time to eliminate cell debris. Supernatants were collected and

(1) purified by sucrose gradient. To this end, three different concentration levels of sucrose were prepared and filled into a 25ml ultracentrifuge tube as follows: 7ml of 60% sucrose at the bottom, 6ml of 30% sucrose in the middle and 3ml of 10% sucrose on top. The virus-containing supernatant was then carefully layered on top and the sample was ultracentrifuged at 25000 rpm for 1 hour. This led to the formation of a virus-containing band, which was then carefully collected using a 3ml syringe and a 20G needle. Aliquots of the produced virus stock were stored at –80°C until use. Viral titers were determined by $TCID_{50}$ analysis.

(2) concentrated gradually using Amicon Ultra-15 Centrifugal Filter Units with a 100kD size cutoff. Samples were centrifuged at a speed of 3,000 RCF (relative centrifugal force) and subsequently purified by sucrose gradient as described above. Viral titers were determined by $TCID_{50}$ analysis.

(3) Cells were treated with 0.01% of Triton-X and incubated at 37°C in a total volume of 10ml of OptiPRO for 1 hour. This was followed by a brief centrifugation at 1800 rpm to eliminate cell debris. All supernatants were then ultracentrifuged at 25000 rpm for 1 hour. The virus-containing bands were collected, and each of them resuspended in 500µl of cold PBS. The suspension was transferred to microcentrifuge tubes and subjected to 3x 30 seconds of sonication. Subsequently, it was purified by sucrose gradient as described above. Viral titers were determined by $TCID_{50}$ analysis.

2.2.5 Indirect immunofluorescence assay

Huh7 cells were plated in 300µl of culture medium in 8-chamber culture slides at a density of 10^5 cells per chamber. The following day, when the cells were approximately 70-90%

confluent, they were either mock-infected with PBS or infected with rVSV, rNDV or rVSV-NDV at an MOI of 0.001 by adding the virus directly to the culture medium. After 24 hours of incubation at 37°C, the infectious medium was removed and the cells were washed with PBS and fixed with 100µl of 4% PFA per chamber for 15 minutes. The cells were then washed again with PBS for three five-minute cycles and subsequently quenched with 100µl of 50mM NH_4Cl per chamber for another 15 minutes. After another three cycles of washing, the cells were covered with 100% ice-cold methanol and transferred to -20°C for 10 minutes to permeabilize them. They were washed again and blocked in 5% goat serum in PBS/Triton (0,3% Triton-X-100) at room temperature (RT) for 60 minutes. The blocking solution was then aspirated and dilutions of the viruses' respective primary antibodies were added. The samples were then incubated at 4°C overnight. The following day, the cells were washed again and incubated together with their respective secondary antibodies (Cy3 anti-mouse for VSV-M and NDV-HN, and FITC-conjugated anti-rabbit for VSV-G) in the dark at RT for 1 hour. All samples were then counter-stained with DAPI for nuclei localization and finally mounted with Vecta-Shield Mounting Medium. Representative images were taken with an Axio Imager under 400x magnification.

2.2.6 Western blot

For protein analysis of the viruses, BHK-21 cells were plated in 6-well plates at a density of $3{,}5x10^5$ cells/well and were mock-infected with PBS or infected with either rVSV, rNDV or rVSV-NDV at an MOI of 0.01. After 14 hours of incubation at 37°C, supernatants were discarded and the cells were lysed on ice by adding 200µl of lysis buffer to each well for 10 minutes. Cell lysates were then collected, briefly vortexed and centrifuged at maximum speed for 5 minutes. Supernatants were collected and protein concentrations were determined using the photometric Pierce BCA Protein Assay. To this end, 200µl of working reagent were incubated with 10µl of each sample at 37°C for 30 minutes. The samples' absorbance was subsequently measured at 590nm. Their protein concentrations were calculated based on a standard curve constructed from serial dilutions of a protein standard. Proteins were separated according to their size by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE): 10µg of protein from each sample were topped up with PBS to a volume of 10µl, mixed with the equal volume of Laemmli-Buffer (containing β-mercaptoethanol) and incubated at 98°C for 5 minutes. Samples, as well as a protein size standard were loaded onto a resolving gel at a polyacrylamide concentration of 7.5%. Gels were run in 1x running buffer at 100V for 1,5 hours. Afterwards, the proteins were transferred from the gel onto a nitrocellulose membrane using a semi-dry transfer system set at 25V for 30 minutes. The membranes were subsequently blocked in 5% skim milk (with TBS/T) for 1

hour and incubated with the primary antibodies at 4°C overnight. The following morning, the cells were washed with TBS/T for three five-minute cycles prior to being incubated with the respective secondary antibody at RT for 1 hour. Membranes were washed again and protein bands were visualized on CL-XPosure Film using the Amersham ECL Prime Western Blotting Detection Kit in accordance with the manufacturer's recommendations. Actin was used as an internal control. If the same membrane needed to be reused to detect a different protein, it was washed with TBS/T, stripped with stripping buffer for 15 minutes, washed again and blocked.

2.2.7 Growth curves

To assess the ability of the viruses to grow and replicate in different cell lines, viral growth curves were analyzed in HCC cell lines (Huh7, HepG2 and McA-RH7777), as well as in primary human hepatocytes and primary mouse neurons. HCC cell lines were seeded in 6-well dishes at a density of $3.5x10^5$ cells per well, while PHH and neurons were plated in collagen-coated 24-well dishes at a density of 10^5 cells per well. The following day, the cells were infected with either rVSV, rNDV or rVSV-NDV at an MOI of 0.01. The infections were performed in a total volume of either 1ml (6-well dishes) or 250µl (24-well dishes) of PBS. After incubation with the infectious solution at 37°C for 1 hour, the cells were washed three times with PBS, and fresh medium was added. At 0, 16, 24, 48 and 72 hours post-infection, supernatants were removed, 600µl (6-well dishes) or 150µl (24-well dishes) of PBS were added, and the cells were collected by scraping in PBS. The cells were then lysed by three cycles of freezing and thawing and intracellular viral titers were determined by $TCID_{50}$ analysis.

2.2.8 Cytotoxicity assays

2.2.8.1 CytoTox 96 Non-Radioactive Cytotoxicity Assay

Cytotoxic effects of the viruses on HCC cell lines (Huh7, HepG2 and McA-RH7777) and PHH were assessed by performing the CytoTox 96 Non-Radioactive Cytotoxicity Assay. This colorimetric assay determines the amount of lactate dehydrogenase (LDH) released from the cells into the supernatant. LDH is an almost ubiquitous cytosolic enzyme that is released upon cell death and can therefore be used to analyze the effectiveness with which the viruses kill cells.

HCC cells and PHH were plated, infected and washed as described for the growth curve experiments. Extra wells were plated in order to be mock-infected or to serve as maximum

LDH-release control. Also, wells containing only culture medium were included in the experimental setup to serve as absorbance background control.

At 24, 48, and 72 hours post-infection, aliquots of supernatants or culture medium only were collected. Supernatants of the non-infected maximum LDH-release control wells were treated with 10x lysis buffer and incubated at 37°C for 45 minutes. Subsequently, all supernatants were centrifuged at 1800 rpm for 5 minutes to separate them from residual cells, and cell debris was discarded. Supernatants from the 24- and 48-hours time-points were temporarily stored at 4°C until the common endpoint of 72 hours, in order to be able to evaluate all samples at the same time. 50µl of each sample were then transferred to a flat bottom 96-well plate and the Cytotoxicity Assay was performed following the manufacturers protocol. LDH-release was determined by measuring the absorbance at 490nm using a 96-well plate reader. The viruses-mediated cytotoxicity was subsequently calculated by first subtracting the mean background absorbance (based on the absorbance values obtained from the medium-alone control-wells) from all other absorbance values, followed by subtraction of the mean baseline LDH-release (released by mock-infected cells) from the residual samples' absorbance values. Then, the cytotoxicity of the experimental wells was calculated as a percentage of the mean maximum LDH-release control.

2.2.8.2 CellTiter96 AQueous One Solution Cell Proliferation Assay

Cytotoxic effects on neurons were analyzed by using the CellTiter96 AQueous One Solution Cell Proliferation Assay. It is another colorimetric assay, which makes use of the fact that one of its compounds, namely MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carbooxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), gets metabolized by viable cells into a formazan product, the absorbance of which can then be detected.

Neurons were seeded in collagen-coated 96-well dishes at a density of $5x10^4$ cells per well. They were mock-treated or infected with rVSV, rNDV or rVSV-NDV at an MOI of 0.01 24, 48 or 72 hours before the assay was performed in order to achieve a common endpoint. Right before the assay was performed, the old culture medium was replaced by 100µl of fresh culture medium, so that equal original conditions were established. Also, 100µl of culture medium was then added to some extra wells to be able to detect background absorbance by culture medium only. After the medium exchange, 20µl of MTS solution were added to the wells and the plate was incubated at 37°C for 2 hours, in accordance with the manufacturers protocol. The absorbance was then recorded at 490nm using a 96-well plate reader. Background absorbance was subtracted from the experimental wells and cell viability was calculated as a percentage of the mean of the mock-treated control wells. Cytotoxicity was subsequently specified as the difference in cell viability between the experimental samples and the uninfected controls.

2.2.9 Microscopic analysis of infected cells

HCC cell lines (Huh7, HepG2 and McA-RH7777), PHH and neurons were plated, infected and washed as described for the growth curve experiments. At 0, 16, 24, 48 and 72 hours post-infection, representative images of 200x magnified cells were taken with an AxioCam ICm1 camera attached to an Axiovert 40CFL microscope.

2.2.10 Live cell movie analysis

Huh7 cells, PHH and healthy mouse neurons were infected with either rVSV, rNDV or rVSV-NDV at an MOI of 0.01 and recorded over a time span of 72 hours using the “JuLI Br” live cell analyzer.

2.2.11 Interferon protection assay

Interferon-sensitive A549 cells were plated in 24-well dishes at a density of 10^5 cells per well and cultured in 1ml of the respective cell medium. The following evening, the cells were pretreated with one of four different concentrations of universal type I Interferon (IFN) (0, 100, 500 or 1000 IU/ml, diluted with OptiPRO), which were added directly to the culture medium. After overnight incubation, fresh medium, again containing the respective IFN concentration, was added, and duplicates were infected with either rVSV, rNDV or rVSV-NDV at an MOI of 0.01. 48 hours post-infection, supernatants were discarded, and the cells were collected by scraping in 100µl of PBS. Subsequently, the cells were lysed by three cycles of freezing and thawing, and the intracellular virus titer was determined by $TCID_{50}$ analysis.

2.2.12 Statistical analysis

Data were plotted and analyzed using GraphPad Prism 6.0. For statistical analysis, a two-sided Student’s t-test was used. P-values of less than 0.05 were considered to be statistically significant.

3 Results

3.1 Establishment of a high-concentration virus stock production protocol

3.1.1 Determination of a suitable host platform

In order to be able to produce high-titered viral stocks of rVSV-NDV, several propagation conditions were tested. First of all, an optimal host platform needed to be determined. Initially, virus replication in BHK-21 cells was investigated, as VSV, rVSV-NDV's backbone, is known to efficiently replicate in this cell line (e.g. Ebert et al. 2003; Altomonte et al. 2009). To this end, BHK-21 cells were infected at several MOIs (0.1, 0.01, 0.001) and viral titers of supernatants as well as cell lysates were determined at several time-points (24, 36, 48 and 60 hours post-infection) by $TCID_{50}$ analysis. Comparing the results, titers measured from cell lysates were at all times found to be higher than those measured from supernatants. Peak results ($5.62x10^5$) were obtained from cell lysates of those samples that were incubated with rVSV-NDV for 48 hours at an MOI of 0.001 (see Figure 8 (A and B)).

Next, replication of rVSV-NDV was studied in embryonated chicken eggs, being the method of choice for NDV production (e.g. Altomonte et al. 2010). For this purpose, 10-day old embryonated chicken eggs were infected with either 10, 100 or 1000 PFU of rVSV-NDV and viral titers were measured from the allantoic fluid collected 24, 48 and 72 hours post-infection. Viral replication turned out to be much less effective than in BHK-21 cells, with titers ranging between 3.16 and $1.78x10^4$ $TCID_{50}$/ml. (see Figure 8 (C)).

Finally, viral growth was compared in four different cell lines, which are all known to be potent replication platforms for virus stock productions: BHK-21 cells are, as mentioned, a common host cell line for the production of VSV. The embryonic chicken fibroblast cell line DF-1 is an established cell line for production of avian viruses (Himly et al., 1998; Schaefer-Klein et al., 1998). Human embryonic kidney (HEK) 293 cells are commonly used for adenovirus propagation, and the human lung carcinoma cell line A549 has also been investigated for this purpose (Smith et al., 1986). The listed cell lines were infected with rVSV-NDV at an MOI of 0.001, which had already been proven to be an efficient application dose. The cells were incubated until CPE was observed in most areas (48 hours in BHK-21 cells, 72 hours in the other cell lines) and viral titers were analyzed from supernatants and lysates, respectively. In analogy with our previous findings, higher titers were measured from cell lysates, and, although titers were comparable, infection in BHK-21 cells resulted in the highest yield (mean $2.47x10^6$) (see Figure 8 (D)).

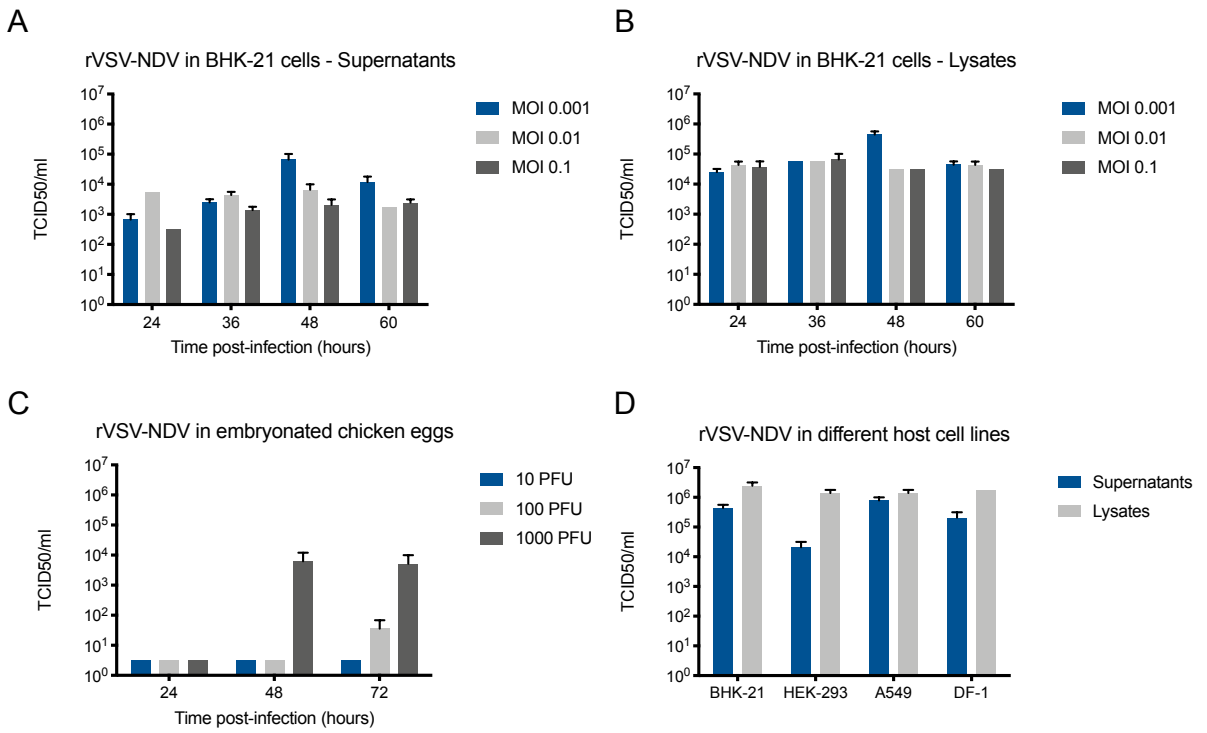

Figure 8 Comparison of different host platforms for virus propagation

*(**A** and **B**) BHK-21 cells were infected with rVSV-NDV at different MOIs of 0.1, 0.01 or 0.001. 24, 36, 48 or 60 hours post-infection, (**A**) supernatants and (**B**) cell lysates were harvested and subjected to $TCID_{50}$ analysis for determination of viral titers. (**C**) 10 day-old embryonated chicken eggs were inoculated with either 10, 100 or 1000 PFU of rVSV-NDV and viral titers were determined at 24, 48 or 72 hours post-infection by $TCID_{50}$ analysis. (**D**) BHK-21, DF1, A549 and HEK293 cells were infected with rVSV-NDV at an MOI of 0.001. When CPE was observed in most areas (48 hours in BHK-21 cells, 72 hours in all other cell lines), supernatants and cell lysates were collected and viral titers of the samples were determined by $TCID_{50}$ analysis. Experiments were performed in duplicate (except for the 48h time-point in (**C**), which was performed in triplicate). Results are presented as means +/- SEM.*

3.1.2 Optimization of cell lysis

Once we had decided on BHK-21 cells as promising host cell line for production of rVSV-NDV-stocks, different methods of cell lysis were investigated, because lysates had been shown to contain more virus than supernatants (see Figure 8). To this end, BHK-21 cells were infected with rVSV-NDV at an MOI of 0.001 and incubated for 48 hours. Subsequently, cells were lysed by either three cycles of freezing and thawing or by incubation with different concentrations of the detergent Triton-X. While 1% Triton-X caused complete cell destruction, incubation with lower percentages of Triton-X led to increased titers compared to standard cell lysis by freezing and thawing (see Figure 9 (A)). Highest titers were achieved by incubation with 0.01% Triton-X for 60 minutes. This approach was further investigated and combined with sonication, which resulted in great improvement of virus yields as shown in Figure 9 (B).

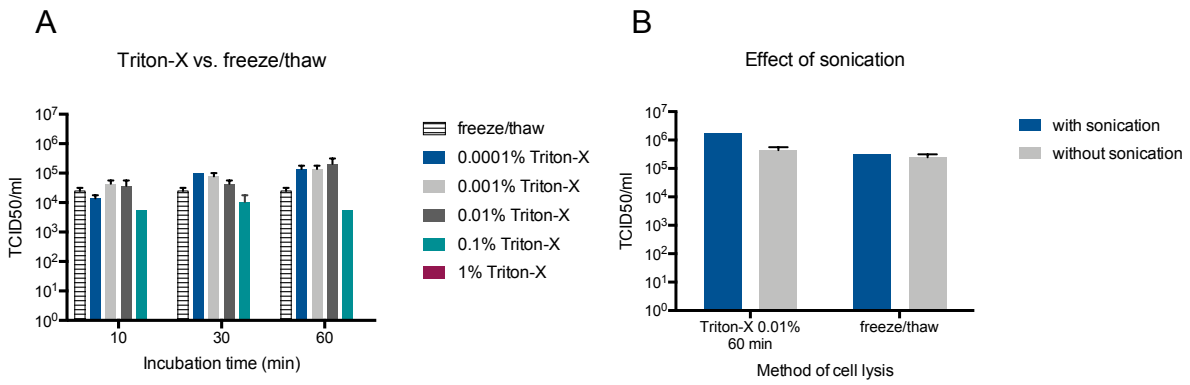

Figure 9 Comparison of different methods of cell lysis

*BHK-21 cells were plated in 6-well dishes and infected with rVSV-NDV at an MOI of 0.001. (**A**) 48 hours post-infection, cells were lysed by three cycles of freezing and thawing or by incubation with different concentrations of Triton-X (1%, 0.1%, 0.01%, 0.001% or 0.0001%) for either 10, 30 or 60 minutes. (**B**) 48 hours post-infection, cells were lysed by three cycles of freezing and thawing or by a 60 minutes-incubation in 0.01% Triton-X. Subsequently, titers were either measured directly from the obtained cell lysates or samples were primarily subjected to three times 30 seconds of sonication. Experiments were performed in duplicate and means +/- SEM are plotted. Viral titers were determined by $TCID_{50}$ analysis.*

3.1.3 Large-scale virus production

With regard to further experiments, especially upcoming *in-vivo* studies, high amounts of high-titered, purified virus stocks were needed. To this end, different protocols for large-scale virus production were evaluated: Based on our previous findings, BHK-21 cells were infected in 15cm^2 dishes with rVSV-NDV at an MOI of 0.001, incubated for approximately 48 hours (until CPE was observed in most areas) and cells were subsequently (1) lysed by three cycles of freezing and thawing (2) lysed by three cycles of freezing and thawing and concentrated using Amicon Ultra-15 Centrifugal Filter Units or (3) lysed by the above mentioned combination of Triton-X 0.01% and sonication. All production methods were followed by purification of the produced viral stock by sucrose gradient as described in section 2.2.4.5. Best results were obtained by method (3) with a final, purified titer measuring 1.78x10^6 $TCID_{50}$/ml (see Figure 10).

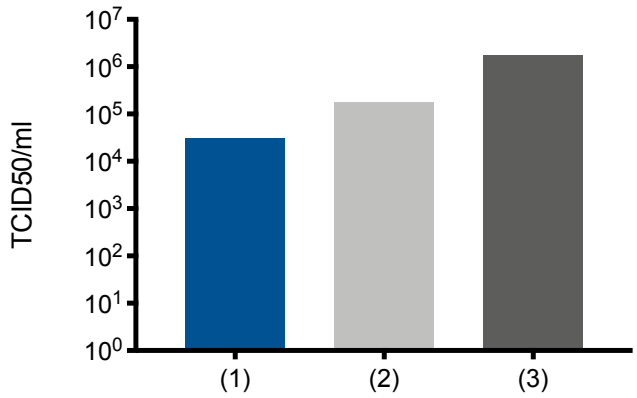

Figure 10 Comparison of different strategies for large-scale virus production

*BHK-21 cells were seeded in 15cm^2 dishes and infected with rVSV-NDV at an MOI of 0.001. Approximately 48 hours post-infection cells were lysed by (**1**) three cycles of freezing and thawing (**2**) three cycles of freezing and thawing followed by concentration using Amicon Ultra-15 Centrifugal Filter Units or (**3**) lysed by 0.01% Triton-X combined with sonication. All production methods were followed by purification of the produced viral stock by sucrose gradient and titers were determined by $TCID_{50}$ analysis.*

3.2 *In-vitro* characterization of rVSV-NDV

3.2.1 Geno- and phenotypic characterization

To investigate whether VSV's glycoprotein G had successfully been replaced with the envelope proteins of rNDV (HN and F), western blot analysis and indirect immunofluorescence staining were performed. Cells were infected with either rVSV, rNDV or rVSV-NDV, and screened for protein expression of VSV-G, VSV-M and NDV-HN. Unfortunately, we did not have access to antibodies able to detect NDV-F.

Samples for western blot analysis were extracted from infected BHK-21 cells while indirect immunofluorescence staining was performed on Huh7 cells. As anticipated, rVSV-infected cells expressed VSV-G and VSV-M proteins, while rNDV-infected cells showed positive results for NDV-HN. With regard to the novel vector, both assays displayed that rVSV-NDV infected cells still express rVSV's matrix protein M, but that they no longer express the glycoprotein G, indicating the successful elimination of the latter. Moreover, immunofluorescence staining confirmed that rVSV-NDV's genome now comprises the rNDV-HN protein. Sadly, western blot analysis for this protein didn't work.

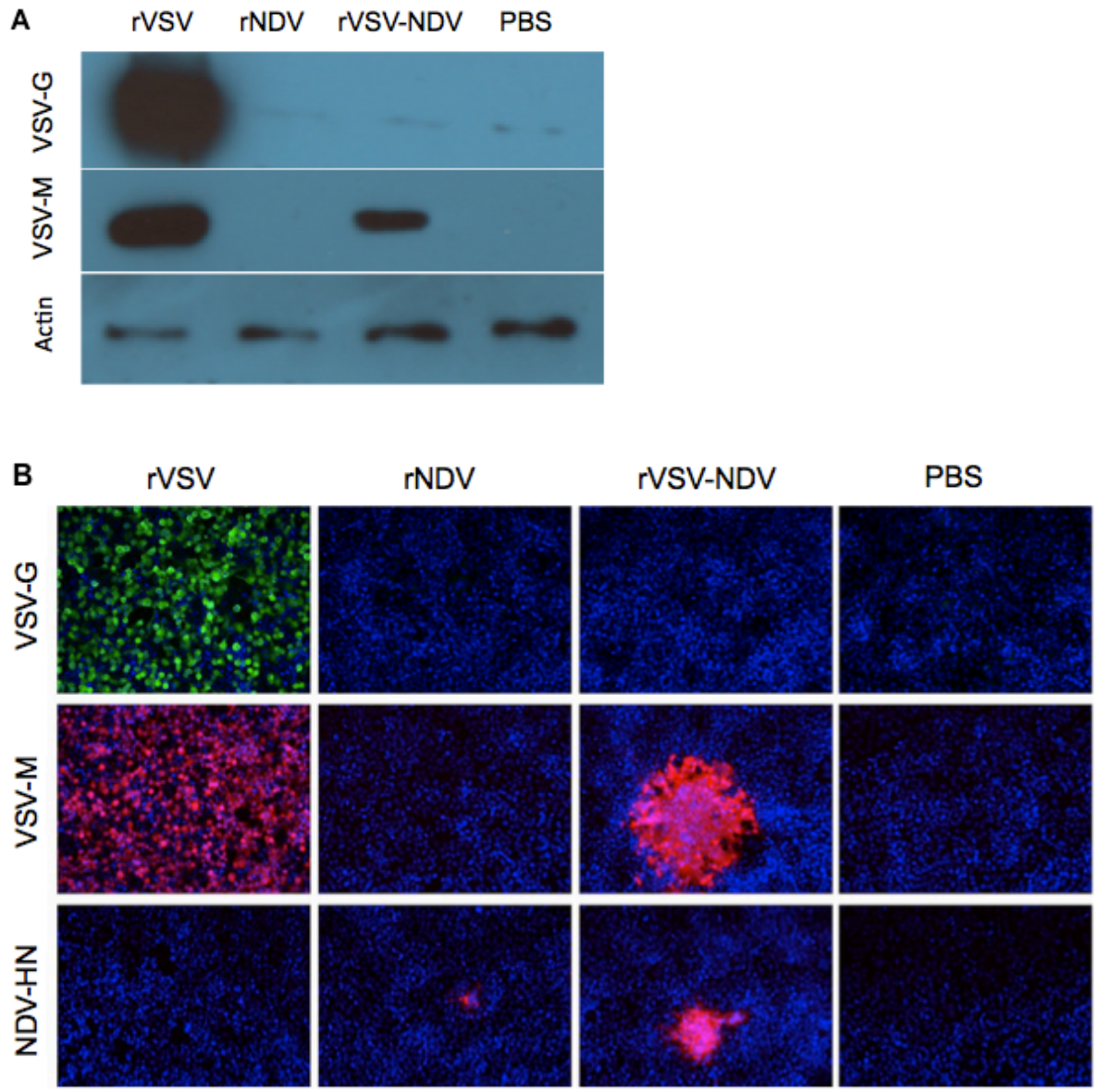

Figure 11 Western blot analysis and indirect immunofluorescence staining

*(**A**) BHK-21 cells were mock-infected or infected with either rVSV, rNDV or rVSV-NDV at an MOI of 0.01. 14 hours post-infection, cell lysates were harvested and 10µg of protein samples were subjected to western blot analysis and screened for expression of VSV-G and VSV-M. Actin served as internal control. (**B**) Huh7 cells were either mock-infected or infected with rVSV, rNDV or rVSV-NDV at an MOI of 0.001. 24 hours post-infection, cells were fixed with 4% PFA and protein expression of VSV-G, VSV-M and NDV-HN was analyzed by indirect immunofluorescence. All samples were counter-stained with DAPI for nuclei localization. Representative images are shown at 400x magnification* (Abdullahi et al., 2018).

3.2.2 Evaluation of rVSV-NDV's oncolytic activity *in-vitro*

To assess the chimeric vector's capability to effectively replicate in and kill liver tumor cells, viral growth curves and LDH-release levels were analyzed in two human HCC cell lines (Huh7 and HepG2) as well as in one rat hepatoma cell line (McA-RH7777). Results upon infection with rVSV-NDV were then compared to those achieved by its parental viruses rVSV and rNDV. The cell lines were infected at an MOI of 0.01 and viral growth curves were plotted based on titers measured from cell lysates 0, 16, 24, 48 and 72 hours post-infection. The quantity of LDH, released by the infected cells into the supernatant upon cell death, was determined at 24, 48 and 72 hours post-infection. For further illustration of the results, representative images of the infected cell lines were taken 0, 16, 24, 48 and 72 hours post-infection. Additionally, infection in Huh7 cells was recorded on video over a time span of 72 hours.

Analysis of the viral growth behavior in human HCC cell lines indicated attenuated replication of rVSV-NDV compared to its parental vector rVSV, but similar replication kinetics as presented by rNDV (Figure 12 (A and B)). As shown, rVSV replicated rapidly, reaching maximum titers in average of 1.0-1.3x10^8 after 16 to 24 hours while rVSV-NDV and rNDV seemed to replicate more slowly and less efficiently, peaking at 48 hours post-infection with maximum titers averaging 2.1x10^6 (rNDV) or 7.5x10^5 (rVSV-NDV) only. LDH-release levels measured from those samples indicated lower levels of cytotoxicity at early time-points upon infection with rVSV-NDV compared to its parental vectors, but nevertheless demonstrated complete cell killing in both cell lines for all viruses by 72 hours post-infection (Figure 12 (C and D)). Also, representative pictures of the infected cells, captured at the indicated time-points, showed clear signs of syncytia formation in rVSV-NDV-infected cells as early as 16 hours post-infection (Figure 13 and 14). Compared to cells infected with the parental viruses, early signs of CPE were observed at the same time-point as they were in rVSV-infected cells, and even earlier than in rNDV-infected cells. Complete cell lysis of both cell lines was observed at 48 hours post-infection for rVSV-NDV- and rNDV-infected cells and at 24 hours post-infection for rVSV-infected cells. These findings of early syncytia induction and effective cell killing by rVSV-NDV were supported by analysis of video material tracking the morphological development of infected Huh7 cells over the course of 72 hours, showing first signs of fusion-mediated CPE after 12 and complete cell killing by 48 hours post-infection.

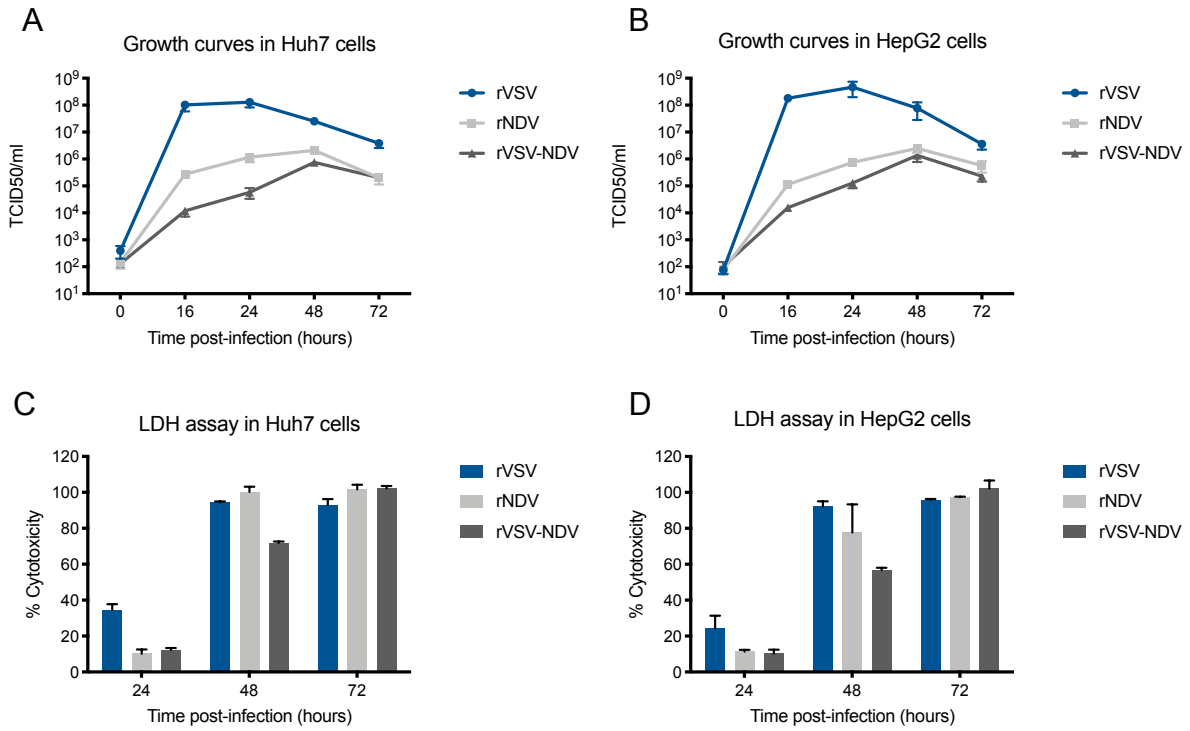

Figure 12 Viral growth curves and LDH-cytotoxicity assays in the human HCC cell lines Huh7 and HepG2

*Huh7 and HepG2 cells were infected with either rVSV, rNDV or rVSV-NDV at an MOI of 0.01. At 0, 16, 24, 48 and 72 hours post-infection, cell lysates from (**A**) Huh7 and (**B**) HepG2 cells were collected and intracellular virus titers were determined by $TCID_{50}$ analysis. Additionally, at 24, 48 and 72 hours post-infection, samples of supernatants from infected (**C**) Huh7 and (**D**) HepG2 cells were harvested and the quantity of LDH-release upon cell death was determined. For each experiment, the cells were infected in duplicate and mean values +/- SEM of three individual experiments are shown (adapted from* (Abdullahi et al., 2018)*).*

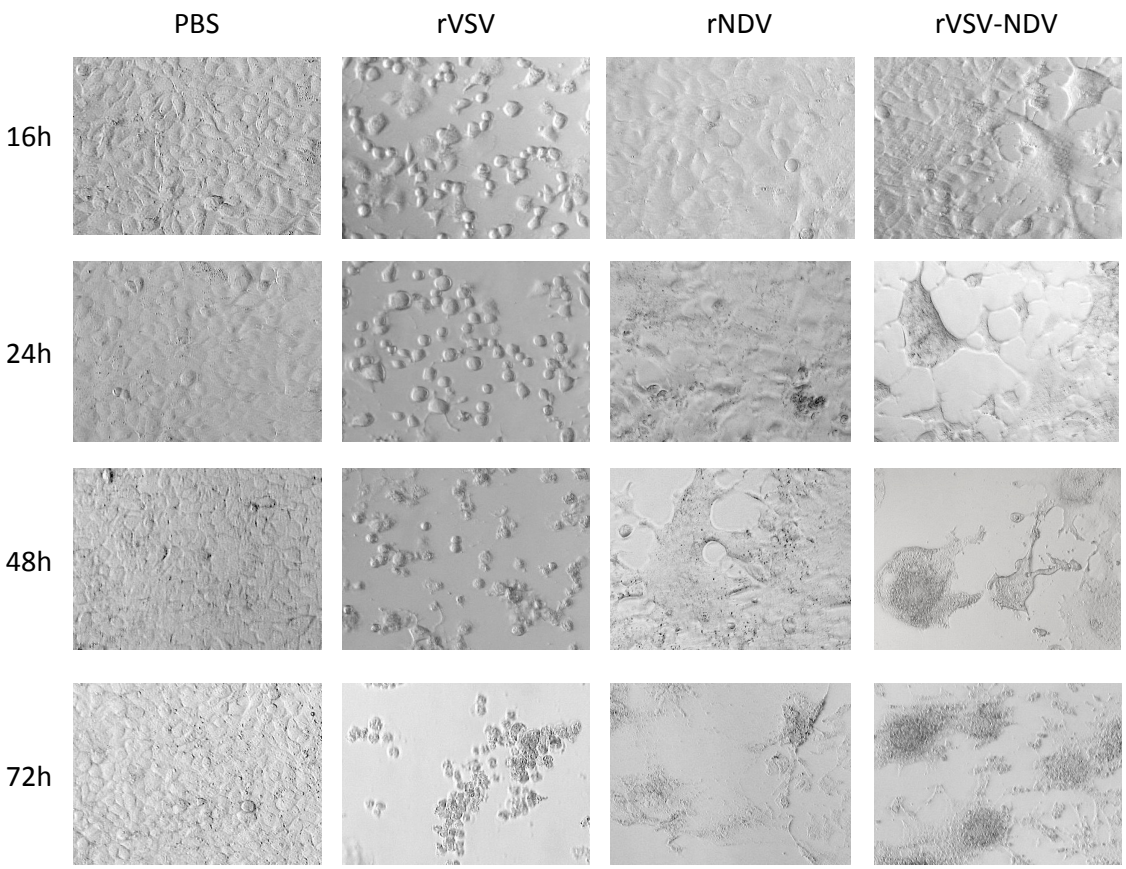

Figure 13 Pictures of infected Huh7 cells

Representative fields of view are shown of pictures taken of infected Huh7 cells at 16, 24, 48 and 72 hours post-infection at 200x magnification.

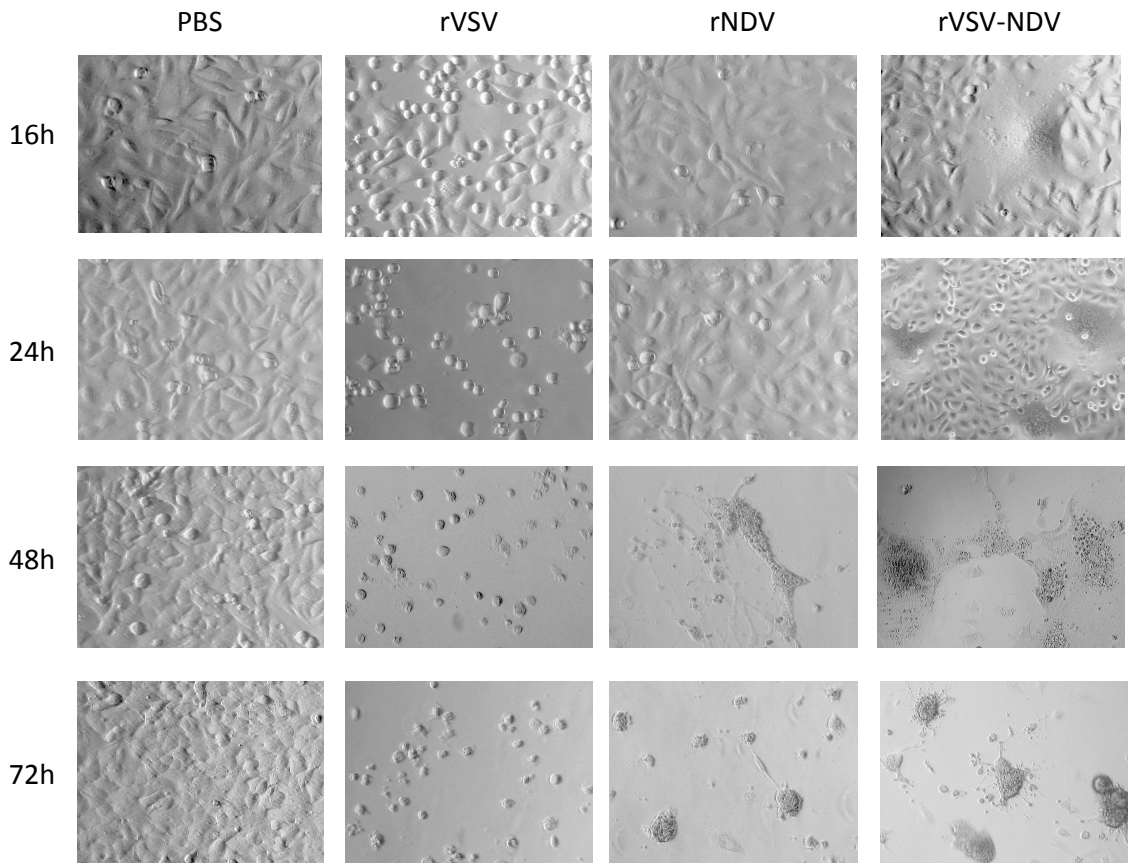

Figure 14 Pictures of infected HepG2 cells

Representative fields of view are shown of pictures taken of infected HepG2 cells at 16, 24, 48 and 72 hours post-infection at 200x magnification.

In contrast to infection in human HCC cell lines, infection with rVSV-NDV in McA-RH7777 cells did not lead to efficient viral replication, nor to efficient cell killing. Analysis of the respective data revealed only low replication titers as well as low levels of cytotoxicity (Figures 15 (A) and (B)). Captured pictures of the infection showed almost no signs of syncytia formation or cell killing (Figure 16). Analysis of the impact of infection with the parental vectors on McA-RH7777 cells showed effective replication and cell killing for rVSV-infected cells, but replication and cell killing abilities of rNDV were also attenuated, compared to results achieved in human HCC cell lines,

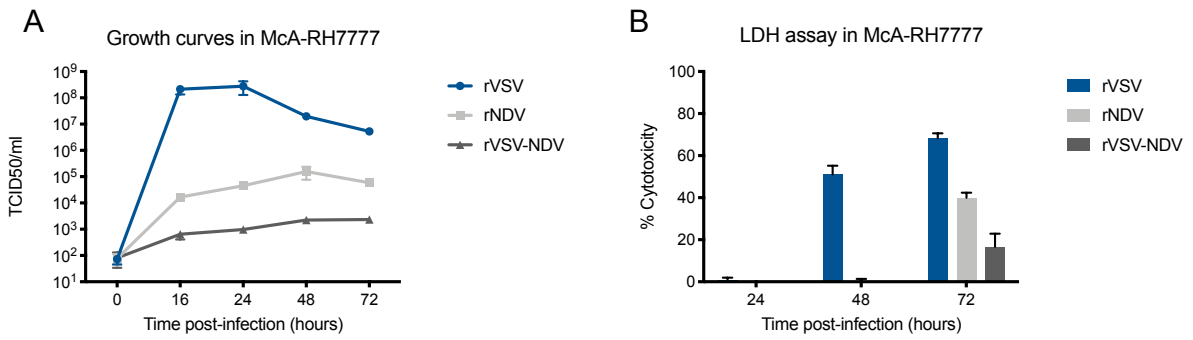

Figure 15 Viral growth curves and LDH cytotoxicity assay in McA-RH7777 cells

McA-RH7777 cells were infected with either rVSV, rNDV or rVSV-NDV at an MOI of 0.01. (A) At 0, 16, 24, 48 and 72 hours post-infection, cell lysates were collected and intracellular virus titers were determined by $TCID_{50}$ analysis. (B) Additionally, at 24, 48 and 72 hours post-infection, samples of supernatants were harvested and the quantity of LDH-release upon cell death was determined. For each experiment, the cells were infected in duplicate and mean values +/- SEM of three individual experiments are shown (adapted from (Abdullahi et al., 2018)*).*

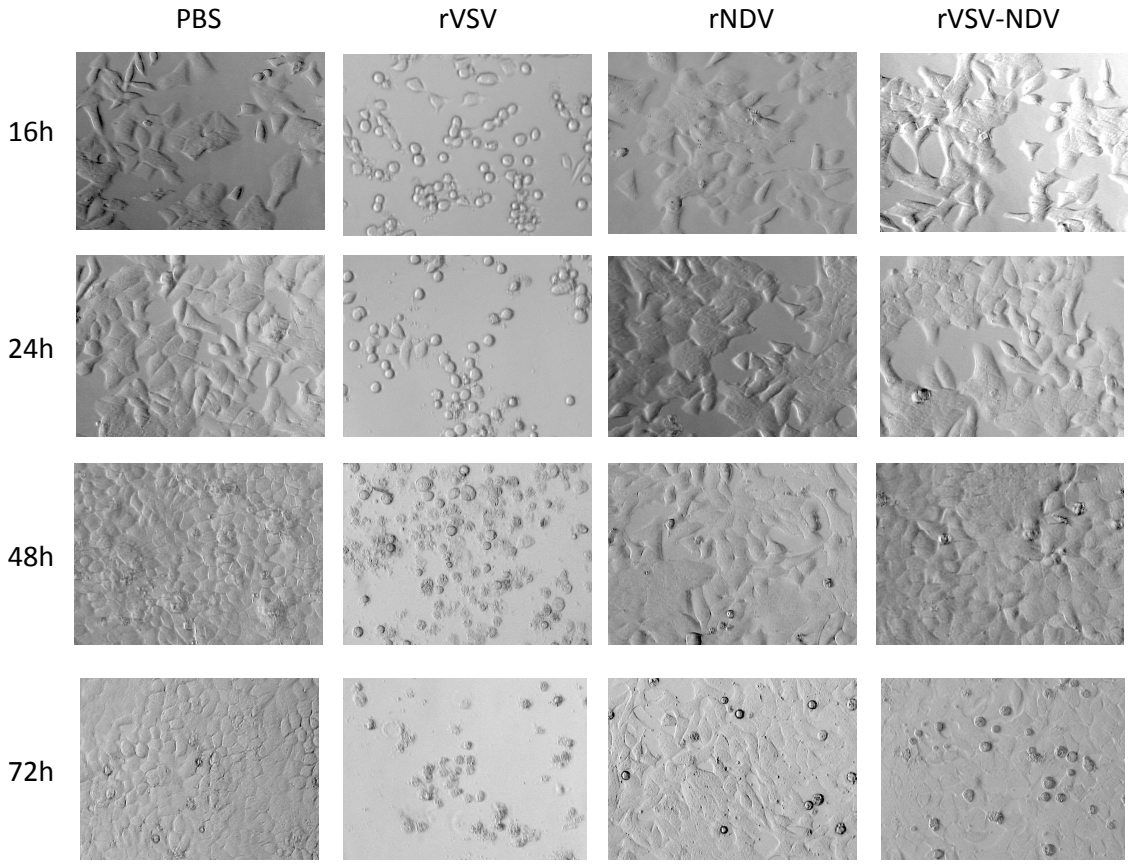

Figure 16 Pictures of infected McA-RH7777 cells

Pictures of infected McA-RH777 cells were captured 0, 16, 24, 48 and 72 hours post-infection at 200x magnification. Representative fields of view are shown.

3.2.3 Evaluation of rVSV-NDV's safety profile *in-vitro*

To evaluate the pseudotyped virus' safety profile, especially in comparison with its parental vector rVSV, which is known to cause severe neurotoxicity, viral replication and cytotoxic effects of the viruses were studied in primary human hepatocytes (PHH) as well as in healthy mouse neurons. Replication of rVSV-NDV in PHH was greatly diminished as compared to the parental vectors, with titers measured at 48 hours post-infection being five logs lower than determined for rVSV-infected hepatocytes and three logs lower than for rNDV-infected ones (Figure 17 A). Also, LDH-release was found to be significantly lower in rVSV-NDV-infected cells as opposed to both rVSV- and rNDV (p-value <0.05 at 72 hours post-infection), with only minimal cytotoxicity levels measured for rVSV-NDV at all investigated time-points (Figure 17 C). Additionally, microscopic analysis of rVSV-NDV infected cells showed no signs of syncytia formation, whereas rNDV-infection caused clearly visible cell fusion.

Investigation of the corresponding data in primary mouse neurons revealed that replication of rVSV-NDV and rNDV was attenuated in comparison with rVSV and statistical analysis proved cytotoxicity levels of both rVSV-NDV and rNDV to be significantly lower at all investigated time-points (p-value < 0.05) (Figure 17 B and D).

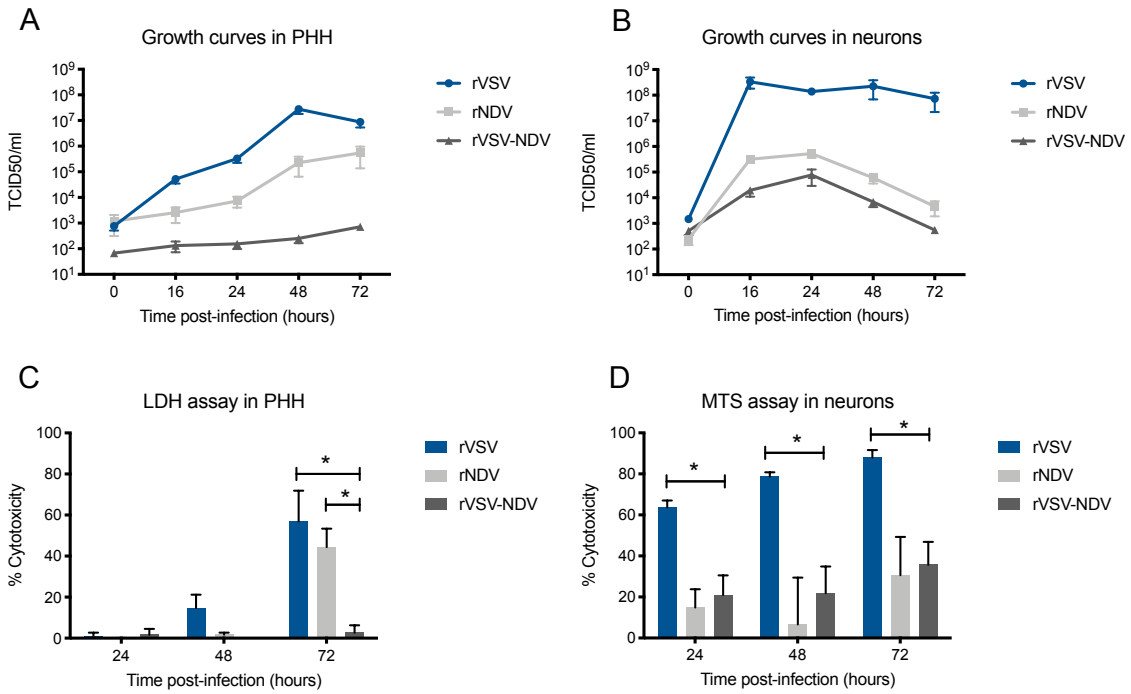

Figure 17 Viral growth curves and cytotoxicity-assays in PHH and healthy mouse neurons

*Primary human hepatocytes and primary mouse neurons were infected with either rVSV, rNDV or rVSV-NDV at an MOI of 0.01. (**A** and **B**) At 0, 16, 24, 48 and 72 hours post-infection, cell lysates were collected and intracellular virus titers were determined by $TCID_{50}$ analysis ((**A**) PHH, (**B**) neurons). (**C** and **D**) At 24, 48 and 72 hours post-infection, samples of supernatants were harvested and levels of cytotoxicity were determined using an LDH-release quantification assay for PHH, (**C**) or an MTS-assay for neurons (**D**). For each experiment, the cells were infected in duplicate and mean values +/- SEM of three individual experiments are shown. * p<0.05. (*adapted from (Abdullahi et al., 2018)*).*

Sensitivity to type I Interferon (IFN) is one of the key mechanisms of tumor selectivity and therefore one of the key mechanisms of safety of oncolytic viruses. This property is especially well characterized for VSV (Balachandran & Barber, 2000; Barber, 2004; Lichty et al., 2004; Stojdl et al., 2000). To assess whether the newly engineered vector had preserved precisely this sensitivity despite the replacement of its glycoprotein, an interferon protection assay was performed. To this end, IFN-sensitive A549 cells were pretreated with increasing doses of universal type I IFN and infected with either rVSV, rNDV or rVSV-NDV at an MOI of 0.01. 48 hours post-infection, viral titers were determined from cell lysates and responsiveness to type I IFN was compared between the viruses. Analysis of the data confirmed rVSV and rVSV-NDV to be susceptible to the actions of type I IFN, with titers dropping three to five logs to a level of 10^2 - 10^3. In contrast, only minimal changes of titers were measured in rNDV-infected cells, revealing a relative insensitivity of the virus to type I IFN in this assay.

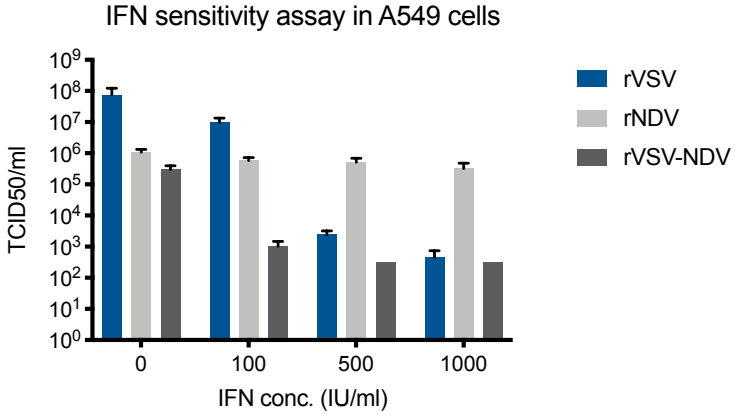

Figure 18 Interferon protection assay

IFN-sensitive A549 cells were plated in 24-well dishes and pretreated or mock-treated with escalating doses of universal type I IFN (0, 100, 500, 1000 IU/ml). The following day, the cells were infected with either rVSV, rNDV or rVSV-NDV at an MOI of 0.01. 48 hours post-infection, viral titers were determined from cell lysates by $TCID_{50}$ analysis. The cells were infected in duplicate and mean values +/- SEM of three individual experiments are shown (adapted from (Abdullahi et al., 2018)*).*

4 Discussion

4.1 Establishment of a high-concentration virus stock production protocol

A well-established production protocol that results in high yields of purified stock is a crucial requirement for the preclinical and clinical applicability of every oncolytic vector. In order to find optimal propagation conditions and purification methods for the newly engineered virus, several approaches were considered. As rVSV-NDV is a hybrid construct of VSV and NDV, we first investigated whether it would preferentially replicate in BHK-21 cells, a cell line often chosen to produce VSV (Jennifer Altomonte, Braren, et al., 2008; Ebert et al., 2003), or in embryonated chicken eggs, the method of choice for the production of NDV (Jennifer Altomonte et al., 2010). Virus production in BHK-21 cells turned out to be much more efficient than production in embryonated chicken eggs (see Figure 8). In fact, in most eggs, no propagation of the virus could be observed at all, and maximum titers achieved from those solitary eggs in which inoculation with rVSV-NDV actually lead to propagation were still 4 logs lower than usually produced by rNDV (Jennifer Altomonte et al., 2010) This might be explained by the fact that rVSV-NDV, despite being a hybrid construct of both VSV and rNDV, is built on the backbone of VSV: Although it now holds NDV's glycoproteins F and HN, replication kinetics are still mediated by VSV's remaining proteins. In particular, the absence of NDV's V protein could be the reason why rVSV-NDV is no longer able to replicate efficiently in embryonated chicken eggs, as this avian specific IFN-antagonist is said to be crucial for its pathogenicity in birds and an important determinant of host cell restriction (Park, García-Sastre, et al., 2003; Park, Shaw, et al., 2003).

A second important finding was that during production studies, titers measured from cell lysates were at all times higher than titers measured from supernatants (see Figure 8). The underlying reason might be that the mechanism of viral spread was altered by the integration of the F protein into rVSV-NDV's genome: Instead of virion release upon cell death, as induced by VSV infection, the F protein allows the hybrid virus to mediate syncytia formation and therefore to preferentially spread intracellularly by direct cell-to-cell fusion (Matveeva et al., 2015).

As higher titers were obtained from cell lysates, different methods for cell lysis were tested to increase virus yields. Cell lysis through the application of 0.01% Triton-X in combination with sonication was shown to be the most efficient and was used for large-scale virus production of the hybrid vector (see Figures 9 and 10).

Finally, especially with regard to upcoming animal studies as well as potential clinical trials, it was crucial to guarantee a purified viral stock. For this purpose the virus was applied to a sucrose gradient, a method that had previously been proven to be effective both for VSV and for NDV purification (Jennifer Altomonte et al., 2010; Jennifer Altomonte, Braren, et al., 2008).

In conclusion, we were able to successfully establish a production protocol for a purified stock of rVSV-NDV. Based on these findings, the protocol has been further improved by our laboratory, resulting now in titers as high as $3x10^8$. This outcome allows for animal studies, and with the possibility of further development of the protocol, even clinical studies would be accomplishable.

4.2 In-vitro characterization of rVSV-NDV

4.2.1 Geno- and phenotypic characterization

By western blot analysis and indirect immunofluorescence staining we were able to show that rVSV-NDV preserved VSVs backbone, as it expresses VSVs matrix protein M, but that it no longer encodes for VSVs glycoprotein G. Instead, we were able to demonstrate the expression of NDVs glycoprotein HN by indirect immunofluorescence staining. Sadly, western blot analysis for this protein didn't work, although several protocols and antibodies were tested. As it worked for neither rVSV-NDV nor rNDV, we assume that this is more likely because of a methodological problem rather than a reflection of the real absence of this protein. One explanation for the assay not to work could be that the antibodies available to us at the time were all directed against the HN protein of the NDV LaSota strain, and maybe therefore didn't work to detect HN protein of the Hitchner B1 strain, at least not for western blot analysis. Also, we did not have access to an antibody to monitor the expression of the F protein. However, analysis of representative pictures taken of immunofluorescent-stained cells suggested that rVSV-NDV-infection leads to fusion-mediated syncytia formation as opposed to a classical CPE as induced by VSV. (see Figure 11). Additionally, the presence of the F- and HN genes was confirmed by reverse transcription-PCR analysis of RNA isolated from infected cells, performed by PD Altomonte.

4.2.2 Evaluation of rVSV-NDV's efficacy and safety profile

The two major properties of a virus, which determine whether it is regarded as a promising oncolytic vector, are its efficacy in killing tumor cells and its safety profile in terms of limited off-target toxicity. rVSV-NDV was developed as a novel oncolytic agent in order to combine

the positive features of VSV and NDV while eliminating the negative ones. It was investigated for the treatment of HCC, as therapeutic options for this tumor entity are still very limited. rVSV and rNDV were chosen for this project, because they both represent very attractive vector platforms as detailed in the introduction. rVSV's key benefits are its well-studied biology, its rapid and strictly cytoplasmatic replication cycle, its exquisite sensitivity to type I IFN and the lack of pre-existing immunity in the general population. Regarding rNDV, there is also almost no preexisting immunity or pathogenicity in humans and its replication cycle is also limited to the cytoplasm. An additional benefit is that, mediated by its glycoproteins, it is able to induce syncytia-formation, which allows for an efficient mode of viral spread and a potent activation of the immune system (Cuadrado-Castano et al., 2015).

However, as detailed earlier, both viruses come with severe safety concerns, which have greatly limited their clinical application. rNDV, although being safe in humans as shown in Phase I-II clinical trials (Freeman et al., 2006; Hotte et al., 2007; Laurie et al., 2006; Lorence et al., 2007), poses a great environmental and economical risk to the poultry industry (Ganar et al., 2014; Lancaster, 1976). Its meso- and velogenic strains have even been classified as select agents by the U.S. Department of Agriculture (www.selectagents.gov), hindering its further clinical development. rVSV was shown to have severe neuro- and hepatotoxic side effects when tested in animal models (Johnson et al., 2007; K. Shinozaki et al., 2005; van den Pol et al., 2002). Also, there has been a reported case of VSV-induced encephalitis in a child (Quiroz et al., 1988).

To facilitate VSV's clinical translation, researchers investigated several recombinant VSV vectors in order to eliminate its inherent toxicities: Amongst these recombinants are vectors that harbor a mutated M-protein (VSV-ΔM51), which results in increased IFN levels and therefore enhanced anti-viral defense responses in IFN-sensitive cells (Ahmed et al., 2003; Coulon et al., 1990; Ebert et al., 2005; Stojdl et al., 2003). Also, a VSV-recombinant encoding INFβ (VSV-INFβ) was shown to lead to great reduction of neurotoxicity (Jenks et al., 2010; Obuchi et al., 2003) and resulted in several ongoing clinical trials (see ClinicalTrials.gov: NCT02923466, NCT03120624 and NCT03017820). Furthermore, the introduction of microRNA target sequences into the viral genome in order to alter the virus' tropism showed promising results (Edge et al., 2008; E. J. Kelly et al., 2010). However, reduced toxicity often comes as a tradeoff for efficacy. A more recent approach is the replacement of the virus' glycoprotein with that of a heterologous safer virus, as VSV-G, the responsible protein for receptor binding and cell entry (Roche et al., 2008), is often seen as the root of VSV's neurotropism (Boritz et al., 1999; Hastie et al., 2013; Alexander Muik et al., 2012; Tani et al., 2007) This process, known as pseudotyping, showed encouraging results: Muik et al. for example pseudotyped VSV with the envelope glycoprotein of the lymphocytic choriomeningitis virus (LCMV), which resulted in enhanced infectivity of malignant glioma

cells but reduced neurotropsim (A. Muik et al., 2011; Alexander Muik et al., 2014). Others replaced VSV-G with heterologous glycoproteins modified in such way to specifically target cancer cells, with pleasing success (Ayala-Breton et al., 2012; Bergman et al., 2007; Gao et al., 2006). We aimed to alleviate VSV's inherent toxicities by pseudotyping it with the two non-neurotropic glycoproteins of NDV, F and HN. By doing so, we hoped to not only detarget VSV from healthy tissue, but to at the same time arm the new vector with additional weapons mediated by NDV's glycoproteins, thereby addressing both safety and efficacy.

4.2.2.1 Evaluation of rVSV-NDV's efficacy profile

Our *in-vitro* experiments in human HCC cell lines, showed attenuated replication of rVSV-NDV compared to rVSV, but similar replication kinetics as presented by rNDV. LDH-levels released from rVSV-NDV-infected cells were initially lower than levels achieved by both its parental vectors, but infection resulted in complete cell killing of the two cell lines by 72 hours (Figure 12). rVSV is known to replicate rapidly, with a replication cycle lasting only 8-10 hours (Wagner & Rose, 1996). It spreads via virion release from infected cells upon cell-death, which can be recognized as cells become small and round when undergoing apoptosis (Kopecky et al., 2001). rNDV and rVSV-NDV showed slower and less efficient replication. However, both viruses were able to induce complete monolayer destruction of the two human HCC cell lines. The underlying reason for their ability to efficiently kill tumor cells although they do not replicate as fast and to as high titers might be that they cause cell death by syncytia formation. As detailed in the introduction, syncytia are multinucleated cells, which are formed by the fusion of infected cells with the plasma membranes of uninfected neighboring cells. By introducing the hyperfusogenic mutant of the F-protein F3aa(L289A), we speculated that the hybrid vector would be able to mediate syncytia formation to an even greater extent, as it had previously been demonstrated for rNDV (Jennifer Altomonte et al., 2010; Vigil et al., 2007). Indeed, analysis of picture and video material taken of rVSV-NDV infected human HCC cells showed clear signs of syncytia formation as early as 12-16 hours post-infection. Complete destruction of the monolayers was observed by 48 hours (see Figures 13 and 14). This proves direct cell-to-cell fusion to be an efficient mode of viral spread, causing effective cytotoxic effects in the two investigated human HCC cell lines - even without the need for high virus titers.

Besides syncytia formation being an effective mode of viral spread and cell killing, another advantage of it is that the virus remains primarily intracellular. It is therefore less exposed to the immune system and the risk of premature clearing *in-vivo* is limited (Higuchi et al., 2000; Matveeva et al., 2015). Additionally, syncytia formation has been identified as a cause of immunogenic cell death (ICD) (Cuadrado-Castano et al., 2015). All viruses naturally act as danger signals, capable of recruitment and activation of antigen-presenting cells. Those

antigen-presenting cells, such as dendritic cells (DCs), can then stimulate adaptive immune responses against specific, tumor-associated antigens (TAAs). This supports the fight against the tumor by helping to overcome the naturally immunosuppressive tumor microenvironment. Syncytia formation is known to trigger TAA presentation by DCs to an even greater extent, as it causes dying cells to release or express damage-associated molecular patterns (DAMPs), which serve as major stimulants of DCs. Important DAMPs are the membrane localization of calreticulin (ecto-CRT) or the release of heat-shock proteins (Hsp70, Hsp90), high mobility group box 1 (HMGB1) protein, ATP and uric acids (A. R. Bateman et al., 2002; Matveeva et al., 2015; Palucka & Banchereau, 2012). Since rVSV-NDV has shown efficient syncytia formation in the experiments completed for this thesis, Sabine Behrend, a member of our laboratory group, did further research and investigated whether it therefore also successfully induces ICD (Abdullahi et al., 2018). Huh7 cells were infected with either rVSV, rNDV or rVSV-NDV and analyzed for the release of ATP, HMGB1 and Hsp70 and 90, as well as for the surface-expression of ecto-CRT. rVSV-NDV and rNDV, being the two vectors holding fusogenic glycoproteins, lead to substantially enhanced expression of ecto-CRT and an increased release of HMGB1, Hsp70, and Hsp90 as compared to rVSV. Furthermore, rVSV-NDV-infected cells released significantly higher concentrations of ATP as cells infected with either of the parental vectors. These results indicate that rVSV-NDV is not only able to efficiently cause direct cell killing, but that, when applied *in-vivo*, it possibly also provides an additional therapeutic mechanism by a successful activation of adaptive immune responses, thus an increased antitumor immunity and therefore a long-lasting tumor regression.

Although it was demonstrated that the L289A mutant of the F protein used here is theoretically able to induce syncytia formation in the absence of HN protein expression (Ebert et al., 2004), we speculated that the inclusion of the HN gene would provide additional benefits: First, it was shown that fusion activity is enhanced if HN remains expressed (Sergel et al., 2000). Second, being the cell binding protein, HN mediates attachment to its target cells via binding to molecules containing sialic acid residues. Those sialoglycoproteins were found to be overexpressed on the cell surface of tumor cells (Büll, den Brok, et al., 2014; Büll, Stoel, et al., 2014), as by forming a thick layer they manage to shield cancer antigens from the immune system, thereby providing an immune escape strategy for malignant cells (Matveeva et al., 2015). Indeed, the invasive potential of tumor cells has been positively correlated with the cell surface sialylation of various types of metastatic cancers (Pearlsteint et al., 1980; Yogeeswaran & Salk, 1981). The binding of HN to sialic acid residues could therefore be advantageous in two ways: First, since tumor cells tend to overexpress sialoglycoproteins, it could provide for a convenient intrinsic tumor-targeting mechanism. Second, since HN proteins possess a sialidase activity, they are able to remove sialic

residues from the tumor cell surface, thereby unmasking tumor antigens, which consequently results in reduced tumor growth and a stimulation of the immune system (Cohen et al., 2010; Matveeva et al., 2015; Powell et al., 1987).

All those aspects indicate that rVSV-NDV might be a potent vector when applied *in-vivo.* Indeed, recent *in-vivo* efficacy studies completed by our laboratory group demonstrated encouraging results: immune-competent mice bearing orthotopic, multifocal HCC that were treated with rVSV-NDV at a dose of 10^7 $TCID_{50}$/ml via tail vein injection showed significant survival prolongation as compared to mice treated with PBS ($p<0.005$), whereas rVSV-treated mice at the same dose did not cause any observable therapeutic benefit (Abdullahi et al., 2018). The relative inefficiency of rVSV in this experiment did not come as a surprise, as systematic OV administration (such as tail vein injection) is known to be an inefficient route for virus delivery. Nevertheless, rVSV-NDV injection by exactly this route resulted in an almost doubling of the median survival time compared to PBS. Based on our *in-vitro* studies, we speculate that the underlying mechanisms allowing the vector to cause such significant survival prolongation, despite such an ineffective administration route, are its above mentioned fusogenicity: Even without accumulating to high titers in the tumor, single virions are able to powerfully kill tumor cells by direct cell-to-cell fusion, syncytia formation and subsequent ICD induction. Also, as mentioned, the virus remains primarily intracellular. It therefore has minimal exposure to neutralizing antibodies, which presumably allows for prolonged replication kinetics. Future mechanistic studies will address this aspect more profoundly.

In contrast to the observations made in human HCC cell lines, infection of the rat hepatoma cell line McA-RH7777 with rVSV-NDV neither led to efficient viral replication, nor to efficient cell killing. rNDV too, showed reduced replication and cell killing abilities. rVSV on the other hand, was not attenuated and led to similar results as in the investigated human HCC cell lines (see Figures 15 and 16). A possible explanation for the attenuation of both rVSV-NDV and rNDV in McA-RH7777 cells is that, as these viruses spread by direct cell-to-cell fusion, they are dependent on a confluent cell-monolayer (A. Bateman et al., 2000). McA-RH7777 cells however usually do not grow to such confluence, making it difficult for the viruses to spread. However, to fully understand the cause of the virus' attenuated behavior in McA-RH7777 cells, further experiments are needed. An interesting approach would be to investigate replication in a 3D culture system. This would reflect conditions faced by the virus *in-vivo* in a much better way, and, especially with regard to the completed *in-vivo* studies, we speculate that the virus would be able to efficiently spread and induce cell lysis after all.

Taken together, rVSV-NDV showed potent cell-killing abilities *in-vitro* as long as long as confluent cell monolayers are given, and first *in-vivo* efficacy studies showed encouraging

results. Although further investigations are needed, we believe rVSV-NDV to be a very promising oncolytic vector, due to the many beneficial features of its extensive fusogenicity.

4.2.2.2 Evaluation of rVSV-NDV's safety profile

As stated, a vector's safety profile is at least as important as its efficacy. In our *in-vitro* studies, we were able to show that the chimeric vector remained susceptible to the antiviral actions of type I IFN, as virus titers dropped to levels similar to those observed for rVSV, which is known to be extremely susceptible to type I IFN (see Figure 18). While our studies revealed a relative insensitivity of rNDV to type I IFN, this is contradictory to previous findings of our group (Jennifer Altomonte et al., 2010) and should be further investigated. When tested in healthy mouse neurons, both rNDV and rVSV-NDV showed attenuated replication as well as significantly lower cytotoxicity levels in comparison with rVSV at all investigated time-points (see Figure 17). In PHH, results were especially striking: replication of rVSV-NDV was greatly diminished as compared to both parental vectors, with titers measured at 48 hours post-infection being 5 logs lower than those determined for rVSV-infected hepatocytes and three logs lower than for rNDV-infected ones. Also, LDH release was found to be significantly lower in rVSV-NDV-infected cells as opposed to both rVSV and rNDV, with only minimal cytotoxicity levels measured for rVSV-NDV and no signs of syncytia formation in the microscopic analysis as opposed to rNDV-infected cells. Taken together, rVSV-NDV showed very little replication or cytotoxic effects in primary healthy hepatocytes and neurons *in-vitro*, suggesting that pseudotyping successfully resulted in reduced off-target toxicity compared to rVSV.

Preliminary *in-vivo* results from immunodeficient NOD-SCID mice treated with either rVSV or rVSV-NDV reinforced this impression: All mice infected with rVSV at doses of 10^5 $TCID_{50}$/ml or higher showed clear outward signs of toxicity such as weight loss, altered posture and neurological symptoms. Histological analysis further demonstrated pathologies such as intrasinusoidal edema, moderate acute hepatitis, and acute brain stem necrosis. On the other hand, the majority of mice treated with rVSV-NDV at even higher doses of 10^7 $TCID_{50}$/ml did not show any pathological signs, neither outwardly nor histologically. The maximum tolerated doses (MTD) in NOD-SCID mice were therefore determined to be 10^4 $TCID_{50}$/ml for rVSV and 10^7 $TCID_{50}$/ml for rVSV-NDV, which signifies an at least 1000-fold elevation of the MTD and implies an important reduction of toxicity of the new chimeric vector compared to rVSV (Abdullahi et al., 2018).

This stands in contrast to a previously developed fusogenic VSV recombinant (rVSV-NDV/F(L289A), herein referred to as rVSV/F), which expresses NDV's modified fusion protein F3aa(L289A) in addition to VSV's endogenous glycoprotein G. This virus, able to

spread both by classic CPE as well as by syncytia formation, was shown to lead to enhanced intratumoral viral spread and significant survival prolongation in different tumor models (Jennifer Altomonte, Braren, et al., 2008; Ebert et al., 2004; Shin et al., 2007). However, as VSV-G remained expressed, the tropism of the vector was not altered compared to rVSV. The expression of NDV-F therefore resulted in enhanced efficacy by syncytia formation, but had no effect on reducing off-target toxicities, and, in contrast to the new vector, the maximum tolerated dose was unaltered compared to parental rVSV (Ebert et al., 2004).

Additionally, due to the mechanism of virus spread by direct cell-to-cell fusion, the virus remains primarily intracellular. We speculate that this could provide for an additional safety mechanism, as fewer infectious particles are released into the surrounding tissue, which could reduce the risk of or prevent viremia. Indeed, no infectious virus could be recovered from the blood, liver, or brain upon euthanasia of the rVSV-NDV infected NOD-SCID mice, indicating that no viremia had occurred (Abdullahi et al., 2018).

Last but not least, rVSV-NDV appears to be safe in its avian hosts. In contrast to rNDV, inoculation of embryonated chicken eggs with rVSV-NDV resulted in almost no propagation of the vector (see Figure 8). Additionally, a standard mean death time assay performed in embryonated chicken eggs by members of our laboratory group revealed that rVSV-NDV could be classified as lentogenic, whereas rNDV, in line with previous findings, was classed as mesogenic (Abdullahi et al., 2018). As mentioned, this might be explained by the fact that NDV's V-protein, an avian specific IFN-antagonist crucial for its pathogenicity in birds, was not included in our chimeric vector. Lentogenic strains are known to be non-/ low virulent (Suarez et al., 2019). It is therefore not to be expected that the hybrid vector will pose a threat to the environment, which signifies an important improvement over rNDV and many other recombinant NDV-vectors.

In summary, the completed experiments suggest enhanced safety of the viral vector compared to its parental viruses, with regard to both reduced off-target toxicities in the recipient and apathogenicity in birds, making rVSV-NDV a promising candidate for further development.

4.3 Clinical relevance, Conclusion and Outlook

Recent successes, particularly the FDA's approval of Talimogene laherparepvec (T-Vec) for the treatment of advanced melanoma, have encouraged interest and research in the field of oncolytic virotherapy. This has resulted in a large number of clinical trials and an even larger number of preclinical studies. Cook and Cauhan as well as Macedo et al. have recently published review papers giving an overview of the current landscape of clinical research on oncolytic viruses (Cook & Chauhan, 2020; Macedo et al., 2020). As detailed by Cook and Cauhan, a large part of the recent research focuses on how to increase a virus' immunogenicity (Cook & Chauhan, 2020). This comes as we have learned that oncolytic viruses do not only develop their anticancer potential by direct tumor cell lysis, but that an important role must be attributed to their ability to induce systemic antitumor immune responses (Lichty et al., 2014). While the conditions that facilitate this ability are still under investigation, fusogenicity seems to play an important part (Krabbe & Altomonte, 2018). As explained earlier, syncytia formation enhances both direct oncolytic as well as immune-stimulating effects, as dying cells undergo immunogenic cell death. This triggers the release of danger signals such as DAMPs and PAMPs, which lead to the activation of adaptive immune responses and elicit adaptive antitumor immunity (Guo et al., 2014; Kroemer et al., 2013; Matveeva et al., 2015; Zelenay & Reis e Sousa, 2013). Paramyxoviridae such as NDV, measles virus, mumps virus and Sendai virus are of special interest, as they are naturally fusogenic viruses. Additionally, as detailed above, their attachment protein, e.g. HN for NDV, could be advantageous in two ways: First, by providing an intrinsic target mechanism, as it binds to sialic acids, which tend to be overexpressed on tumor cells (Büll, den Brok, et al., 2014; Büll, Stoel, et al., 2014). Second, by provoking an extra stimulation of the immune system by unmasking tumor antigens using its sialidase activity (Cohen et al., 2010; Matveeva et al., 2015; Powell et al., 1987). Paramyxoviridae and fusogenic viruses in general therefore seem to represent promising OV platforms (Krabbe & Altomonte, 2018).

By pseudotyping rVSV with the glycoproteins of rNDV, we therefore hoped to not only eliminate the viruses' respective safety issues, but to simultaneously generate an oncolytic vector with efficient cell killing abilities. Although rVSV-NDV did not replicate to titers as high as those achieved by its parental vectors, we were able to demonstrate that it causes effective cell lysis in human HCC cell lines by rapid and efficient syncytia formation. Less successful results in a rat hepatoma cell line might be explained by the need for a confluent cell monolayer to enable efficient viral spread. *In-vitro* safety studies indicated significantly reduced cytotoxicity in primary human hepatocytes and healthy mouse neurons, as well as a preserved susceptibility to the anti-viral actions of type I IFN. Additional experiments completed by our laboratory group demonstrated almost no pathogenicity in embryonated

chicken eggs and substantially reduced toxicity in immunodeficient NOD-SCID mice when comparing rVSV-NDV to rVSV, suggesting that pseudotyping had successfully resulted in an enhanced safety profile for the new vector. The group was also able to show that the virus potently induces immunogenic cell death, which, as detailed above, could potentially result in an increased and long-lasting anti-tumor immunity. Additionally, due to the vector's spread and infection mode by direct cell-to-cell fusion, the virus remains primarily intracellular. It is therefore less exposed to neutralizing antibodies, allowing for prolonged replication kinetics *in-vivo*. Spreading via cell-to-cell fusion should also mean that viremia can be reduced or prevented, as fewer infectious particles are released into the surroundings. Preliminary *in-vivo* efficacy studies showed a significant survival prolongation in orthotopic HCC-bearing mice treated with rVSV-NDV, even though the vector was administered via systemic injection, an administration route known to be inefficient for OV delivery.

Taken together, these results indicate preserved cell-killing abilities and an increased safety profile of rVSV-NDV compared to its parental vectors. Although further investigations are needed, we think that the new vector could represent a promising platform for further clinical translation, especially because of its potent syncytia induction and the resulting immunogenicity.

One successful strategy to increase a virus' immunogenicity even further is to introduce immunostimulatory genes into the vector's genome. Such transgenes are commonly cytokines or chemokines, such as interleukins, tumor necrosis factor alpha (TNF-α) or, most frequently, GM-CSF (de Graaf et al., 2018). The expression of GM-CSF results in the recruitment and activation of antigen-presenting cells and the subsequent induction of tumor-specific T-cell responses (Kaufman et al., 2014). The aforementioned T-Vec is probably the most prominent example, but several other OVs have also been armed with GM-CSF (Burke et al., 2012; Grossardt et al., 2013; Kanerva et al., 2013). Therefore, one idea for the further development of rVSV-NDV is the integration of immunostimulatory genes into the vector's genome. This should be possible without further attenuation of the virus, as indicated by a proof of principle study completed by our laboratory group.

Immunotherapy in general has become of great interest to both researchers and clinicians over the last decade. Immune checkpoint inhibitors (ICIs) are currently the most popular immunotherapeutics. They are monoclonal antibodies that block receptor-ligand interactions which the tumor would otherwise use to create an immunosuppressive tumor microenvironment and escape the immune system. By inhibiting those checkpoints, immunosuppressive signals are reduced and adaptive immune responses are reactivated (Pardoll, 2012). Although they revolutionized cancer therapy and are part of the standard therapy regimen for multiple malignancies, they can cause severe adverse events (Postow et

al., 2015). Furthermore, some tumors are non-responsive to these immunotherapeutics, at least when no additional therapeutic agents are applied (Kelderman et al., 2014). Therefore, combination therapies are under intense investigation and oncolytic viruses have emerged as attractive candidates, as they seem to help overcome the tumor's resistance, enhance ICIs' therapeutic efficacy and allow for lower doses of the used agents, resulting in reduced adverse effects (de Graaf et al., 2018; L. Russell et al., 2019). Krabbe and Altomonte postulate that fusogenic viruses in particular could serve as optimal partners for ICIs, leveraging their strong immunogenicity as well as their improved spread and enhanced safety (Krabbe & Altomonte, 2018). ICIs are being assessed as a treatment option for HCC (El Dika et al., 2019; Zongyi & Xiaowu, 2020), so investigating a combination therapy consisting of rVSV-NDV and an ICI would be of great interest.

Last but not least, testing rVSV-NDV in different tumor entities warrants investigation, as theoretically there is nothing limiting its applicability to HCC.

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6 Publication

Please note that parts of this thesis were previously published under my birth name, **Sarah Abdullahi** (now Sarah Conway).

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