DEVELOPMENT OF RECOMBINANT ONCOLYTIC COXSACKIEVIRUS B3 FOR TREATMENT OF COLORECTAL CARCINOMA

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LIST OF ABBREVIATIONS

5-FU	5-fluorouracil
APC	Adenomatous Polyposis Coli
BSA	Bovine Serum Albumin
CAR	Coxsackie and Adenovirus Receptor
CIMP	CpG Island Methylator Phenotype
CIN	Chromosomal Instability
COX-2	Cyclooxygenase-2
CPE	Cytopathic Effect
CRC	Colorectal Carcinoma
CVA21	Coxsackievirus A21
CVB3	Coxsackievirus B3
DAF	Decay-Accelerating Factor
DAMP	Danger Associated Molecular Pattern
DCC	Deleted in Colorectal Cancer
EGFR	Epidermal Growth Factor Receptor
FDA	Food and Drug Administration
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor
GSK3β	Glycogen Synthase Kinase-3β
HS	Heparan Sulphate
HS6ST2	Heparan Sulphate 6-O-Sulfotransferase 2
HSV1	Herpes Simplex Virus-1
ICAM-1	Intercellular Adhesion Molecule 1
IGF2R	Insulin-like Growth Factor-2 Receptor
IRES	Internal Ribosomal Entry Site
LOH	Loss of Heterozygosity
LV	Leucovorin

mAb	Monoclonal Antibody
MAPK	Mutagen-activated Protein Kinase
MCS	Multiple Cloning Site
MiRNA	MicroRNA
MiR-TS	MicroRNA Target-Site
MMR	Mismatch Repair Genes
MOI	Multiplicity of Infection
MSI	Microsatellite Instability
ORF	Open Reading Frame
PAMP	Pathogen Associated Molecular Pattern
PBS	Phospate Buffered Saline
PFU	Plaque Forming Units
PI3K	Phosphoinositide 3-kinase
Pre-miRNA	Precursor MicroRNA
Pri-miRNA	Primary MicroRNA
T-VEC	Talimogene Laherparepvec
TAA	Tumor Associated Antigen
TCA	Tricholoracetic acid
TGFBR2	Transforming Growth Factor-B Receptor 2
TNM	Tumor-Node-Metastases
TP53	Tumor Protein 53
UTR	Untranslated Region
VEGF	Vascular Endothelial Growth Factor
VSV	Vesicular Stomatitis Virus

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ABSTRACT

DEVELOPMENT OF RECOMBINANT ONCOLYTIC COXSACKIEVIRUS B3 FOR TREATMENT OF COLORECTAL CARCINOMA

Ahmet HAZINI

PhD Thesis

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Colorectal cancer is one of the most death related cancer type in worldwide. Conventional cancer therapeutics show curable effect only in the patients at early stage of the disease. In case of the diagnosis at late stages, the survival rate decreases dramatically up to 10%. Therefore, novel cancer therapeutics are urgently needed. Oncolytic virotherapy is a new treatment approach which exploits the viruses against cancer. Clinical trials prove the efficiency of various oncolytic viruses in treatment of different tumors. Coxsackievirus B3 (CVB3), a single-stranded RNA virus of the picornavirus family, has been described as a novel oncolytic virus against lung carcinoma. However, CVB3 can induce severe diseases, such as pancreatitis, myocarditis and encephalitis, but the course of infections is decisively influenced by the particular strain of CVB3.

In this thesis, firstly, it was aimed to investigate different laboratory strains of CVB3 (Nancy, 31-1-93, H3) which use the coxsackievirus and adenovirus receptor (CAR) and the strain PD which uses N- and 6-*O*-sulfated heparin sulfate (HS) for entry into

colorectal tumor cells, for their potential to lyse these cells and for their safety profile. *In vitro* investigations showed variable infection efficiency and lysis of colorectal carcinoma cell lines by the CVB3 strains. The most efficient strain was PD, which was the only one that could lyse all five of the investigated colorectal carcinoma cell lines. Lytic activity of CAR-dependent CVB3 did not correlate with CAR expression on colorectal carcinoma cells, whereas there was a clear correlation between lytic activity of PD and its ability to bind to HS at the cell surface of these cells. Intratumoral injection of CVB3 Nancy, 31-1-93 or PD into subcutaneous colorectal DLD1 tumors in BALB/c nude mice each resulted in strong inhibition of tumor growth. The effect was seen in the injected tumor, as well as in a non-injected, contralateral tumor. However, all animals treated with 31-1-93 and Nancy developed systemic infection and died or were moribund and sacrificed at 6 and 8 days post intratumoral virus injection. In contrast, five of the six animals treated with PD showed no signs of a systemic viral infection and PD was not detected in any organ. These results indicate that PD has potential to be a potent and safe oncolytic CVB3 strain for use in the treatment colorectal carcinoma.

To increase the tumor selectivity, so that to abolish the possibility of undesirable virus infection in healthy organs, target sites of pancreas-specific microRNA was inserted into the CVB3 genome and recombinant CVB3 was analyzed *in vitro* and *in vivo*. Detargeting of the pancreas, which is the most susceptible tissue for CVB3 infection, by exploiting pancreas-specific corresponding miRNAs, increased the survival rate of treated animals significantly. The virus was unable to replicate in the pancreas and all animals survived. In contrast, colorectal carcinomas were destroyed as efficient as the wild-type CVB3.

Finally, it was aimed to increase the efficiency of CVB3 against infection resistant colorectal cancer cell line by using directed viral evolution strategy. Results showed that CVB3 can specifically adapted to colorectal carcinomas leading to breaking the resistance of the tumor cells against CVB3 infection.

Keywords: Colorectal Carcinoma, Coxsackievirus B3, Oncolytic Virotherapy, RNA interference, Virus adaptation

ÖZET

KOLOREKTAL KANSER TEDAVİSİ İÇİN REKOMBİNANT ONKOLİTİK KOKSAKİ B3 VİRÜSLERİNİN GELİŞTİRİLMESİ

Ahmet HAZINI

Doktora Tezi

Danışman: Prof. Dr. Peter NEUBAUER
Danışman: Prof. Dr. Ibrahim ISILDAK

Kolorektal kanser, dünya genelinde ölüm oranı en yüksek kanser türüdür. Geleneksel kanser terapötikleri yalnızca erken kanser evresindeki hastalarda etki göstermekte, teşhisin geç dönemde yapıldığı durumlarda ise hayatta kalım oranı dramatik şekilde %10 seviyelerine düşmektedir. Bu nedenle yeni tedavi yöntemlerine acil ihtiyaç duyulmaktadır. Onkolitik viroterapi, virüsleri kansere karşı kullanarak yapılan yeni bir tedavi türüdür. Klinik deneylerde birçok farklı virüs türünün çeşitli kanser tiplerine karşı etkinliği kanıtlanmıştır. Pikornavirüs ailesinin tek sarmallı RNA virüsü olan üyesi olan Coxsackievirus B3 (CVB3), akciğer kanserine karşı yeni bir onkolitik virüs olarak tanımlanmıştır. Ancak, CVB3 miyokardit, pankreatit veya ensefalit gibi ciddi hastalıklara neden olabilmekte ve bu yan etkilerin seyri direk olarak CVB3′ün belirli suşu tarafından belirlenmektedir.

Bu tez çalışmasında, ilk olarak, hücrelere girmek için CAR reseptörü kullanan farklı CVB3 laboratuvar suşlarını (Nancy, 31-1-93 ve H3) ve heparan sülfatı reseptör olarak kullanan CVB3 PD suşunun, kolorektal karsinomları liz etme potansiyeli ve güvenlik profilleri araştırılması hedeflendi. *İn vitro* deneyler, CVB3 suşlarının, kolorektal karsinoma hücrelerini enfekte etmekte değişkenlik gösterdiğini ve sonuç olarak farklı

etkinlikte hücre lizisine etken olduklarını ortaya koydu. En etkili suş, incelenen kolorektal karsinoma hücre dizilerinin beşini de liz edebilen CVB3-PD idi. CAR-bağımlı CVB3 suşlarının litik aktiviteleriyle hücre yüzeylerinde ifade edilen CAR oranları arasında herhangi bir korelasyon gözlenmemesine rağmen, PD suşunun litik etkinliği hücrelerde ifade edilen HS miktarıyla doğru orantılı bir ilişkilendirme göze çarptı.

CVB3 Nancy, 31-1-93 veya PD'nin, BALB/c immün yetmezliği bulunan farelerde bulunan subkutanöz kolorektal DLD1 tümörlerine intratümöral enjeksiyonu, her deney grubunda da tümör büyümesinde güçlü bir gerilemeye neden oldu. Etki, enjekte edilen tümörde olduğu gibi, enjekte edilmemiş kontralateral tümörde de görüldü. Bununla birlikte, 31-1-93 ve Nancy ile tedavi edilen tüm hayvanlar, şiddetli sistemik enfeksiyon sonucu olarak, virüs enjeksiyonundan 6 ve 8 gün sonra öldürüldü. Buna karşılık, PD ile tedavi edilen altı hayvandan beşinde sistemik bir viral enfeksiyon belirtisi görülmedi ve herhangi bir organda PD tespit edilmedi. Bu sonuçlar, PD'nin kolorektal kanser tedavisinde kullanılmak üzere potansiyel ve güvenli bir onkolitik CVB3 türü olabileceğini göstermektedir.

CVB3′ün güvenliğini arttırmak için, pankreasa spesifik bir mikroRNA'nın bir mikro RNA hedef bölgesi (miR-TS) virüs genomuna eklendi ve rekombinant CVB3 *in vitro* ve *in vivo* koşullarda analiz edildi. miRNA'ların kullanılarak CVB3'e karşı en duyarlı organ olan pankreasın hedef olmaktan çıkarılması, hayvanlara yaşam sürelerini anlamlı bir biçimde yükseltti. Virus pankreasta replike olamadı ve tüm hayvanlar sağlıklı olarak hayatta kaldı. Tersine, kolorektal karsinomlar doğal fenotip CVB3 ile enfekte edilmiş tümörler kadar verimli şekilde yok edildi.

Son olarak, yönlendirilmiş evrim stratejisi kullanılarak, enfeksiyona dirençli kolorektal kanser hücre dizisine karşı CVB3'ün etkinliğinin artırılması amaçlandır. Sonuçlar, CVB3'ün spesifik olarak tümör hücrelerine adapte olabileceğine ve bu adaptasyonun tümör hücrelerinin CVB3 enfeksiyonuna karşı direncinin kırılmasına yol açtığını ortaya koydu.

Anahtar Kelimeler: Kolorektal Karsinoma, Coxsackievirus B3, Onkolitik Viroterapi, RNA interferansı, Virüs adaptasyonu

CHAPTER 1

INTRODUCTION

1.1 Review of Literature

1.1.1 Colorectal Cancer

Colorectal cancer (CRC) is one of the most common cancers worldwide, as it is the second most common in women with 614,000 cases per year and the third most common in men with 764,000 cases per year [1]. CRC kills nearly 700,000 people every year and makes it the fourth cancer related death after lung, liver and stomach cancer. The highest rates of incidence and 55% of cases are observed in western countries, but developing countries such as China and Brazil have rising incidence of CRC in parallel to their economic growth indicating the strong relation of CRC and modern lifestyle [2, 3].

CRC starts on the inner surface of colon with excess non-cancerous cell growth which is called adenomatous polyposis. However, these polyps are usually benign and do not cause cancer. In the case of continuous growth of polyps in the tissue layers, the non-malignant adenomas become malignant and called as adenocarcinoma **(Figure 1.1)**. The occurrence of cancer from polyps is a very slow process and usually takes around 10 to 20 years [4, 5].

Colorectal polyps types can be variable and some of them are more tend to cause malignant tumor. Hyperplastic polyps and adenomatous polyps are the most common histologic types of colorectal cancer. Hyperplastic polyps were supposed to have no potential of malignancy, however, lately it has been suggested as precursor of CRC in some cases [6, 7]. Adenomatous polyps are more often malignant and the risk of CRC is notably increased with the number of these polyps in colon [8].

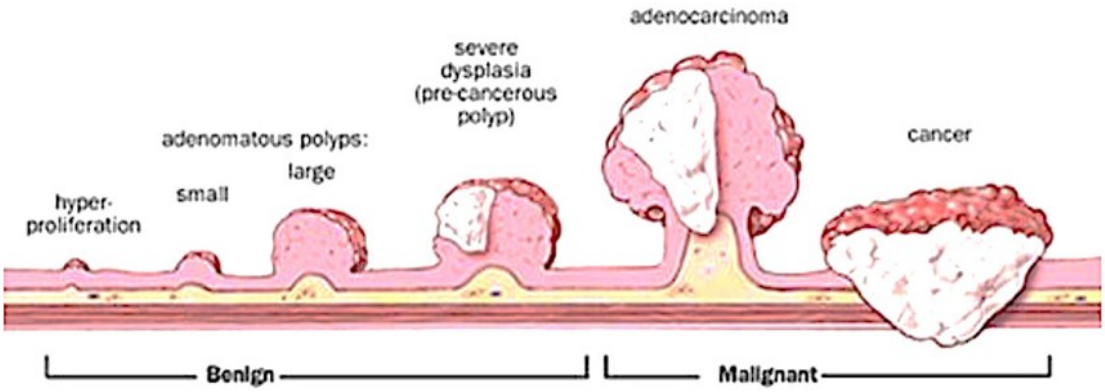

Figure 1.1 Progression of CRC from non-cancerous polyps [9].

1.1.1.1 Molecular Mutations in Colorectal Cancer

Cause of the CRC like other cancers is related to mutations in oncogenes that basically causing induction of cell proliferation or survival and tumor suppressor genes which are primarily responsible for regulation of cell growth and motility [10, 11]. The most common and well identified mutations which are responsible for CRC genesis are mutations in the adenomatous polyposis coli (APC) gene, tumor protein 53 (TP53), KRAS and DCC genes [4, 12]. Point mutation originated sporadic CRC types are 70% of all CRC and around 30% of CRC cases are caused by inherited and familial cancers, however only 10% of inherited mutations related genes are well known and the rest still are not identified [13, 14, 15]. Inherited CRC are subdivided into two groups which are the polyposis and non-polyposis colorectal cancer (HNCC). Familial adenomatous polyposis (FAP) that is identified with accumulation of hundreds of polyps from the early ages, is an example of polyposis form of inherited CRC [16, 17]. On the other hand, the most common HNCC is Lynch syndrome which is related to inherited mutations of DNA mismatch repair genes (MMR) [18].

Three main pathogenic mechanisms lead to colorectal carcinogenesis, so-called chromosomal instability (CIN), microsatellite instability (MSI) and CpG island methylator phenotype (CIMP) **(Figure 1.2)** [19]. Genomic instabilities dramatically increase the rate of mutations in the genome so that resulting in the formation of cancer [20].

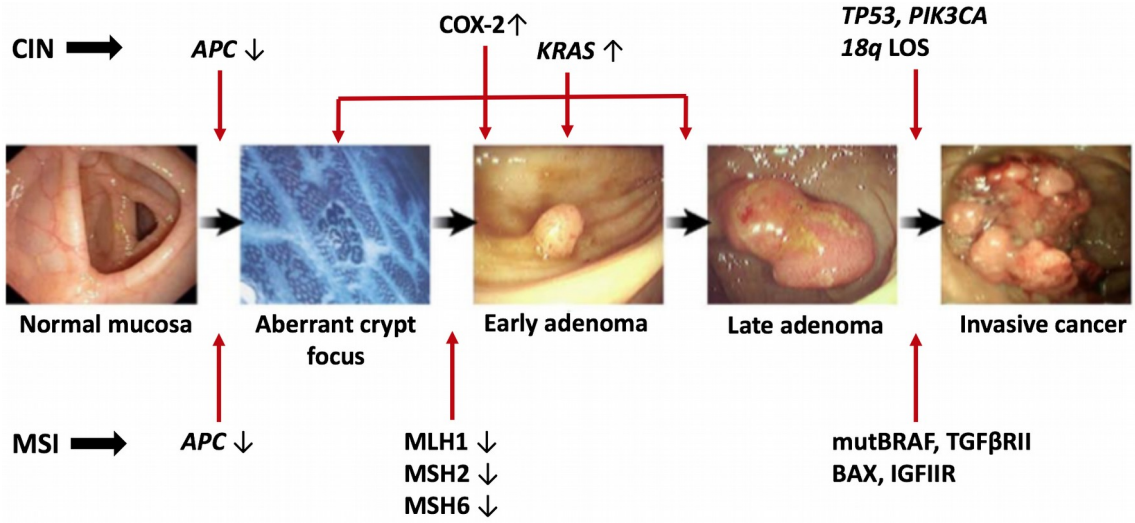

Figure 1.2 Molecular, genetic and epigenetic alterations according to CRC cancer progression from normal mucosa [19, 21]. Three genomic pathways are identified in CRC which are CIN, MSI and CIMP pathways. The CIN pathway is characterized by imbalance in chromosome number and loss of heterozygosity. The MSI pathway is caused by defect in DNA mismatch repair activity. Upper side and lower side of the figure indicates the mutations which are correlated to the CIN pathway and MSI pathway, respectively. Red arrows show the correlation of respective mutation on CRC stage.

The CIN pathway is also termed as classical pathway because it is correlated with 80% of CRC tumors. Chromosome segregation, telomere dysfunction and the DNA damage response are the main underlying reasons of CIN. The CIN pathway causes imbalance in chromosome number, genomic amplifications and loss of heterozygosity (LOH) at important tumor suppressor genes and oncogenes [21]. The firstly described genomic alteration in colon tumors is the tumor suppressor APC gene mutation resulting in dysfunctionality of APC protein. It is found 70-80% of CRC and suggested as tumor initiator due to detection even in the earliest stages of adenoma [22]. The most crucial function of this protein is its interaction with glycogen synthase kinase-3β (GSK3β) and β-catenin. Disruption of APC causes activation of WNT signaling; in turn, β-catenin translocates to the nucleus where it induces transcription of oncogenes implicated to tumor growth and invasion [23]. Mutational activation of KRAS and phosphoinositide 3-kinase (PI3K) oncogenes which are found in 30-50% and 20% of CRC, respectively, cause cell proliferation by activation of mutagen-activated protein kinase (MAPK) [24], [25] . P53 is defined as “guardian of the genome” has several functions in cells for prevention of cancer, such as activation of DNA repair, holding the cell cycle in case of DNA damage and activation of apoptosis [26]. Loss-of-function mutations in TP53 gene, resulting inactivation of P53, is found in 50% of all tumors and 50-75% of CRC

and has a role in the transformation from adenoma to carcinoma [27, 28, 29]. Allelic loss of chromosome18q, which is a region of tumor suppressor genes DCC, SMAD2 and SMAD4, is described to occur in 70% of CRC especially in advanced stage of disease [30, 31]. Finally, cyclooxygenase-2 (COX-2), which is found to be involved in invasion, metastasis, proliferation of CRC tumors and also angiogenesis, is overexpressed in 43% of adenomas and 86% of carcinomas [32, 33], [34].

Defect in DNA mismatch repair system resulting in a hypermutable phenotype which is called MSI. This pathway is found in 15% of colorectal carcinomas. 12% of these CRC are sporadic cases and 3% are associated with Lynch syndrome [18, 35]. It is considered to be an early event in adenoma formation and the mutated genes in tumors are mainly found to be MLH1, MSH2 and MSH6 [36]. Inactivation of MLH1 by promoter hypermethylation is the 90% of all MSI cases [37]. Additionally, mutations in BRAF gene, which encodes RAF protein playing a role in MAPK/ERKs signaling pathway, is also detected in MSI tumors [38]. Frameshift mutations are usually observed in MSI tumors, particularly in non-coding regions of genes that have small runs of nucleotide repeats, such as transforming growth factor-β receptor 2 (TGFBR2) and insulin-like growth factor-2 receptor (IGF2R) [39, 40].

The third pathway which is described to be involved in progression of CRC, is the CpG island hypermethylation of tumor suppressor genes which result in transcriptional inactivation of these regulatory proteins. CIMP is found around 35% of all CRC cases and separated in two groups as CIMP-high and CIMP-low tumors [41, 42]. While BRAF mutations and MLH1 methylation are linked to CIMP-high tumors, KRAS mutations mainly occur in CIMP-low tumors [43].

1.1.1.2 Stages of Colorectal Carcinogenesis

Staging of colorectal carcinoma is very crucial process as the therapy is determined considering this information. This process is done by physical examination, colonoscopy, ultrasound or computed tomography, positron emission tomography or magnetic resonance imaging scan [44]. Recently, the tumor-node-metastases (TNM) staging system is used to stage CRC by classifying the range of cancer based on size of tumor (T), regional lymph node status (N) and the status of metastasis (M) **(Figure 1.3) (Table 1.1)** [45]. By grouping these classifications, the stage of cancer determined as follow;

Stage 0 (in situ); This is also called as tumor in situ, which means the cancer cells are only located in the mucosa of colon or rectum. This tumor is small and harmless usually can be removed by colonoscopy without surgery.

Stage I (local); The tumor has grown through the mucosa and has invaded the colon layers without reaching outside of the wall of colon. The survival rate is 90%.

Stage II (local); The tumor has grown through the colon layers into the nearby tissue or organ without spreading to lymph nodes. The survival rate is around 70%

Stage III (regional); The tumor has spread to the lymph nodes without invading other organs. The survival rate is around 40%.

Stage IV (distant); The final stage which indicates that tumor has spread to one or more distant organ (liver, lung). The survival rate is around 9-15% [46].

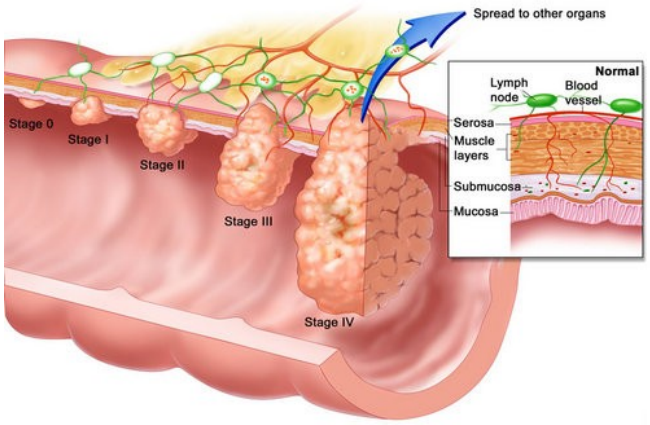

Figure 1.3 Stages of colorectal cancer [47].

Table 1.1 Tumor node metastasis stage grouping and clinical classifications [48].

Stage 0	Tis	N0	M0
Stage I	T1, T2	N0	M0
Stage II	T3, T4	N0	M0
Stage IIA	T3	N0	M0
Stage IIB	T4a	N0	M0
Stage IIC	T4b	N0	M0
Stage III	Any T	N1, N2	M0
Stage IIIA	T1, T2	N1	M0
	T1	N2a	M0
Stage IIIB	T3, T4a	N1	M0
	T2, T3	N2a	M0
	T1, T2	N2b	M0
Stage IIIC	T4a	N2a	M0
	T3, T4a	N2b	M0
	T4b	N1, N2	M0
Stage IVA	Any T	any N	M1a
Stage IVB	Any T	any N	M1b

TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma *in situ*: intraepithelial or invasion of lamina propria
T1	Tumor invades submucosa
T2	Tumor invades muscularis propria
T3	Tumor invades subserosa or into non-peritonealized pericolic or perirectal tissues
T4	Tumor directly invades other organs or structures and/or perforates visceral peritoneum
T4a	Tumor perforates visceral peritoneum
T4b	Tumor directly invades other organs or structures
Nx	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in 1-3 regional lymph nodes
N1a	Metastasis in 1 regional lymph node
N1b	Metastasis in 2-3 regional lymph nodes
N1c	Tumor deposit(s), *i.e.*, satellites, in the subserosa, or in non-peritonealized pericolic or perirectal soft tissue without regional lymph node metastasis
N2	Metastasis in 4 or more regional lymph nodes
N2a	Metastasis in 4-6 regional lymph nodes
N2b	Metastasis in 7 or more regional lymph nodes
M0	No distant metastasis
M1	Distant metastasis
M1a	Metastasis confined to one organ [liver, lung, ovary, non-regional lymph node(s)]
M1b	Metastasis in more than one organ or the peritoneum

1.1.1.3 Treatment Methods for Colorectal Cancer

Colorectal cancer treatment has improved significantly together with new imaging technology, surgical techniques, chemotherapy and biological therapeutics [49]. Treatment type is selected considering the stage of the disease, location and molecular characteristic of the tumor [50]. 5-year survival rate of CRC is around 90% when the cancer is diagnosed at the localized stage. However, the survival rates dramatically decline to 71.7% and 13.6% when diagnosis is made at regional and distant stage, respectively [51]. Tumor location within the tumor is also important criteria as tumors in the proximal colon are found to show higher risk of death [52]. Additionally, primary tumor location is suggested to be a strong prognostic factor for the patients with metastatic colorectal cancer. Further investigations are under progress for better understanding of treatment response considering tumor location [53].

Surgery is the first treatment and the most common option for stage 0 to stage II of colorectal cancer. The detected polyps at stage 0 and stage I are removed with surgery by polypectomy and in the case of large polyps the segment of the colon may be removed by local excision. In the stage II and stage III partial colectomy is used to remove portion of colon which includes cancerous tissue, some part of surrounding tissue and lymph nodes. Adjuvant chemotherapeutics such as 5-fluorouracil (5-FU) can be also used when the possibility of recurrence of the cancer [54, 55]. Use of chemotherapy for stage II colorectal cancer patients is a controversial theme in medical oncology and usually it is not recommended by international guidelines due to strong side effect on patients without any improvement in survival rates [56, 57]. However, adjuvant chemotherapy is recommended to be considered by physicians at stage II patients with high risk features [58].

Colon cancer recurrence following surgery is very common within stage III diagnosed patients and the rate is around 35% seven years following operation [59]. Therefore, use of chemotherapeutics such as 5-FU and Oxaliplatin are recommended to clinicians [60]. Many studies showed prolonged survival rates in patients who were treated with adjuvant chemotherapy [61, 62]. Surgical treatment of rectal cancer is more complicated operation compared to colon cancer and additionally, rectal cancer is suggested to have higher recurrence rate. Therefore, to shrink size of tumor and to prevent local recurrence, radiotherapy may also be used after or before surgery combined with chemotherapy [63].

When the cancer has already reached to stage IV, which means the tumor has spread to one or more distant organs and tissues, treatment of CRC become a more complex challenge for patients and clinicians. At time of diagnosis 20% of CRC patients are found to be in stage IV and 12% of these patients have liver metastasis followed by the peritoneum and the lung with 6.4% and 1.6%, respectively [64, 65]. However, almost 50% of all CRC patients develop metastases [46]. The majority of patients with stage IV are not suitable for curative resection (75-90%). In some cases, when patients show positive respond to chemotherapy regimen, resection can be potentially curative [66]. Systemic therapy with cytotoxic chemotherapy agents and biological targeted agents have significantly improved survival rate of advanced colorectal cancer patients [67]. Treatment patterns of colon and rectal cancer according to stage of disease is shown in **Figure 1.4**.

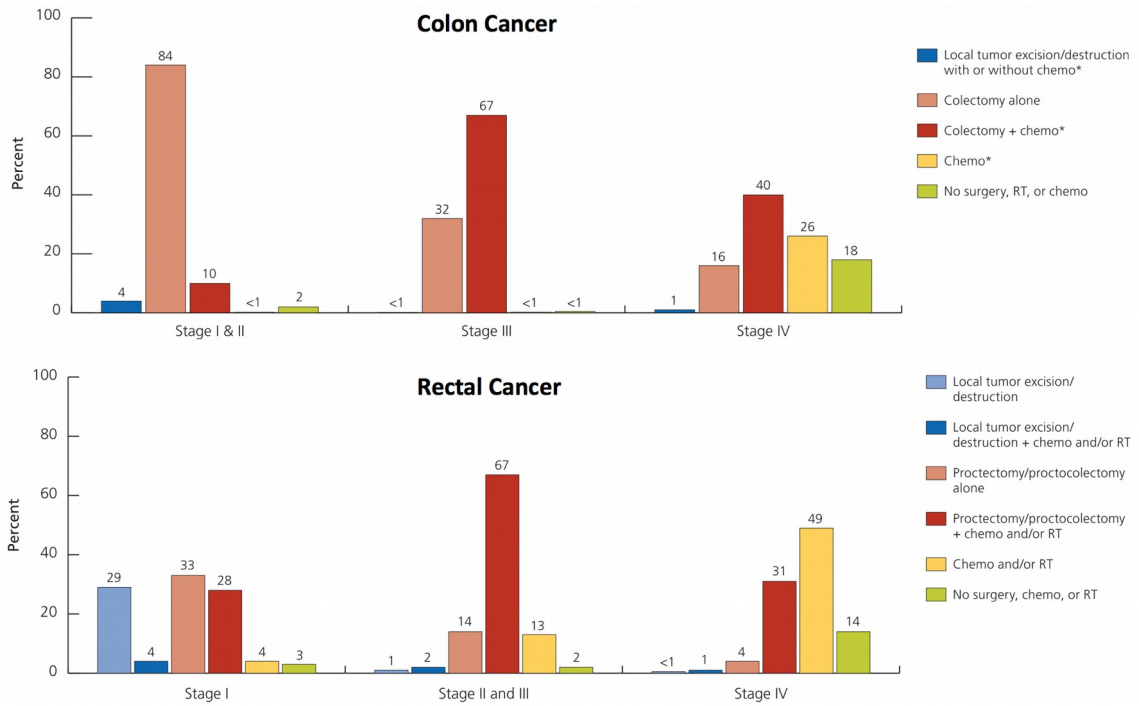

Figure 1.4 Colon and rectal cancer treatment patterns in 2013 by stage. Chemo, chemotherapy (includes immunotherapy and targeted therapy); RT, radiation therapy. *A small number of these patients received RT [68].

Cytotoxic Chemotherapy

Systemic therapeutics for CRC treatment has been evolved during last two decades. However, the backbone of advanced CRC treatment is still based on cytotoxic agents, particularly fluoropyrimidines. The first approved fluoropyrimidine is 5-FU, which causes to cell death by blocking thymidylate synthase [69]. Use of 5-FU has improved the overall survival around 12 months. However, following the addition of leucovorin (LV) lower side effects together with higher response rate and overall survival has been achieved. LV is known as folinic acid and it helps to stabilize the interaction of 5-FU with thymidylate synthase [70].

Capecitabine is an oral fluoropyrimidine, which turns into active form of 5-FU after three enzymatic reactions, can be also used together with LV as an alternative [71].

Topoisomerase I inhibitor Irinotecan was approved by US Food and Drug Administration (FDA) in 2000 as first line treatment for advanced CRC as it increased overall survival up to 20 months when used together with 5-FU [72]. Irinotecan is converted to SN-38 by carboxylesterases which results in DNA fragmentation and apoptosis through the stabilization of DNA-topoisomerase complex [73]. It is suggested to be used in combination with infusional 5-FU/LV (FOLFIRI) due to higher efficiency and lower toxicity. Administration of Irinotecan together with Capecitabine was found to be more toxic compared to FOLFIRI [74].

Another important development was the introduction of platinum containing agent Oxaliplatin in the early 2000s. Oxaliplatin is a platinum compound which forms DNA adducts that causes to impaired DNA replication [75]. While it has limited efficiency as a single agent, when it is combined with infusional 5-FU/LV (FOLFOX) it shows significantly higher response rate and higher survival [76].

Different phase III trials have proved that FOLFOX and FOLFIRI have similar activity with different toxicity profile as first-line treatment of CRC [77]. Second-line chemotherapy is only suggested to patients with competent organ function and good performance status. Moreover, patients with FOLFOX refractory will receive FOLFIRI in second-line treatment and in patients refractory to FOLFIRI will receive Oxaliplatin consisting second-line treatment [78].

In addition to conventional cytotoxic chemotherapy, less toxic treatment method which is called targeted therapy increased overall survival of advanced CRC patients. FDA

approved cytotoxic and biological targeted agents with their basic characteristics is shown in **Table 1.2**.

Table 1.2 The US Food and Drug Administration approved agents for treatment of metastatic colorectal cancer [79, 80].

Class	Agent	Year	Characteristics
Cytotoxic Chemotherapy	5-fluorouracil	1962	It is listed as one of the essential medicines of World Health Organization's (WHO's). Inhibits the thymidylate synthase which synthesizes pyrimidine thymidine required for DNA replication.
	Capecitabine	2001	This is product form of 5-Fu. It is metabolized to active form of thymidylate synthase inhibitor.
	Irinotecan	2000	It leads inhibition of DNA replication by the inhibition of topoisomerase I.
	Oxaliplatin	2004	Prevents DNA synthesis by causing DNA to crosslink (platinum based).
Inhibits growth of new blood vessels	Bevacizumab	2004	A monoclonal antibody which is one of the WHO's essential medicines.
	Aflibercept	2012	It acts as VEGF trap, firstly used for treatment of wet macular degeneration.
EGFR antibody	Cetuximab	2004	It binds to EGFR receptor however, it cannot be applied in case of KRAS mutation.
	Panitumumab	2006	It has similar activity to cetuximab.
Kinase inhibitor	Regorafenib	2013	The most lastly approved agent for colorectal cancer, it inhibits several different kinases.

Targeted Therapy

Together with improvement in molecular biology, target-specific agents have been developed for treatment of the cancer. Over last decade, overall survival of metastatic CRC patients has further improved with introduction to biological targeted therapies. Differently from cytotoxic agents, targeted agents inhibit tumor growth by interrupting specific molecules which are needed for carcinogenesis [81]. Three main focus of targeted therapy are as follow; (i) inhibition growth of new vessels by targeting vascular endothelial growth factor (VEGF), (ii) interfering cancer cell growth by targeting epidermal growth factor receptor (EGFR), (iii) blocking receptor tyrosine kinases by multikinase inhibitors [82].

Tumor vascularization (angiogenesis) is an essential process for tumor growth due to excess need of nutrient for cancer cells and metastasis. Therefore, blocking angiogenesis has been found to have a strong therapeutic effect on cancer. Between several signaling molecules, VEGF is identified as the most important regulator which promoting angiogenesis and highly expressed in CRC cells [83].

Bevacizumab is a FDA approved humanized monoclonal antibody (mAb) which binds to VEGF-A (member of receptor VEGF activating ligand) and inhibit endothelial cell proliferation. When bevacizumab was combined with irinotecan or oxaliplatin based regimen, overall survival prolonged up to 5 months. Moreover, it has been also found that continuing with bevacizumab while changing cytotoxic agent backbone in second-line improved the overall survival [84].

Aflibercept is another VEGF inhibitor, which is used for treatment of CRC by binding all isoforms of VEFG-A, VEGF-B and placental growth factor. It is a recombinant fusion protein consisting of extracellular domain of VEGF receptors attached to Fc region of human IgG1 immunoglobulin [85]. An International randomized-blind study (VELOUR trial) in 2012, showed that patients who received aflibercept together with FOLFIRI had significantly higher response rate of 20% versus 10% and longer overall survival of 13.5 versus 12.1 months [86].

EGFR is one of the most studied therapeutic target for treatment of advanced cancer. EGFR is highly expressed in many tumor types such as colorectal, lung, breast, head and neck. Attachment of endothelial growth factor (EGF) to EGFR stimulates pathways which promote cell growth and division [87].

Cetuximab and Panitumumab are two FDA approved monoclonal antibodies which bind to EFGR and prevent further ligand binding. It has been shown that administration of these mAbs alone or together with a chemotherapy regimens resulted in improved overall survive [88, 89]. However, anti-EGFR agents cannot be used within the tumors which have KRAS mutations because of their EGFR-independent signal activation [90]. As KRAS mutations have been found in 40-45% of CRC cases, RAS analysis is a mandatory precondition for cetuximab or panitumumab therapy [91].

Regorafenib is an oral multikinase inhibitor that blocks several protein kinases which are involved in tumor growth and angiogenesis. It was approved by FDA following a pivotal phase III multinational trial (CORRECT) which showed the efficacy of regorafenib for patients with chemorefractory CRC by increased overall survival of 6.4 versus 5 months [92].

The most recently FDA approved mAb for the treatment of CRC is ramucirumab which binds to human VEGF-R2 and its ligand. The approval was based on RAISE trail which compared two groups received FOLFIRI plus placebo or FOLFIRI plus ramucirumab.

Administration of ramucirumab increased overall survival of 13.3 versus 11.7 months [93].

Over last two decades, treatment of advanced CRC has improved continuously with chemotherapy and biological targeted agents. However, there are still many limitations such as severe side effects of chemotherapy, resistance to cytotoxic agents, low response rate to targeted therapy due to high heterogeneity of the disease [94]. After curative treatment, recurrence rate of stage IV CRC patients is around 65% and even after application of chemotherapy, radiotherapy and targeted therapy the survival rate is still around 10% [80]. Potent and more efficient therapeutic approaches are needed to treat CRC patients who are still suffering, even with the help of conventional therapeutic strategies.

1.1.2 Oncolytic Virotherapy

Oncolytic virotherapy is a novel form of therapy for treatment of cancer, whose efficacy has been proven in several clinical trials in the last two decades [95]. Oncolytic viruses selectively replicate in cancer cells, spread within tumor tissue and lead to tumor destruction. In addition, viruses also induce the innate and adaptive immune responses, resulting in immune-mediated tumor cell killing [96].

The power of viruses as oncolytic agent started to be discovered in the beginning of 20th century. First reports were based on some naturally occurred infections which resulted in detected tumor regression [97]. However, between 1950 and 1960 many researches were conducted with the aim of treating cancer patients by using several different viruses such as hepatitis, measles, West Nile fever, dengue fever or pox viruses from infectious body fluids or infected tissue extracts of other patients [98]. It is important to note that the first study which described the oncolytic power of group B Coxsackie viruses run across these years [99]. Many of cases didn't respond to the treatment because of the immune system. Viruses were eliminated by immune cells and therefore no tumor regression was observed. Regression was only detected in the limited number or patients, who are immunosuppressed, but strong side effects were also observed in a consequence of normal tissue infection. Because of limited potency, safety concerns and discovery of new chemotherapeutics during that time, interest to oncolytic virotherapy decreased dramatically between 1970s and 1980s [100].

Breakthrough achievements in molecular biology techniques and extensive understanding of virology and oncology together, took the attention of researchers to oncolytic virotherapy again. In 1991, with the help of recombinant DNA technology, the modern age of the oncolytic virotherapy started [96]. Genetically engineered, neurovirulence attenuated thymidine kinase-negative herpes simplex virus was generated and tested for the treatment of human glioblastoma [101]. This significant progress was followed by several studies which aimed to design and construct wide range of viruses to increase safety and targeting them against different types of tumors [102].

Another important finding which opened a new sight to the area was that anti-tumor immune response of host is stimulated in a consequence of virus infection. In addition to direct cell lysis caused by virus infection, activation of adaptive and innate immune system increase the therapeutic efficacy significantly [103]. After direct cell lysis, tumor associated antigens (TAAs), pathogen associated molecular patterns (PAMPs) and danger associated molecular patterns (DAMPs) are released to tumor microenvironment from dying tumor cells. These molecules activate dendritic cells, subsequently activation of T cells lead to immunogenic cell death **(Figure 1.5)** [104]. Therefore, scientists started to arm the viruses by inserting transgenes to enhance effect of the immune system on treatment of cancer [105].

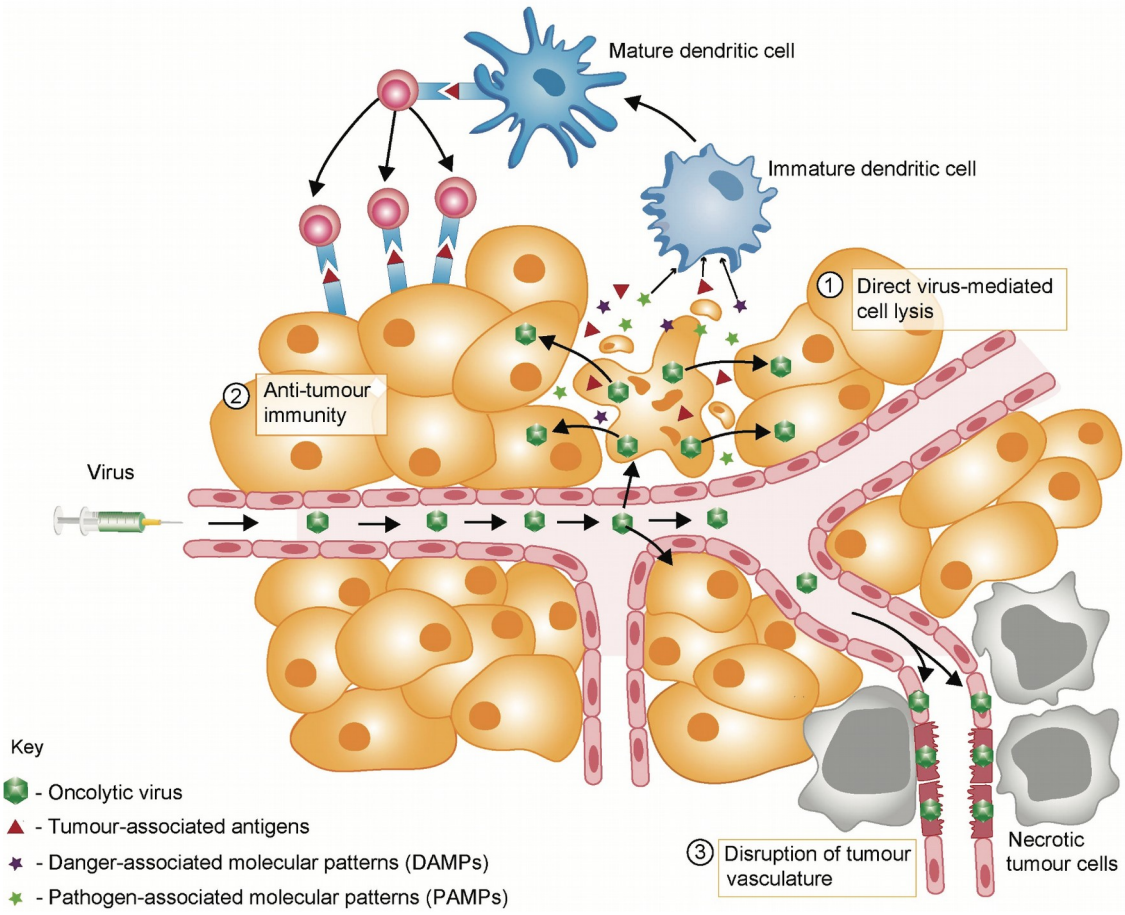

Figure 1.5 Mechanism of viro-oncolysis. (i) Oncolytic viruses infect and replicate in the cells, induce cell lysis and spread over not infected tumor cells. (ii) Viral particles stimulate anti-tumor immunity. (iii) Destruction of tumor vessels [104].

In 2005 a significant milestone was achieved in history of oncolytic virotherapy when China approved the use of genetically engineered adenovirus (H101) for the treatment of head and neck cancer as first oncolytic virus to be used in clinic [106]. Recently, in October 2015, FDA approved use of talimogene laherparepvec (T-Vec) for treatment of melanoma [107]. T-Vec is a genetically engineered herpes virus which has two viral gene deletions and encodes granulocyte macrophage colony-stimulating factor (GM-CSF). However, heterogeneity of tumors and also specific features of each oncolytic virus such as different cellular entry, lifecycle mechanisms and lytic properties result in different antitumor activities in various cancer types. Therefore, even if there are many success stories so far, scientists in this field are working to find a new virus candidate or to improve the existing virus properties for a more efficient oncolytic virus to be tailored in all cancer types.

Various DNA viruses such as adenovirus, herpes virus, vaccinia virus, parvovirus and reovirus have been evaluated as oncolytic viruses for treatment of cancer [108] **(Figure**

1.6). Adenoviruses and herpes viruses among them are the most extensively investigated virus types in the clinic [109, 110]. DNA viruses have been usually selected due to their high transgene capacity and genome stability which let to researches construct a specific genetically engineered virus. Additionally, tumor specificity for DNA viruses is determined at transcriptional level and gene expression can be controlled by promoters. On the other hand, different oncolytic properties of RNA viruses open promising scope to oncolytic virotherapy field [111].

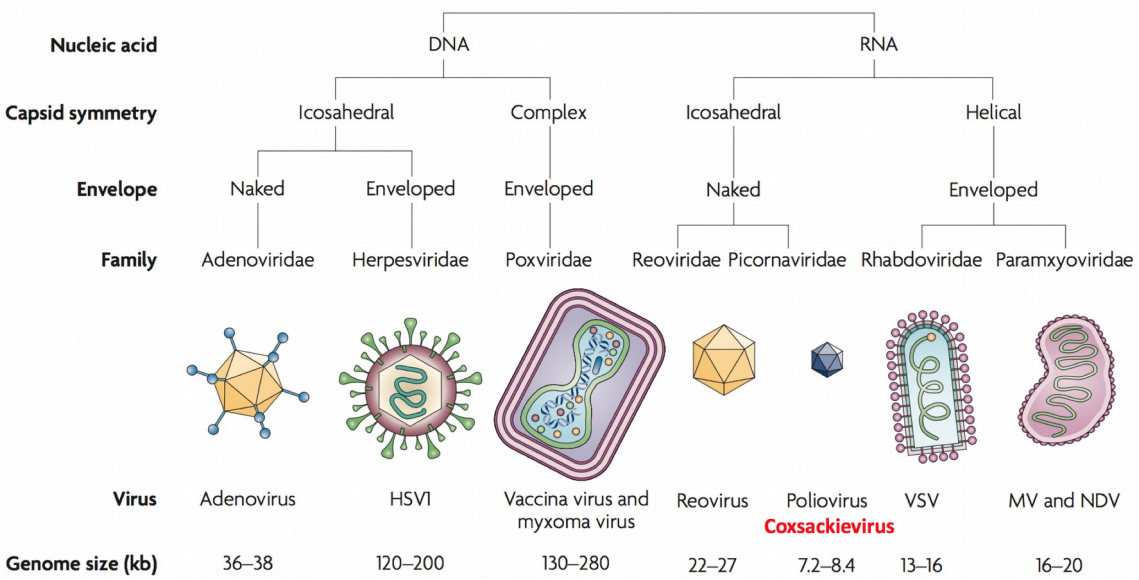

Figure 1.6 Oncolytic viruses which are recently under clinical trials [112].

RNA virus differ from DNA virus with several features which give them a special aspect as oncolytic virus. Firstly, RNA viruses have inherent natural tumor selectivity without need to genetic engineering. The formation of double stranded RNA in normal cell cytoplasm induces cellular antiviral mechanism which are activation of PKR and release of interferons. However, many tumor types have mutations in RAS signaling pathway, therefore these cells do not activate PKR and provide a permissive environment for virus replication. Moreover, RNA viruses have a short replication cycle and produce a very large number of progeny, which gives them another advantage relative to DNA viruses in oncolytic virotherapy. Another important advantage is that RNA viruses are considered to be safer, because they replicate in cytoplasm so that lack the potential for genotoxic effects caused by integration into the host genome [113].

In the course of the last fifteen years, enteroviruses such as coxsackie virus A21 (CVA21), echovirus 1 and poliovirus, which are single-stranded RNA-viruses and belong to the *Picornaviridae* family, were evaluated for their potential as oncolytic

agents against melanoma, breast and prostate cancer [114, 115, 116]. Enteroviruses are potent agents for treatment of cancer due to their ability to bind specific cell receptors which are overexpressed on tumor cells [117]. As an example, one of the most widely investigated enterovirus, CVA21 (trade name; CAVATAK) uses intercellular adhesion molecule 1 (ICAM-1) and decay-accelerating factor (DAF) to enter host cells. It can cause common cold like upper respiratory tract infections and muscle inflammation infections in humans [118]. The virus has a natural tropism to some cancer types such as breast cancer, gastric cancer and melanoma due to overexpression of ICAM-1 and DAF. CVA21 is considered as wild type oncolytic virus [119] and currently under clinical phase II study for malignant melanoma (NCT01227551). Similarly, poliovirus which utilizes the CD155 receptor to bind and infect the host cell, has a specific cell tropism for glioma cells due to overexpression of CD155. Poliovirus is highly pathogenic in humans as it causes human paralytic poliomyelitis in 1% infected individuals. Therefore, it was attenuated by replacing its internal ribosome entry site (IRES) site with human rhinovirus serotype 2 (HRV2) IRES site. This new engineered virus called PVS-RIPO, is currently under clinical phase II studies for treatment of malignant glioma [120] (NCT02986178).

Recently, another member of this group, coxsackievirus B3 (CVB3), strain Nancy, was described as a novel oncolytic virus for treatment of lung carcinomas. Importantly, CVB3 induced tumor destruction is not only a result of direct virus-induced cell lysis but also as a consequence of virus-mediated stimulation of innate immunity, leading to the recruitment of cytolytic natural killer (NK) cells into the tumor. CVB3 caused cell lysis results in release of DAMPs and TAAs such as HMGB-1, calreticulin and ATP which induce infiltration of immune effector cells. No treatment-related mortality, but moderate hepatic dysfunction and mild myocarditis were reported to be the main side effects of CVB3 Nancy treatment of lung carcinoma in mice [121].

1.1.3 Coxsackievirus B3

Group B coxsackieviruses have been identified in the middle of 20th century [122]. Between 6 serotypes CVB3 is the most widely investigated one due to its role on viral myocarditis. CVB3 is a nonenveloped icosahedral particle which is around 30 nm and consist of 4 capsid proteins. Among them VP4 is an inner protein and VP1, VP2, VP3 compose outer layer of virus particle. The viral genome is around 7.5 kb and comprises an open reading frame (ORF) flanked by 5′ and 3′ untranslated regions (UTR). ORF

encodes 11 proteins which are VP1-4 capsid proteins, 2A and 3C viral proteases, 3D RNA polymerase, 2B and 2C proteins for RNA synthesis, 3A and 3B proteins for initiation of RNA synthesis [123]. Virus infection starts with binding to host cell receptor, therefore receptor expression is needed for entry to target cell [124]. Interaction with receptor ends up with endocytosis of virus and subsequently uncoating of viral RNA to cytoplasm. Afterwards positive strand RNA is translated into large polyprotein which is subsequently cleaved into structural and nonstructural proteins. 3D RNA polymerase transcripts + RNA to – RNA. The negative strand RNA acts as template for further + RNA translation to be packed by capsid proteins to form new virions. The life cycle ends by release of new viruses for further infections [125] **(Figure 1.7)**.

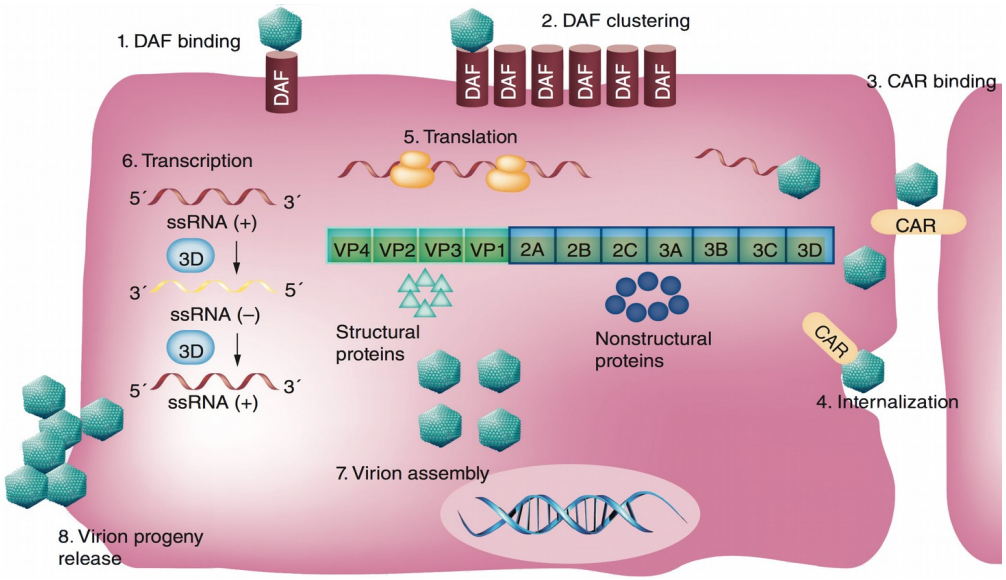

Figure 1.7 The life cycle of CVB3 [123].

CVB3 like many other RNA viruses, has high mutation rates and can easily adapt to new cellular environments [126]. This important feature resulted in the emergence of several clinical and laboratory strains with varying infection and growth properties. A number of CVB3 strains have been characterized for their tissue tropism and organ toxicity in order to better understand of virus-host interaction and pathogenesis caused by viral infection **(Figure 1.8)**. Among these CVB3 strains, there are strains which are highly cardiotropic, such as CVB3 H3, 31-1-93, M2, HA or H310A1 [127, 128, 129, 130], whereas a number of other strains have been found to be low or non-cardiotropic, e.g. Nancy and PD [130, 131]. There are also CVB3 strains that preferentially infect the liver [132, 133]. Moreover, almost all known CVB3 strains are able to infect the pancreas [134]. The difference in pathogenicity is believed to be attributable to viral

capsid proteins, which are directly involved in virus-cell attachment and virus uptake. In general, most of CVB3 strains utilize the coxsackievirus and adenovirus receptor (CAR) as primary and the decay accelerating factor (DAF) as co-receptors to infect cells [135, 136]. It is indicated that DAF plays a role in virus attachment and improvement of virus access to CAR but not in internalization [137]. However, the strain PD has a unique receptor tropism as it uses N- and 6-O-sulfated heparan sulfates (HS) to enter the host cells [138]. Nevertheless, the exact molecular mechanism which underlying the pathogenesis is still not fully understood [139].

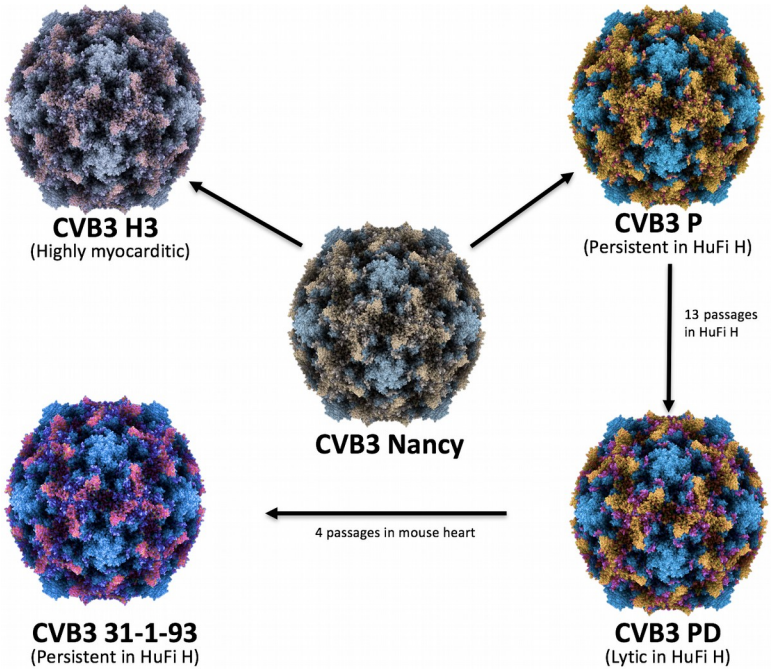

Figure 1.8 Relations of CVB3 Nancy, H3, PD and 31-1-93 strains. CVB3 PD was derived from CVB3 P as a result of several passaging in HuFi H cell line. Myocarditic strain 31-1- 93 was produced from PD by four passages in mouse heart [129, 140].

1.1.4 Increasing Tumor Selectivity by MicroRNA-mediated Targeting

One important limitation of CVB3 as a therapeutic agent in oncolytic virotherapy of tumors becomes evident: in some cases, replication of CVB3 is not exclusively restricted to tumor cells, but also takes place in healthy tissue and may therefore lead to severe adverse effects, such as inflammation in the heart, pancreas and brain [141, 142, 143]. Using murine models, a similar progression of disease as in humans could be observed [144]. The prevention from those adverse effects is the main criterion in the development of a safe therapy using CVB3 as viro-oncolytic agent.

Many different approaches have been used to improve tumor selectivity and to prevent unwanted virus replication in healthy organs. Some viruses can be adapted or

engineered for specific receptor which expressed selectively in tumor. For example, Measles virus have been adapted to use CD46 which is frequently highly expressed in cancer on cancer cells [145]. Another approach is that engineering the virus to use defective antiviral pathways of cancer cells and functional in normal cells, such as activated RAS pathway. [146]. Influenza virus has been attenuated by deleting NS1 gene which let the virus infect cancer cells with aberrant RAS signaling [147]. Alternatively, restriction of virus replication in transcriptional level by insertion of tumor specific promoters [148]. More recently, RNA interference mechanism is adapted to viruses to improve tumor selectivity by insertion of microRNA target sequences (miR-TS) to viral genome [149].

MicroRNAs (miRNAs) are small, non-coding, ~22 nt RNA molecules which are expressed in all eukaryotic cells. They regulate several cellular processes such cell cycle, apoptosis, migration and metabolism by inhibiting or catalyzing mRNA translation over recognition and binding to complementary sequence [150]. It is found that while some miRNAs are expressed in all tissues many of them are highly expressed in specific tissues and more importantly some miRNA levels are deregulated in cancer cells [151] **(Figure 1.9)**.

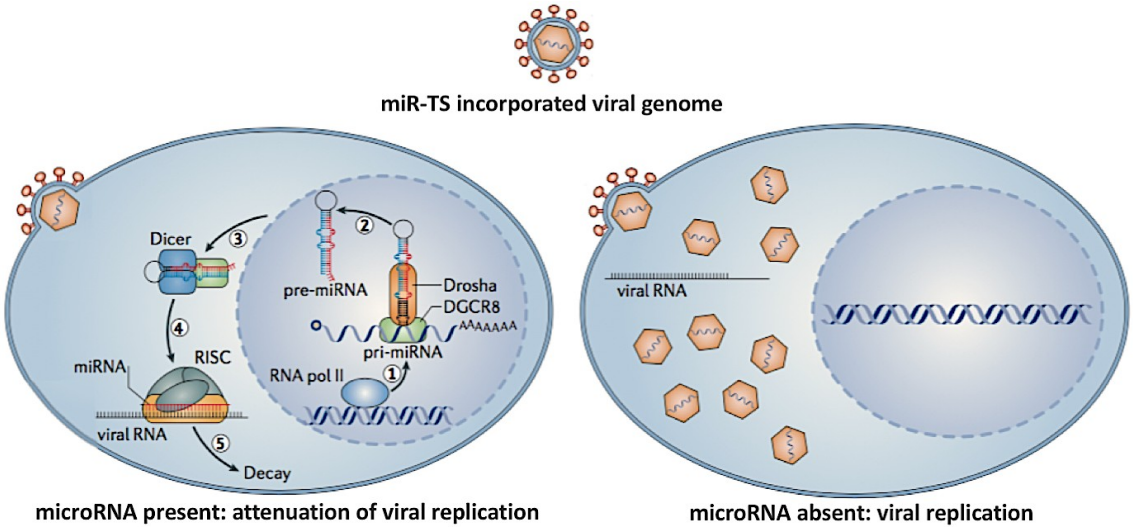

Figure 1.9 Modification of virus tropism via microRNA regulation. Abundant expressed microRNAs are selected for tissue specific targeting. Introduction of miR-TS to the genome of virus resulting in attenuation of engineered virus only in the presence of cognate microRNA. Primary microRNA (pri-miRNA) is transcribed by RNA pol II (step 1). The pri-miRNA is processed to precursor microRNA (pre-miRNA) by Drosha and DGCR8 and exported to the cytoplasm by Exportin-5 (step 2, 3). The pre-miRNA is cleaved by Dicer and loaded to RNA induced silencing complex (RISC) (step 4). Finally, the miRNA directs the RISC to the complementary site in viral RNA and induces the degradation viral genome (step 5) [152].

This new approach has been used by Kelly et al. [118] for CVA21, which is strongly related to CVB3. Kelly et al. inserted target sites (TS) of muscle-specific microRNAs (miR-133 and miR-206) into the oncolytic CVA21 genome which causes fatal myositis in mice. Both target sites were incorporated into the 3′UTR of virus genome. Thus, virus pathogenicity was eliminated by the corresponding miRNAs as result of TS binding and subsequently degradation of the viral genome via RNA interference mechanism. Independent from this, the engineered virus was still able to replicate without limitation in tumor cells, as these cells did not express abundant level of miR-133 or miR-206. Using this approach, Kelly et al., were able to suppress the CVA21 replication in skeletal muscle cells in infected mice. All animals which normally died by CVA21 infection of the skeletal muscles, survived in this experiment while subcutaneously inoculated tumors were efficiently destroyed. Meanwhile, this microRNA-TS technology is widely used as a gene therapeutic and viro-oncolytic practice to improve the specificity of vectors and viruses [153, 154].

1.1.5 Directed Evolution Strategy for Personalized Therapy

In spite of great results in preclinical or clinical studies of oncolytic viruses, the complexity of the disease and the virus-tumor cell interactions lead to necessity of addressing one of the main concerning issue of virotherapy, which is efficacy of the viruses against tumors that varied within the patients [155]. As mentioned also before, many different strategies have been used to overcome this issue. Nowadays, arming the viruses or manipulating their genes with genetic engineering techniques are most widely preferred approaches [156]. However, modification of viruses mostly results in attenuation in replication capability and restricting the efficient respond from the therapy. The natural ability of the viruses gives another important opportunity to generate more effective viruses. Oncolytic viruses differ from conventional therapeutics as they self-replicate and mutate, so that have the possibility to evolve. Directed evolution strategy gives chance to generate a more efficient virus together with genetic engineering strategies [157].

For instance, even though it was indicated that CVB3 has high oncolytic efficiency, it was also shown by Miyamoto et al. that some lung tumor cells have different responses to virus. It was also found that while CVB3 shows high oncolytic efficiency, this efficiency differs from 100 to 1000 fold in different lung tumor cell lines [121]. The fact that, virus-host interactions are still not clearly understood. This point causes the

challenge within the scope of virus and tumor cells interactions, as tumor cells differs from patient to patient. Consequently, a generation of oncolytic virus which specific for each tumor cell becomes evident. RNA viruses have great potential to adapt to new environment due to their high mutation rate [158]. Additionally, their small genome size compare to DNA viruses makes possible and easier the detection and evaluation of mutations responsible for better oncolytic efficiency. Recently, this ability of viruses has been started to be used as directed evolution in several applications [159, 160]. Wollmann et al. showed that by repeated passaging of wild type vesicular stomatitis virus (VSV) in human glioblastoma cells, a new variant which has higher lytic activity and replication efficiency was established [161]. In another viro-therapy study, Garijo et al., adapted the VSV to p53-knockout mouse embryonic fibroblasts (MEF) by serial passaging the VSV in p53 -/- MEF cells. Finally, while he was able to increase viral fitness of VSV in p53 -/- cells, virus was still not able to infect p53 +/+ cells [162].

According to ecological theories it is predicted that serial passaging of virus in a selected tumor cell types will increase the fitness for these cells but decrease the fitness for normal cells which virus is not evolved **(Figure 1.10)** [163]. However, there is some important steps need to be considered for a successful evolution of viruses. The population size must be effective, so that the multiplicity of infection, which means the number of infectious virus per cell, should be determined attentively. While low population size is not efficient for evolution, high population size would cause to formation of defective interfering particles, the evolution of hyperparasites [164]. Additionally, the number of serial of passages needs to be considered as a few passages would be adequate for sufficient adaptation particularly with high mutation rate of RNA viruses. In summary, directed evolution of viruses offer another tool to increase viral fitness of CVB3 against resistant tumors which are derived from each patient individually.

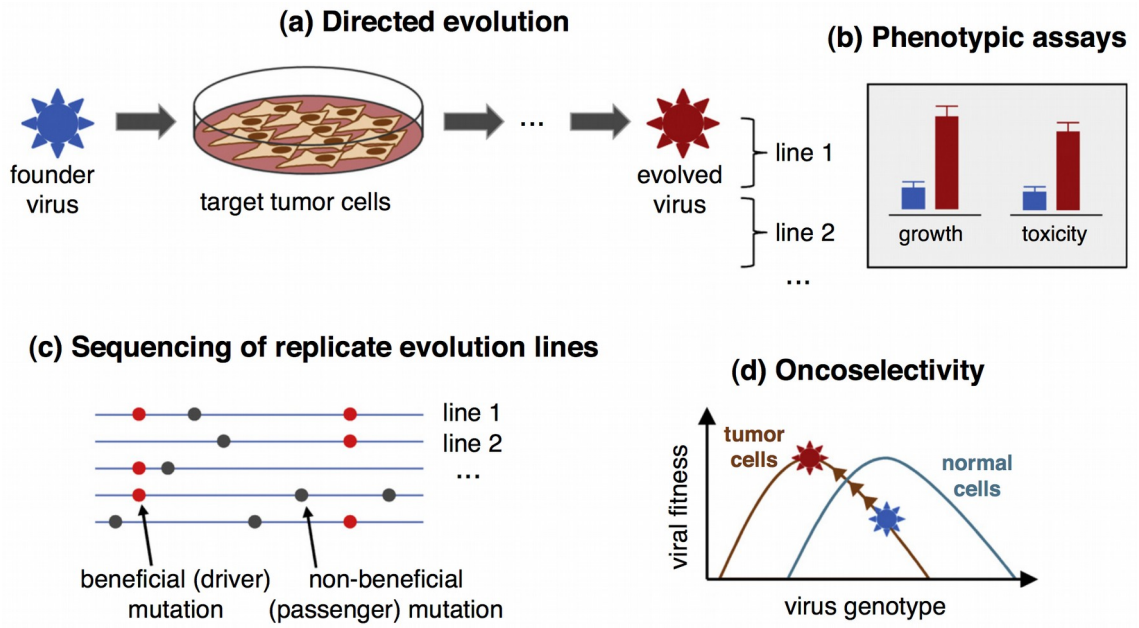

Figure 1.10 Directed evolution of oncolytic viruses. (a) Founder virus is which is relatively resistant by target tumor cells. Serial passages may be done to obtain more effective viruses after each transfer. The amount of passage depends on RNA or DNA virus type. b) Phenotypic assays can be done to analyze differences such as growth, toxicity rates compared to founder virus and evolved virus. c) Sequencing would give an important information about the essential mutations which increase the oncolytic fitness. d) In many cases adaptation to tumor cells come with loss of fitness to normal cells. [163].

1.2 Aim of the Study

The aim of this study was as follow;

(i)- To analyze and compare the oncolytic efficiency and safety profile of the CAR-binding CVB3 strains Nancy, H3, 31-1-93 and HS-binding PD in treatment of human colorectal cancer.

(ii)- Construction of miRNA-regulated CVB3 by insertion of miR-TS into the CVB3 genome to enhance tumor selectivity and to avoid unintended virus replication in healthy organs.

(iii)- Enhancement of viro-oncolytic efficiency of CVB3 by serially passaging of virus through the less susceptible colorectal carcinoma cells. So that, first step of personalized oncolytic virotherapy would be performed.

1.3 Hypothesis

Colorectal cancer is one of the most death causing cancer type in worldwide. While significant improvement in treatment by using combination of chemotherapy and

biological targeted therapy, 80% of the patients with stage IV CRC cancer cannot be cured. Oncolytic virotherapy is a novel treatment method for cancer by using wild-type or genetically engineered viruses. A broad range of viruses has been investigated for many type of cancers in preclinical and clinical phase. CVB3 is one of these viruses which was up to today evaluated only for lung cancer in murine animal model. However, this small RNA virus may be suitable to kill also other cancer types. Additionally, the existence of many CVB3 variants with different receptor tropism and growth properties offer an important opportunity to identify a more effective and safe oncolytic CVB3.

In the light of this information, it was hypothesized that one of the laboratory strain which has different receptor tropism may have a higher tumor selectivity together with higher safety profile. Additionally, by engineering the virus with miRNA target-sites and adapt them to resistant tumor cells, may enhance the efficiency and safety which are the most important concerns of virotherapy. Moreover, adapting the virus to different cancer tumor cells may give a solution to overcome the difficulties of treatment which causing from heterogeneity of the disease and give a chance for personalized therapy.

CHAPTER 2

MATERIAL AND METHODS

2.1 Material

2.1.1 Chemicals

Product	Manufacturer
Ampicillin	Sigma-Aldrich, Munich, DE
Bovine serum albumin (BSA)	Roche Diagnostics, Mannheim, DE
Crystal violet	Carl Roth GmbH & Co. KG, Karlsruhe, DE
DEPC water	Life Technologies GmbH, Darmstadt, DE
EDTA	Carl Roth GmbH & Co. KG, Karlsruhe, DE
Ethanol	Carl Roth GmbH & Co. KG, Karlsruhe, DE
Ethidium bromide	Sigma-Aldrich, Munich, DE
Formaldehyde (37%)	Merck, Darmstadt, DE
Fatal calf serum	C.C. Pro, Oberdorla, DE
Glycerin	Carl Roth GmbH & Co. KG, Karlsruhe, DE
Heparin	Sigma-Aldrich, Munich, DE
Isopropanol	Carl Roth GmbH & Co. KG, Karlsruhe, DE
Kanamycin	Carl Roth GmbH & Co. KG, Karlsruhe, DE
LB-Medium (Lennox)	Carl Roth GmbH & Co. KG, Karlsruhe, DE
LB-Agar (Lennox)	Carl Roth GmbH & Co. KG, Karlsruhe, DE

L-Glutamine	Sigma-Aldrich, Munich, DE
Natrium pyruvate	Sigma-Aldrich, Munich, DE
Non-Essential amino acids	Life Technologies GmbH, Darmstadt, DE
Magnesium chloride	Carl Roth GmbH & Co. KG, Karlsruhe, DE
Penicillin/Streptomycin	Sigma-Aldrich, Munich, DE
Sodium Chloride	Carl Roth GmbH & Co. KG, Karlsruhe, DE
Trichloroacetic acid (TCA)	Carl Roth GmbH & Co. KG, Karlsruhe, DE
Trypsin/EDTA	Sigma-Aldrich, Munich, DE
Triton X-100	Carl Roth GmbH & Co. KG, Karlsruhe, DE
Tris-HCL	Carl Roth GmbH & Co. KG, Karlsruhe, DE
Universal Agarose	Rapidozym GmbH, Berlin, DE

2.1.2 Kits and Reagents

Product	**Manufacturer**
Cell Proliferation Kit (XTT)	Roche Diagnostics, Mannheim, DE
Dig RNA labeling Kit	Roche Diagnostics, Mannheim, DE
Dual-Luciferase Reporter System	Promega GmbH, Mannheim, DE
EndoFree Plasmid Maxi Kit	QIAGEN GmbH, Hilden, DE
High Pure Viral RNA Kit	Roche Diagnostics, Mannheim, DE
High Capacity cDNA Reverse Transcription Kit	Life Technologies GmbH, Darmstadt, DE
HistoGreen	Linaris GmbH, Dossenheim, DE
INT Reagent	VWR International Gmbh, Darmstadt, DE
MTT Reagent	VWR International Gmbh, Darmstadt, DE
OPTI-MEM Medium	Life Technologies GmbH, Darmstadt, DE

QIAquick Gel Extraction Kit	QIAGEN GmbH, Hilden, DE
Pertex Mounting Medium	Medite GmbH, Burgdorf, DE
Plasmid Midi Kit	QIAGEN GmbH, Hilden, DE
Plasmid Miniprep Kit	PEQLAB Biotechnolgy GmbH, Erlangen, DE
Polyethylemine HCI Max Transfection Reagent	Polysciences Inc, Warrington, US
TRIzol Reagent	Life Technologies GmbH, Darmstadt, DE
Sso FastTM EvaGreen Supermix	Bio-Rad Laboratories, Munich, DE

2.1.3 Buffers and Solutions

Buffer	**Manufacturer**
1x PBS	PAA Laboratories GmbH, Colbe, DE
10x TBE	Carl Roth GmbH & Co. KG, Karlsruhe, DE
TaqMan®Gene Expression Master Mix	Life Technologies GmbH, Darmstadt, DE
DNA-Loading Buffer	PEQLAB Biotechnologie GmbH, Erlangen, DE
HEPES buffer solution	Life Technologies GmbH, Darmstadt, DE

Solution	**Preparation**
10 % TCA	25 g TCA 250 ml DEPC Water
Crystal Violet 5x	50 % EtOH 50 % 1x PBS Crystal Violet
Lysis Buffer	20mM Tris-HCl (pH 8) 10mM NaCl 0.5% Triton X-100 5mM EDTA, 3mM MgCl2

2.1.4 Enzymes

Enzyme	Manufacturer
Restriction Enzymes	New England Biolabs GmbH, Frankfurt, DE
T4 DNA Ligase	New England Biolabs GmbH, Frankfurt, DE
Taq DNA Polymerase	Rapidozym GmbH, Berlin, DE
RNase H	New England Biolabs GmbH, Frankfurt, DE
DNase I	PEQLAB Biotechnologie GmbH, Erlangen, DE
Klenow Fragment	New England Biolabs GmbH, Frankfurt, DE

2.1.5 Antibodies

Antibody	Manufacturer
Monoclonal mouse anti-CAR/IgG1, clone RmcB	Merck, Darmstadt, DE
Monoclonal mouse anti-DAF/CD55	Merck, Darmstadt, DE
Alexa Fluor® 488 Donkey Anti-Mouse IgG Antibody	Life Technologies GmbH, Darmstadt, DE
Anti-Digoxigenin antibody (HRP)	Roche Diagnostics, Mannheim, DE

2.1.6 Cell Lines and Medium

Cell Line	Medium
HeLa	MEM (1x) (Life Technologies GmbH, Darmstadt, DE)
HEK293T	DMEM High Glucose (Biowest, Darmstadt, DE)
CHO-K1	DMEM / F12 (Life Technologies GmbH, Darmstadt, DE)
DLD1	RPMI 1640 (C.C. Pro, Oberdorla, DE)
Colo680h	RPMI 1640 (C.C. Pro, Oberdorla, DE)

Colo205	RPMI 1640 (C.C. Pro, Oberdorla, DE)
Colo320	RPMI 1640 (C.C. Pro, Oberdorla, DE)
Colon-26	RPMI 1640 (C.C. Pro, Oberdorla, DE)
LS174T	EMEM (Lonza, Basel, CH)
CaCo-2	EMEM (Lonza, Basel, CH)
HCT116	RPMI 1640 (C.C. Pro, Oberdorla, DE)
SW480	Leibovitz L15 (Biowest, Darmstadt, DE)
SW620	Leibovitz L15 (Biowest, Darmstadt, DE)

HeLa cells were cultured in Minimum Essential Medium (MEM) (Life Technologies) supplemented with 5% fetal calf serum (FCS), 5% HEPES buffer solution, 1% non-essential amino acids and 1% penicillin-streptomycin.

HEK293T cell line was cultured in DMEM High Glucose (Biowest) supplemented with 10% FCS, 1% penicillin-streptomycin, 1% L-Glutamine and 1mM Na-Pyruvate.

CHO-K1 cell line was maintained in DMEM/F12 Medium (Life Technologies) supplemented with 10% FCS and 1% penicillin-streptomycin.

Human CRC cell lines (DLD1, Colo205, Colo680h, Colo320, HCT116) and murine CRC cell line (Colon-26) were grown in RPMI 1640 (C. C. Pro) supplemented with 10% FCS, 1% penicillin-streptomycin, 1% L-Glutamine and 1mM Na-Pyruvate.

LS174T cells were maintained in EMEM (Lonza) and supplemented with 10% FCS, 1% penicillin-streptomycin, 1% L-Glutamine, 1% non-essential amino acids and 1mM Na-Pyruvate.

SW480 and SW620 cell lines were cultured in Leibovitz L15 (Biowest) supplemented with 10% FCS, 1% penicillin-streptomycin, 1% L-Glutamine.

2.1.7 Equipment

Device	**Manufacturer**
Advanced Primus 25 Thermal Cycler	PEQLAB Biotechnologie GmbH, Erlangen, DE

C1000 Thermal Cycler	Bio-Rad Laboratories GmbH, Munich, DE
NanoDrop2000	PEQLAB Biotechnologie GmbH, Erlangen, DE
Fluorescent Microscope Observer Z1	Carl Zeiss GmbH, Jena, DE
V-650 Spectrophotometer	Jasco Inc., Easton, Maryland, US
Sigma 3-16PK Centrifuge	SIGMA Laborzentrifugen GmbH, Osterode am Harz, DE
Heraeus Fresco 17 Microcentrifuge	Fisher Scientific GmbH, Schwerte, DE
Avanti J-E Centrifuge	Beckman Coulter GmbH, Krefeld, DE
Tristar LB 942 Microplate Reader	Berthold Technologies GmbH, Bad Wildbald, DE
UV solo Transilluminator	Analytic Jena AG, Jena, DE

2.1.8 Viruses

Virus	**Source**
CVB3-Nancy	ATCC VR30
CVB3-PD	M. Schmidtke, Institute of Virology and Antiviral Therapy, University Jena, DE
CVB3-31-1-93	M. Schmidtke, Institute of Virology and Antiviral Therapy, University Jena, DE
CVB3-H3	A. Henke, Institute of Virology and Antiviral Therapy, University Jena, DE
$CVB3_{miR\text{-}375TS(3x)}$	pMKS1 [165]
$CVB3_{miR\text{-}690TS(3x)}$	pMKS1
$CVB3_{miR\text{-}690TS(6x)}$	pMKS1
$CVB3_{miR\text{-}39TS(3x)}$	pMKS1
$CVB3_{miR\text{-}39TS(6x)}$	pMKS1

2.1.9 Plasmids

Plasmid	Application	Manufacturer
pCMV-miR-375-GFP	Expression of miR-375	Origene Technologies, Rockville, Maryland, US
pCMV-miR-690-GFP	Expression of miR-690	Origene Technologies, Rockville, Maryland, US
psiCHECK2	Dual Luciferase Reporter Assay	Promega GmbH, Mannheim, DE
*pMK-RQ-miR-TScas	Cloning of miR-TS	Life Technologies GmbH, Darmstadt, DE
*pMKS1-3′MCS	Cloning of miR-TS bearing CVB3	
*pMKS1-3′miR-TScas	Construction of miR-TS bearing CVB3 cDNA clones	
*pMKS1-miR-375TS(3x)	Production of miR-375TS(3x) bearing CVB3	
*pMKS1-miR-690TS(3x)	Production of miR-690TS(3x) bearing CVB3	
*pMKS1-miR-690TS(6x)	Production of miR-690TS(6x) bearing CVB3	
*pMKS1-miR-39TS(3x)	Production of miR-39TS(3x) bearing CVB3	
*pMKS1-miR-39TS(6x)	Production of miR-39TS(6x) bearing CVB3	

* Plasmid cards can be found in the appendix.

2.1.10 Primers

Primer	Sequence 5′→3′	Application
5′UTR-s	TTAAAACAGCCTGTGGGTTG	Sequencing
5′UTR-as	TCCTCTACTGTGGGGGAGTT	Sequencing

VP-part1-s	ACCACTTAGCTTGAGAGAGG	Sequencing
VP-part1-as	ACCAATGGGTGTAATAGTT	Sequencing
VP-part2-s	TTCAGTCGGACGCTCCTAG	Sequencing
VP-part2-as	TACACCGACATCTGGCTAT	Sequencing
miR-TS-s	CCATAGATGCGTCTTTGCT	Sequencing
miR-TS-as	CCGTTGTCTAGTTCGGTT	Sequencing
CVB3-s	CCCTGAATGCGGCTAATCC	qPCR analysis
CVB3-as	ATTGTCACCATAAGCAGCCA	qPCR analysis
HS6ST2-s	CCAAGTCAAATCTGAAGCACA	qPCR analysis
HS6ST2-as	TCTGGAAATGGGTCTGAAGGA	qPCR analysis
18S rRNA-s	GTGGAGCGATTTGTCTGGTT	qPCR analysis
18S rRNA-as	GGACATCTAAGGGCATCACAG	qPCR analysis

2.1.11 TaqMan™ microRNA Assays

Assay Name	Assay ID	Mature microRNA sequence 5′→3′
hsa-miR-16	000391	UAGCAGCACGUAAAUAUUGGCG
hsa-miR-34a	000426	UGGCAGUGUCUUAGCUGGUUGU
mmu-miR-690	001677	AAAGGCUAGGCUCACAACCAAA
mmu-miR-124a	001182	UAAGGCACGCGGUGAAUGCC
hsa-miR-1	002222	UGGAAUGUAAAGAAGUAUGUAU
hsa-miR-375	000564	UUUGUUCGUUCGGCUCGCGUGA

As control assay, U6 snRNA was purchased (assay ID, 001973). TaqMan microRNA assays were purchased from Life Technologies with respect to mentioned assay ID.

2.2 Methods

2.2.1 Virology Methods

2.2.1.1 Viral Plaque Assay

HeLa cells were cultured in 24-well cell culture plates as confluent monolayers. After 24 h, medium was removed and cells were overlaid with serial ten-fold dilutions of supernatant (300µl) harvested from virus-infected cell lines or from homogenized mouse organs after 3 freeze/thaw cycles. Cells were then incubated at 37° C for 30 min and, after removal of the supernatant, overlaid with agar containing Eagle's minimal essential medium (MEM) (500µl). Three days later, the cells were stained with 1x 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide iodotetrazolium chloride (MTT/INT) solution (20µl). Virus titers were determined by plaque counting 1h after staining. Each sample analyzed in duplicate.

Eagle overlay medium was prepared as follow;

9.61 g MEM and 1.7 g $NaHCO_3$ dissolved in 750 ml pure water containing 1% Penicillin/Streptomycin. Afterwards, medium was transferred to sterile bottle through the filter and aliquoted to 100 ml sterile bottles and stored at +4℃.

2.2.1.2 Virus Propagation

The CVB3 strain PD was derived by serial passages of the CVB3 strain P on HuFi cells [166]. The cardiotropic CVB3 strain 31-1-93 was isolated from heart tissue after four heart passages of PD in outbred NMRI mice [128]. Both virus strains PD and 31-1-93 were a kind gift from Michaela Schmidtke (Institute of Virology and Antiviral Therapy, University Jena, Germany). CVB3 PD and 31-1-93 were propagated in CAR/DAF-negative CHO-K1 and in HeLa cells, respectively, before use. CVB3 strain Nancy (ATCC VR30) was propagated in HeLa cells. CVB3 H3 was generated from pBKCMV-H3 which was kindly provided by A. Henke (Institute of Virology and Antiviral Therapy, University Jena).

For propagation, cells were cultured in T75 flasks as confluent monolayers. Next day, medium was discarded and cells were infected with MOI of 10 (in 5 ml FKS free medium) virus. Following 30 min incubation in 37 ℃, 10 ml fresh medium was added

and the flask was incubated till the observation of complete cell lysis (24h to 48h). Then the lysed cells were subjected to three thaw/freeze cycles. Supernatant was collected following removal of cell debris by centrifugation. 500 µl aliquots were stored in -80 ℃.

2.2.1.3 Plasmid Based Virus Propagation

Viruses were produced in HEK293T cells. For this purpose, HEK293T cells were seeded to 6 well plate for 60% confluency. Next day, 4 µl PEI Max (2 mg/ml) and 3.2µg plasmid DNA were added to 200µl Opti-MEM medium (Life Technologies) separately. Following 5 min incubation time, DNA and PEI Max was mixed and incubated for another 20 min. Lastly, this mixture was applied to cell culture medium in 6 well plate and incubated till all cells were lysed. After the observation of cell lysis, cells were subjected to three freeze/thaw cycles and supernatant was collected after centrifugation of the samples at 12,000 rpm for 5 min.

2.2.1.4 Single Step Growth Curve

Single step growth curves were prepared for analyzation of virus kinetics and determination of the time required to reach a plateau. For this purpose, selected colorectal carcinoma cell line was seeded to 6 well plate for full confluency and next day, cells were inoculated with CVB3 at MOI of 0.1 (900 µl) and incubated at 37℃ and 5% CO_2 for 30 minutes. Afterwards, virus solution was removed, cells were washed with PBS and 3 ml fresh medium was added. Cells were incubated at 37℃ and 100 µl supernatant was collected at indicated time points (0, 12, 24, 36, 48 and 72 hours post infection). Supernatants were stored at -80℃ and plaque assay was performed for all collected samples in the same time.

2.2.1.5 Serial Passaging of Virus for Cell Adaptation

Cells were seeded to 6 well plates. After 24h when cells were completely confluent, medium was removed and cells were infected with viruses at an MOI of 0.1 and cells were incubated for 30 minutes at 37℃. Medium was removed, following washing step with PBS, 3 ml fresh medium was added and cells were incubated at 37℃ and 5% CO_2. Cells were daily monitored and after 72h incubation, the cell lysate was collected and centrifuged at 12000 rpm for 5 min to clear cell debris and supernatant was collected for

plaque assay. For next passage, same number of cells were seeded and infection was performed with consistent amount of virus (MOI of 0.1).

2.2.2 Cell Biology Methods

2.2.2.1 Cell Killing Assay

Colorectal carcinoma cells were seeded in 96-well plates and, on the following day, when cells reached complete confluence, the medium was carefully removed and 100 µl virus solution was added at multiplicity of infection (MOI) of 1, 10 and 100. After 30 min incubation at 37° C and 5% CO_2, virus-containing medium was removed and fresh medium was added and cells incubated for different periods. To fix the cells, the medium was removed, the cells were washed with PBS and 100 µl 10% trichloroacetic acid (TCA) (Carl Roth) was added. Following an incubation of 10 min, TCA was removed and 30 µl crystal violet solution (Carl Roth) was added. After 5 min incubation, wells were washed with PBS several times, the plate was allowed to dry overnight and photographed.

2.2.2.2 Cell Viability Assay (XTT assay)

The viability assay with XTT was used to quantify the oncolytic effect of viral infection on human CRC cell lines. This assay is based on the reduce of the yellow tetrazolium salt XTT to orange formazan dye by metabolic active cells. As the dye is soluble in water, the density of the occurred dye can be analyzed with spectrophotometer. Therefore, after virus infection, tetrazolium salt was added to the cells indicated time points. The absorption level at 492 nm of optically active reaction product was measured. The procedure was carried out according to the manufacturer's specifications of the Cell Proliferation Kit (XTT) (Promega). Briefly, cells were seeded to 96 well plate with 50 µl medium. Next day when the cells reached full confluency, cells were infected with the viruses at MOI of 1, 10 or 100 in 50µl PBS solution. In indicated time points, 50µl of XTT labeling mixture was added to the cells. Following 4h incubation period in humidified atmosphere, absorbance level was measured using V-650 Spectrophotometer, Jason Inc. All measured values were determined fourfold. As negative control one sample of uninfected cells was treated with 5% Triton X-100

solution. The mean value of the absorption of these wells considered as a background for all other measurements of this cell line.

2.2.2.3 Heparin Inhibition Assay

Colorectal carcinoma cells were seeded in 96 well plates to reach full confluence. HS analogue heparin (porcine mucosal intestinal; Sigma) was dissolved in serum free DMEM to reach stock concentration of 10 mg/ml and stored at 4℃. Later, heparin was diluted to different concentrations in serum free DMEM and each dilution incubated with MOI 3 of CVB3-Nancy or PD for 1h at 37℃. Heparin free DMEM was used as control. Afterwards, media was removed from plates and cells were infected with HS treated virus solutions and incubated for 30 min at 37℃. Following incubation time, the solution was removed and 100µl fresh medium was added. Plates were incubated for 48h and from one plate, 50µl supernatant of each sample was collected for analysis in plaque assay.

2.2.2.4 Flow Cytometric Analysis of CAR and DAF Expression

Colorectal carcinoma cells were washed with PBS and detached from cell culture plates using PBS-2mM EDTA solution. After washing with PBS the cells were stained on ice with monoclonal mouse anti-CAR/IgG1 (clone RmcB) antibody (Merck) or with monoclonal mouse anti-DAF/CD55 (Merck) for 1 h at a dilution of 1:200. Cells were washed again with PBS and resuspended in Alexa Fluor 488-conjugated donkey anti-mouse IgG antibody (Life Technologies), which was diluted 1:400 in PBS, and incubated for 45 min. After a further wash step with PBS, the cells were resuspended in PBS + 1 % formaldehyde and analyzed by flow cytometry using a MACSQuant® Flow Cytometers (Miltenyi Biotec) and FlowJo, Data Analysis Software (Tree Star). The mean fluorescence intensity was calculated by determining the geometric mean of CAR or DAF-expressing cells minus the geometric mean of a negative control sample.

2.2.2.5 Dual Luciferase Reporter Assay

This assay based on measurement of stimulation of two reporter enzymes which are *firefly* and *renilla* luciferases, in the same system. Firstly, to generate stabilized luminescent signal as a normalization control, *firefly* luciferase substrate was added with the reagent which was provided with the kit. After quenching this reaction, second

reagent with *renilla* luciferase substrate was added to the wells. The two luminescence values were compared with each other for rapid quantification of inhibition or expression of plasmid in the host cells.

Cells were seeded to 24 well plate for 60% confluency and next day cells were transfected with 500 ng of reporter plasmid using 2 µl PEI Max (2 mg/ml) transfection reagent. 24h post transfection, medium was removed from the wells and 100 µl lysis buffer was added. Plates were incubated for 1h room at temperature incubation. Afterwards, plate was stored in -80℃ and before measurement samples were centrifuged for 3 min at 300 rpm and 10 µl of supernatant was transferred to microplate. Lastly, the luminescence levels were measured with Tristar Microplate Reader (Berthold Technologies) according manufacturer instructions.

2.2.2.6 Inhibition Assays with miRNA Expression Plasmids

Functionality of inserted miR-TS were analyzed with inhibition assays using relative miRNA expression plasmid. 60% confluent HEK293T cells were transfected with 800 ng miRNA expression plasmid by using PEI Max transfection reagent (2 mg/ml). As the expression plasmids were equipped with GFP reporter, transfection efficiency was observed with fluorescent microscope before virus infection. Twenty-four-hour after transfection, medium was discarded slowly and 300 µl virus solution (MOI of 0.1 or 0.01) were added to the cells and incubated for 30 minutes at 37℃. Fresh medium was added after discarding virus solution and cells were incubated for 18h at 37℃ and 5% CO_2. Lastly, cells were subjected to 3 freeze / thaw cycles and cell lysate was centrifuged to clear cell debris at 12000 rpm for 5 minutes. Collected supernatant was used for determination of virus titer by plaque assay.

2.2.3 Molecular Biology Methods

2.2.3.1 RNA Isolation

RNA isolation was performed from colorectal cancer cells and healthy mouse tissues such as pancreas, brain and heart. For this purpose, cultured colorectal cancer cells were firstly trypsinized and transferred to falcon tube with medium. Following 300 g x 5 min centrifugation, cell pellet was washed twice with PBS. Lastly, cell pellets were resuspended with 1 ml TRIzol reagent (Life Technologies).

Mouse tissues were directly transferred to Eppendorf tube with TRIzol reagent and stored at -80℃. Tissues were homogenized with pestle following thawing process. RNA ısolation was performed according to the manufacturer's instructions as follow; chloroform was added to suspensions and centrifuged at 12,000 g x 15 min, 4℃. Upper phases were transferred to new Eppendorf tube and RNA precipitation was done by adding isopropanol. Following washing step with 75% ethanol, the RNA pellet was dried and dissolved with 50µl DEPC water, incubated at 55℃ for 10 min and RNA concentration was measured with Nano-Drop.

2.2.3.2 cDNA Synthesis

cDNA was synthesized with 500 ng isolated RNA. Reaction volumes and PCR conditions are shown in **Table 2.1** and **Table 2.2.**

Table 2.1 Reaction volume and program for cDNA synthesis

Component	**Volume**	**Time**	**Temperature**
RNA (100 ng/µl)	5 µl	60 min	50℃
10x RT Buffer	2 µl	5 min	85℃
dNTPs (100 mM)	0.8 µl		
Reverse Primer (10 µM)	1 µl		
MultiScribe Reverse Transcriptase 50 U/µl	1 µl		
DEPC water	11.2 µl		
Total volume	**20 µl**		

2.2.3.3 PCR Amplification of cDNA Fragments

PCR amplification was performed with 2 µl cDNA, using relevant forward primer and reverse primer **(2.1.10)**. PCR product was run on the agarose gel for purification and extracted from gel as described under 2.2.4.5.

Table 2.2 Reaction volume and PCR program for DNA amplification

Component	Volume		Time	Temperature
Forward primer (10 µM)	1 µl		2 minutes	95℃
Reverse primer (10 µM)	1 µl	---35 cycles---	15 seconds	95℃
10x Buffer	5 µl		30 seconds	50℃
$MgSO_4$ (25 mM)	4 µl		1 minute	65℃
dNTPs (100 mM)	1 µl		5 minutes	72℃
Taq DNA Polymerase	1 µl			
cDNA	2 µl			
DEPC water	37 µl			
Total volume	**50 µl**			

2.2.3.4 Quantification of CVB3 RNA Genome

Colorectal carcinoma cells were seeded in 96 well plates and infected with CVB3 strains. Twenty-four hours later plates were subjected to three freeze/thaw cycles and the collected supernatant centrifuged for 20 min at 2,000 x g and 4° C to remove cellular debris. Viral RNA was isolated from the supernatant with High Pure viral nucleic acid kit (Roche) according to the manufacturer's instructions, followed by DNase I digestion (Peqlab). The viral RNA was reverse transcribed using the High-Capacity cDNA reverse transcription kit (Applied Biosystems). For quantification of viral RNA, real-time PCR was performed using CFX96 Real-Time System combined with a C1000 Thermal Cycler (Bio-Rad Laboratories), using the CVB3-s and CVB3-as primers in Sso Fast™ EvaGreen Supermix (Bio-Rad). Cycle times were as follows: one cycle at 50 °C for 2 min followed by 94 °C for 10 min, 40 cycles at 94 °C for 15s, and 60 °C for 60s. A standard curve was used to calculate the number of CVB3 genome copies. The standard curve was prepared using 10-fold serial dilutions of 146bp PCR fragment of CVB3 plasmid (pMKS1), which was amplified with the forward and reverse primers described in 2.1.10.

2.2.3.5 Quantification of microRNA Levels

cDNA was synthesized with High Capacity Reverse Transcription Kit (Life Technologies) by using appropriate miRNA primers. Master mix was prepared for PCR reaction as follow;

Table 2.3 Reaction volumes and PCR program for cDNA synthesis

Component	**Volume**	**Time**	**Temperature**
10x RT Buffer	1.5 µl	30 minutes	16℃
dNTPs	0.15 µl	30 minutes	42℃
Reverse Transcriptase	1 µl	5 minutes	85℃
5x RT primer U6	3 µl	∞	4℃
5x RT primer miRNA	3 µl		
DEPC	1.35 µl		
RNA (10ng/µl)	5 µl		
Total Volume	**15 µl**		

Following cDNA synthesis TaqMan qPCR was performed using CFX96 Real-Time System combined with a C1000 Thermal Cycler (Bio-Rad) according to manufacturer instructions **(Table 2.4)**.

Table 2.4 Reaction volumes and TaqMan qPCR program for miRNA analysis

Component	**Volume**		**Time**	**Temperature**
TaqMan miRNA Probe	1 µl		2 minutes	50℃
TaqMan Master Mix	10 µl	--- 40 cycles---	10 seconds	95℃
cDNA product	1.33 µl		15 seconds	95℃
DEPC water	7.67 µl		60 seconds	60℃
Total Volume	**20 µl**			

The data was analyzed by using ΔC_T method and results were normalized against U6 snRNA levels of cell lines and tissues.

2.2.4 Cloning

2.2.4.1 Production of Chemically Competent Bacteria Cells

For production of chemically competent bacteria cells, 5 µl of E. Coli X-gold (Stratagene) were incubated in 5 ml of antibiotics-free LB medium at 37 ° C and 120 rpm for overnight. The next day, 2 ml of the overnight culture was added to 250 ml antibiotic-free LB medium and incubated at at 37 ° C till the medium reach an OD (595 nm) of 0.5. The suspension was transferred to cold centrifuge tubes and kept on ice for 10 min. Afterwards, the tubes were centrifuged for 5 min at 5000rpm and 4 ° C and the supernatants were discarded. The cell pellet was carefully resuspended in 50 ml of ice-cold 0.1 M $MgCl_2$ solution. Cell culture was centrifuged at 0 ° C, 3000rpm for 5min and then the supernatant was discarded. The cell pellet was gently resuspended in 50 ml of ice-cold 0.1 M CaCl 2 solution and kept on ice for 20 min before last centrifugation step at 0 ° C, 3000 rpm for 5 min. Supernatant was discarded again and the cell pellet was resuspended in 5 ml of ice-cold, sterile filtered (0.2 µm) 0.1 M $CaCl_2$ solution containing 14% glycerol and aliquoted to 100 µl each into precooled Eppendorf tubes. The cells were stored at -80 ° C until the use.

2.2.4.2 Restriction

DNA samples were cut with restriction enzymes to isolate the desired part of the plasmid or to control the accuracy of new produced plasmids according to cutting size. All restriction enzymes were purchased from New England Biolabs and reactions were carried out according to manufacturer instructions with recommended buffer solution.

2.2.4.3 Ligation

Ligation was performed in room temperature with T4 DNA ligase (New England Biolabs) according to manufacturer instructions. If required, for ligation of plasmid, blunt ends was produced by fill-in reaction with Klenow Fragment (New England Biolabs) according to manufacturer instructions. Briefly, 1 unit of enzyme was added per µg DNA and incubated with 1.5mM dNTPs for 15min at 25℃.

2.2.4.4 Transformation

E. coli XL-10 Gold Ultracompetent Cells (Stratagene) were transformed with plasmids using heat shock transformation method. 50µl of competent cells were thawed on ice, 200ng plasmid was added and incubated on ice for 30 min. The cells were then heated to 42℃ for one minute and directly transferred to ice for 2 min incubation. Afterwards, 500µl LB medium was added and cells incubated at 37℃ for 45 min with shaking at 300 rpm. The tube was then centrifuged for 10 min at 2500 rpm and 350µl of supernatant was discarded. Lastly, the cell pellet was resuspended with the remaining supernatant and cell suspension was coated on LB agar with 0.1% ampicillin (1 mg/ml) and incubated overnight at 37℃.

2.2.4.5 Plasmid-DNA isolation

Colonies were picked up with pipet tip and transferred into 3 ml LB medium with 0.1% ampicillin containing tubes and incubated overnight at 37℃ for 120 rpm. Next day, DNA was isolated with Plasmid Miniprep Kit (PEQLAB Biotechnolgy). For higher production of bacteria, midi preparation and maxi preparation was performed by transferring 100µl of mini culture to 100ml LB-medium and 300µl of mini culture to 300 ml LB-medium with 0.1% ampicillin (1 mg/ml), respectively. DNA isolation was performed with Midiprep Kit (QIAGEN) and Endofree Maxiprep Kit (QIAGEN) according to manufacturer instructions.

2.2.4.6 Gel electrophoresis

To separate DNA fragments gel electrophoresis was done as follow; agarose was dissolved in 1.5% (w/v) in TBA buffer with heating and ethidium bromide (Sigma) was added to solution after 10 min. The samples were loaded when the gel was solid and electrophoresis was carried out at 120V for 40 min. The bands were monitored using UV transilluminator (Analytic Jena).

2.2.4.7 Isolation of DNA Fragments from Agarose Gel

For DNA isolation, the desired band was removed from agarose gel with razor blade and the gel slice was transferred to tube. Spin column extraction was done using QIAquick Gel Extraction Kit (QIAGEN) according to manufacturer instructions.

2.2.4.8 Sanger Sequencing

For sequencing, appropriate primers were used **(2.1.11)** and samples were sent to LGC genomics, Berlin. Briefly, Sanger sequencing method based on detection of used labeled di-deoxynucleotide triphosphates (ddNTPs) which lack 3′-OH group. The ddNTPs cause termination of DNA elongation by DNA polymerase when incorporated to the template DNA [167].

2.2.4.9 Construction of miR-TS Bearing Plasmid

Various miR-TS which are complementary to mature sequences of cognate miRNA were obtained from Life Technologies in a single plasmid which was named pMK-RQ-miR-TScas **(Appendix 1)**. The miR-TS cassette was generated by GeneArt and it flanked by *Asis*I and *Pme*I restriction sites and contains seven different miR-TS (miR-690TS, miR-200aTS, miR-39TS, miR-375TS, miR-124aTS, miR-1TS and miR-122TS) **(Figure 2.1)** This plasmid was also used for other investigations as source of various miR-TS. MiRNA mature sequences were determined from "miRBase" database website **(Table 2.5)** [168].

Table 2.4 Exploited miRNA mature sequences and inserted target sites

miR-375 mature sequence	5′- UUUGUUCGUUCGGCUCGCGUGA -3′
miR-375 target site	5′- TCACGCGAGCCGAACGAACAAA -3′
miR-690 mature sequence	5′- AAAGGCUAGGCUCACAACCAAA-3′
miR-690 target site	5′- TTTGGTTGTGAGCCTAGCCTTT-3′
miR-39 mature sequence	5′- UCACCGGGUGUAAAUCAGCUUG-3′
miR-39 target site	5′- CAAGCTGATTTACACCCGGTGA-3′

In this study, miR-TS cassette was firstly inserted into the pMKS1-3′MCS plasmid between *Asis*I and *Pme*I restriction sites. Afterwards unneeded target sequences were removed by restriction digestion to obtain final CVB3 cDNA clone with desired miR-TS. The plasmid pMKS1-3′MCS was generated at Fechner Lab. by insertion of multiple cloning site (MCS) containing *Asis*I and *Pme*I restriction sites into the pMKS1 [165], downstream of the stop codon of CVB3 open reading frame **(Appendix 2)**.

Figure 2.1 Synthesized miR-TS cassette. Each miR-TS is shown in different color. *Asis*I, *Nru*I, *Xba*I, *Nde*I, *Afe*I, *Mlu*I, *BssH*II, *Pac*I, *Swa*I and PmeI are unique restriction sites and are not present in pMKS1 plasmid backbone. Between each miR-TS, 6 random nucleotides were inserted to enhance the miRNA binding. Some miR-TS which were not used in this study such as miR-122, miR-1, miR-124a and miR-200a were also inserted to the cassette for other investigations.

2.2.4.10 Construction of miR-TS Bearing CVB3 cDNA Clones

A) miR-375TS CVB3 cDNA clone

Firstly, pMKS1-3′MCS was digested with *Asis*I and *Pme*I and the miR-TS cassette **(Figure 2.1),** harvested from pMK-RQ-miR-TScas by *Asis*I and *Pme*I digestion, was inserted. This plasmid was termed pMKS1-miR-TScas **(Appendix 3)**.

For construction of miR-375TS(3x) bearing plasmid, pMKS1-miR-TScas was at first digested with *Pme*I - *Pac*I, leading to deletion of unnecessary miR-TSs, which located downstream of miR-375TS and relegated. Next, this plasmid was digested with *Mlu*I and *Nru*I and relegated, to delete the rest of the undesired miR-TSs from miR-TS cassette **(Figure 2.2)**. This plasmid was termed pMKS1-miR375TS(3x) **(Appendix 4).**

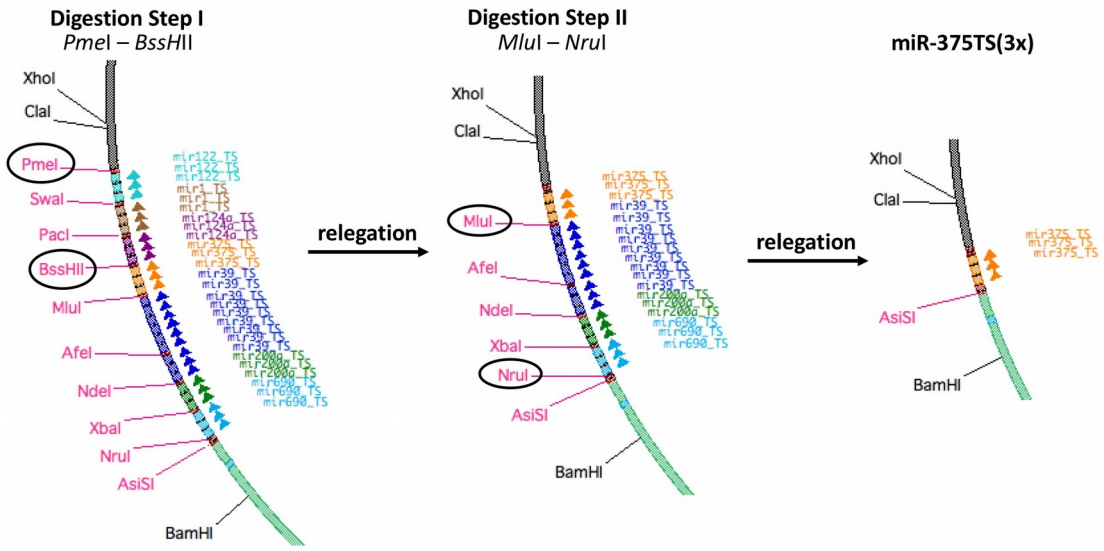

Figure 2.2 Construction of miR-375(3x) containing CVB3 cDNA clone by digestion of miR-TS cassettes. Restriction enzymes which were used during digestion step are shown in circle.

B) miR-375TS CVB3 cDNA clone

The miR-690TS(3x) containing CVB3 cDNA clone was constructed by single digestion of pMKS1-miR-TScas with *Xba*I and *Pme*I and relegation. This plasmid was termed pMKS1-690TS(3x) **(Appendix 5)**.

To produce miR-690TS(6x) bearing CVB3 cDNA clone containing 6 copies of miR-690TS, miR-690TS(3x) was amplified. PCR amplification was performed with 3 µl plasmid DNA, forward primer incorporating an *Xba*I restriction site 5′-CTGTTCTAGATCGCGAGCA-3′ and reverse primer incorporating *Nde*I 5′-TCTACATATGGCGACTAGA-3′. The PCR program was as follow; 1 cycle at 95℃ for 5 min, 8 cycles at 95℃ for 10 sec followed by 30 sec at 45℃ and 1 min at 72℃, 32 cycles at 95℃ for 10 sec followed by 30 sec at 56℃ and 1 min at 72℃ and lastly 1 cycle at 72℃ for 5 min. PCR products were gel purified and digested with *Xba*I and *Nde*I and inserted into the *Xba*I/*Nde*I digested pMKS1-miR-TScas plasmid. The resulted plasmid was digested with *NdeI* and *Pme*I to delete unnecessary miR-TSs and relegated. This plasmid was termed pMKS1-miR-690TS(6x) **(Appendix 6)**.

C) miR-375TS CVB3 cDNA clone

miR-39 is not expressed in mammalian cells therefore its complementary target site was used for construction of control viruses. To produce 3 copies miR-39TS containing CVB3 cDNA clone, pMKS1-miR-TScas was at first digested with *Afe*I/*Pme*I and relegated. Resulted plasmid was digested with *Nde*I/*Nru*I and relegated. This plasmid

was termed as pMKS1-miR-39TS(3x) **(Appendix 7)**. Six copies miR-39TS encoding CVB3 cDNA clone was generated by relegation of *Afe*I/*Nru*I digested pMKS1-miR-TScas. Subsequently, resulted plasmid was digested with *Mlu*I/*Pme*I and relegated. This plasmid was named pMKS1-miR-39TS(6x) **(Appendix 8)**.

2.2.5 *In vivo* experiments

2.2.5.1 Development of Human CRC Tumor Bearing Nude Mouse Model

Colorectal carcinoma cell lines were grown in T175 flask. When cells reached full confluency, medium was removed, cells were washed with PBS and 3 ml Trypsin was added for 3 min. Nine ml medium was added and cell lysate was collected for centrifugation at 300 g for 5 min. After that medium was aspirated, cells were washed with 10 ml PBS and were centrifuged at 300 g for 5 min. Lastly, cell pellet was resuspended with fresh PBS and 50 µl of suspension was used for cell counting. Cell suspension was diluted for 5 x 10^4 cells per µl and 150 µl aliquots were prepared.

DLD1 cells (5 x 10^6 cells) were inoculated subcutaneously into the right and left flanks of 6 weeks old BALB/c nude mice. Tumor burdens were measured daily by hand caliper, and tumor size was calculated as length x width x width/2. CVB3 strains or miR-TS inserted CVB3 clones was injected intratumorally at a dose of 3 x 10^6 plaque forming units (pfu) per animal into one of the tumors when the tumor size reached around 50 mm^3. Animals were sacrificed when tumor size reached 500 mm^3 as it was the highest limit for animal welfare. Animal experiments were performed in accordance with the principles of laboratory animal care and all German laws regarding animal protection.

2.2.5.2 Preparation of Harvested Tissues for Analysis

Harvested tissues was cut to two parts and one part was transferred immediately to liquid nitrogen for plaque assay and the other part was stored in 4% formalin for *in situ* hybridization and histopathological analysis. One part of pancreas tissue stored in 500 µl TRIzol reagent (Life Technologies) in liquid nitrogen due to high enzymatic activity.

Tissues were weighted before homogenization and transferred to 5 ml centrifuge tube with 2 ml PBS. Homogenizer was cleaned in 3% corsoline and PBS between two different samples. Homogenized samples were subjected to freeze-thawing cycles for

three times and were centrifuged at 12000 rpm for 5 min. Supernatant was collected for determination of virus titer by plaque assay.

Pancreas tissues was homogenized in TRIzol (Life Technologies) with pellet pestles and directly was processed for viral RNA isolation according to manufacturer instructions.

2.2.5.3 *In situ* Hybridization

Probes for the detection of CVB3 RNA by *in situ* hybridization were generated using the Dig RNA labeling Kit (Roche) using the pCVB3-R1 plasmid, which was linearized with *Sma*I as previously described [169]. Pretreatment, hybridization and washing steps were done as described previously [170]. Detection of the DIG-labeled RNA-probe was performed with a horseradish peroxidase-conjugated anti-Digoxigenin antibody (Roche, 1:100) and HistoGreen (Linaris) as substrate. Sections were counterstained with hematoxilin and mounted with Pertex mounting media (Medite).

2.2.5.4 Histopathological Analysis

The mouse tissues and explanted human tumors were fixed in 4% paraformaldehyde, embedded in paraffin, and 5 µm thick tissue sections were cut and stained with hematoxylin and eosin (H&E) to visualize and quantify cell destruction and inflammation.

2.2.6 Statistical Analysis

Statistical analysis performed with GraphPad Prism 5.03 (GraphPad Software, Inc., La Jolla, CA, USA). Results are shown as mean ± SEM for each group. Statistical significance was determined by use of the two-tailed unpaired Student *t* test for cell culture investigations and by use of the one-tailed Mann-Whitney U-test for *in vivo* investigations. Differences were considered significant at $P<0.05$. Survival curves were plotted according to Kaplan-Meier method (log-rank-test).

CHAPTER 3

RESULTS

3.1 Oncolytic Efficiency of CVB3 Strains in Human Colorectal Carcinoma

In the first part of the study, it was focused on the evaluation of oncolytic activity of various CVB3 strains on different human CRC cell lines. Each CVB3 strain was selected because of their different tissue tropism and growth property. Especially, virus-receptor interaction and subsequently uptake of virus by cell is the major factor of virus pathogenesis. Therefore, this interaction is also considered to be the main determinant of tumor selectivity of RNA viruses in oncolytic virotherapy. For that reason, firstly, to make a correlation between receptor and virus infection, main CVB3 receptor CAR and the co-receptor DAF expression was analyzed in different CRC cell lines. Afterwards, oncolytic effect and replication level of four different CVB3 strains (H3, 31-1-93, Nancy and PD) was evaluated *in vitro* in five different CRC cell line. Finally, oncolytic efficiency of the CVB3 strains Nancy, 31-1-93 and PD was investigated in immunocompromised DLD1 tumor bearing mouse model.

3.1.1 CAR and DAF Expression in Colorectal Carcinoma Cell Lines

The CVB3 strains Nancy, 31-1-93 and H3 use CAR and DAF for infection, whereas the strain PD uses N- and 6-sulfated HS, but can also bind to CAR and DAF [129, 171]. To determine the expression of the CVB3 receptors on colorectal carcinoma cells, the level of CAR and DAF of nine colorectal carcinoma cell lines (Colo680h, Colo205, DLD1, Colo320, Caco-2, HCT116, SW480, SW620, LS174T) were measured using flow cytometry [172]. Seven cell lines expressed moderate levels of CAR, while two cell lines (Colo680H and Colo205) expressed CAR only at low level. Four cell lines showed high (Colo680, DLD1, Caco2, HCT116) and moderate (Colo320, SW480, SW620, LS174T) levels of DAF expression, whereas one cell line (Colo205) almost completely lacks DAF

on the cell surface (**Figure 3.1)**. These data demonstrate that CAR and DAF are expressed at variable levels on the cell surface of colorectal carcinoma cell lines, with the majority of cell lines expressing CAR and DAF at high or moderate levels.

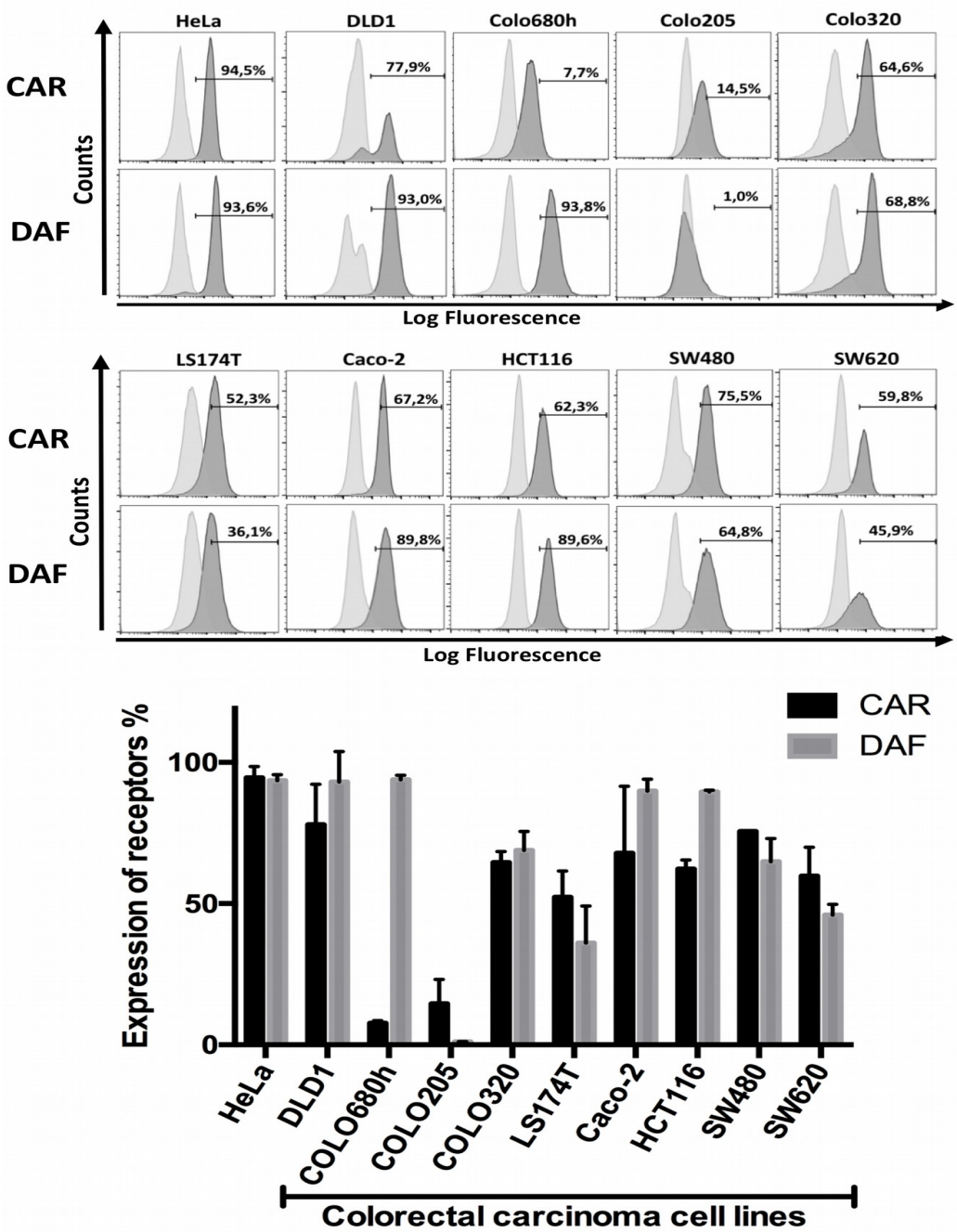

Figure 3.1 Expression level of surface CAR and DAF in colorectal carcinoma cell lines and HeLa cell line. Cell surface expression of CAR and DAF (dark gray graphs) was measured by flow cytometry using anti-CAR (RMCB) and anti-DAF (CD55) antibodies, respectively. As negative control (light gray graph), cells were stained only with the secondary antibody (Alexa Fluor® 488). HeLa cells were used as positive control due to high level expression of CAR and DAF. The black highlighted area represents the set gate. The percentages indicate the proportion of CAR or DAF positive cells. Lower panel: Diagram of cell surface expression of CAR and DAF in colorectal carcinoma cells. Columns shown means ± SEM of three independent experiments [172].

3.1.2 CVB3 Strains Differentially Infect and Lyse Colorectal Carcinoma Cell Lines

To determine whether CAR/DAF expression may influence the ability of CVB3 Nancy, 31-1-93, H3 and PD to infect and lyse colorectal carcinoma cell lines, five human colorectal carcinoma cell lines (DLD1, Colo680h, Colo205, Colo320 and LS174T) which exhibiting different levels of cell surface CAR and DAF expression were used. These cell lines were infected with CVB3 Nancy, H3, 31-1-93 or PD at an MOI of 1 or 10, and viral infection was determined by measurement of the amount of viral RNA genomes 24 h after infection by quantitative RT-PCR. Each virus strain was detected in its target cell line, but there were significant differences in the infection rates. High and moderate infection rates were detected for PD in all cell lines. Strain 31-1-93 showed also moderate and high infection rates, but only in some of the colorectal carcinoma cell lines (LS174T and DLD1). In the other cell lines, it was low. The infection rates for Nancy and H3 were generally low (**Figure 3.2**).

To determine cytolytic activity of the CVB3 strains, colorectal carcinoma cell lines were infected with the viruses at an MOI of 1, 10 or 100, and analyzed 24 h, 48 h and 72 h for cytotoxicity using crystal violet staining. The cytolytic efficiency of the CVB3 strains was highly variable. Much like the infection data, PD showed strong cytolytic activity in DLD1, Colo680H and Colo205 and moderate activity in Colo320 and LS174T. The strain 31-1-93 induced cell lysis in four of the five investigated cell lines, but high cytolytic activity was only detected in the cell line DLD1. Nancy and H3 had comparatively low cytolytic activity. Cytolysis was only observed at high MOIs in DLD1 and Colo320 cells. **(Table 1) (Figure 3.3)**.

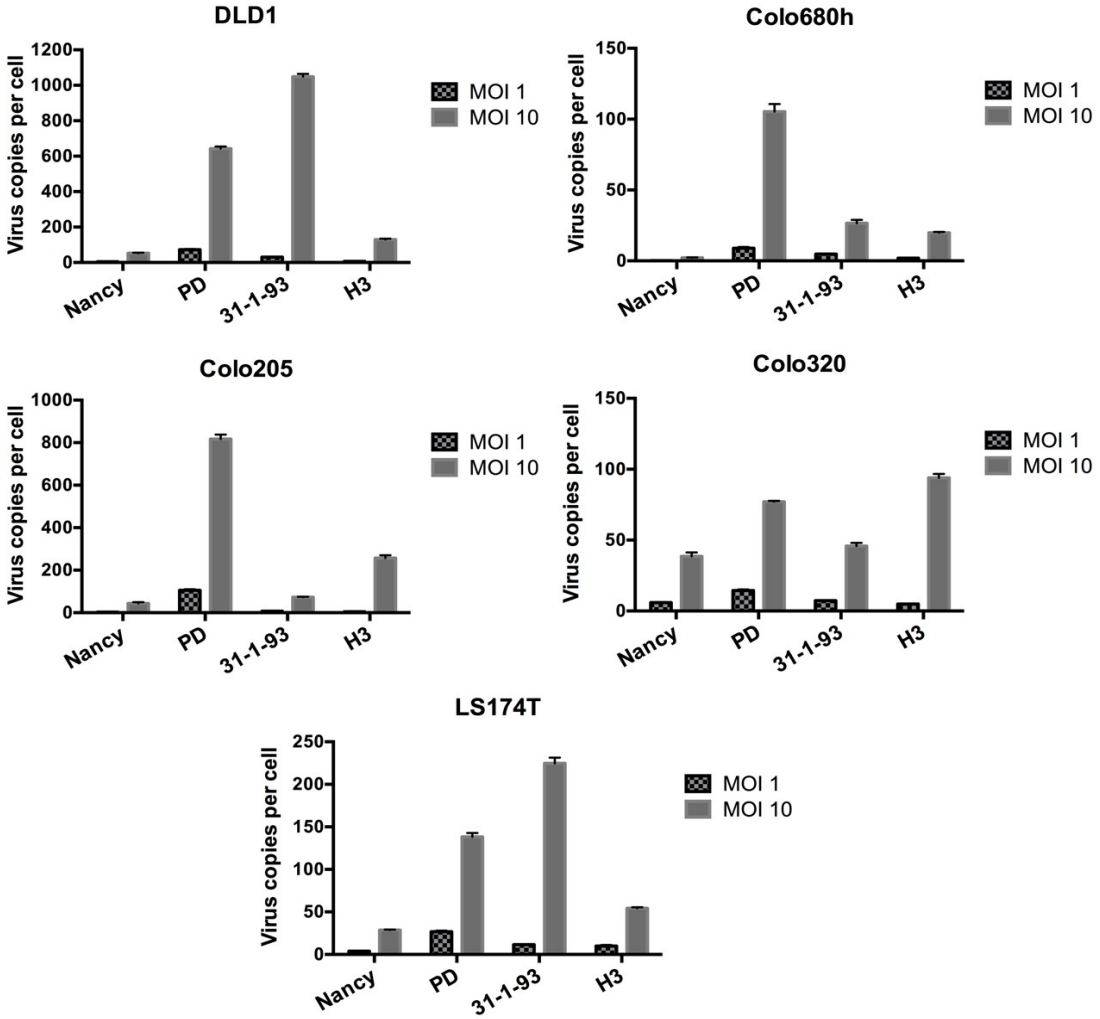

Figure 3.2 Replication of different CVB3 strains in colorectal carcinoma cell. DLD1, Colo680h, Colo205, Colo320 and LS174T colorectal carcinoma cell lines were infected with 1 and 10 MOI of the CVB3 strains Nancy, PD, 31-1-93 and H3. Twenty-four hours post infection; the amount of progeny virus was determined by detection of virus RNA genomes by quantitative RT-PCR. Data were generated in two independent experiments, each of three replicates. Data are shown as mean ± SEM

There was no clear correlation between CAR/DAF expression levels and infection/lysis of colorectal carcinoma cell lines by the CVB3 strains. These data demonstrate that the CVB3 strain PD most efficiently infected and lysed each of the colorectal carcinoma cell lines.

Table 3.1 CAR and DAF expression and lytic activity of CVB3 strains in different colorectal carcinoma cell lines.

	Average Expression [%]		Sensitivity of Cell Lines Against CVB3 Variants			
Colorectal Carcinoma Cell Lines	*CAR*	*DAF*	CVB3-Nancy	H3	31-1-93	PD
DLD 1	77,9 ± 14,3	93,0 ± 10,8	++	++	++++	+++++
Colo680H	7,7 ± 0,9	93,8 ± 1,6			++	+++
Colo205	14,5 ± 8,6	1,0 ± 0,1		+	+	+++++
Colo320	64,6 ± 3,8	68,8 ± 6,7	+	+	+	+
LS174T	52,3 ± 9,3	36,1 ± 13,1				+

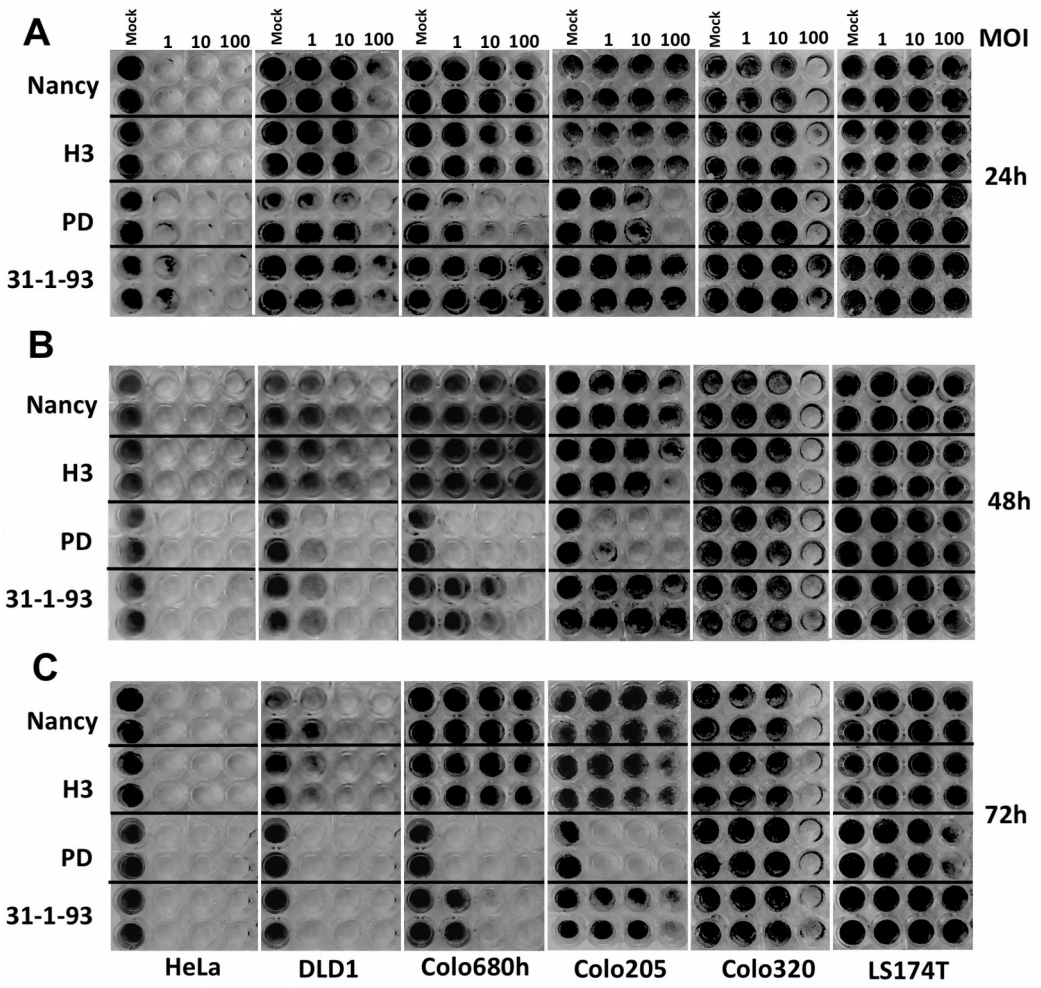

Figure 3.3 CVB3 strains have different oncolytic efficacy against colorectal carcinoma cells *in vitro*. The cell killing assay was performed with CVB3 Nancy, H3, PD and 31-1-93 strains. HeLa cells and DLD1, Colo680H, Colo205, Colo320, and LS174T colorectal carcinoma cells were infected with either 1, 10 or 100 MOI of virus. Cell viability was determined **A)** 24 h, **B)** 48 h and **C)** 72 h post infection by crystal violet staining.

3.1.3 Heparan Sulfate Binding Mediate Oncolytic Efficiency of PD

As distinct from other strains, PD can use N- and 6-O-sulfated HS to attach and infect cells. To ascertain the importance of the interaction of PD with HS for infection and lysis of colorectal carcinoma cell lines, the expression level of HS6ST2 was analyzed by quantitative RT-PCR. HS6ST2 catalyses the transfer of sulfate groups to position 6 of the N-sulfoglucosamine residue in HS and leads to production of 6-O-sulfated HS [173]. The investigations were carried out with DLD1, Colo205 and Colo680h cell lines, which are susceptible to PD, and with the LS174T cell line, which is resistant to PD **(Table 3.1, Figure 3.3)**. As shown in **Figure 3.4**, HS6ST2 mRNA expression was detected in DLD1, Colo205, Colo680h cells, but not in LS174T cells, indicating a correlation between HS6ST2 expression and susceptibility of colorectal carcinoma cell lines for PD.

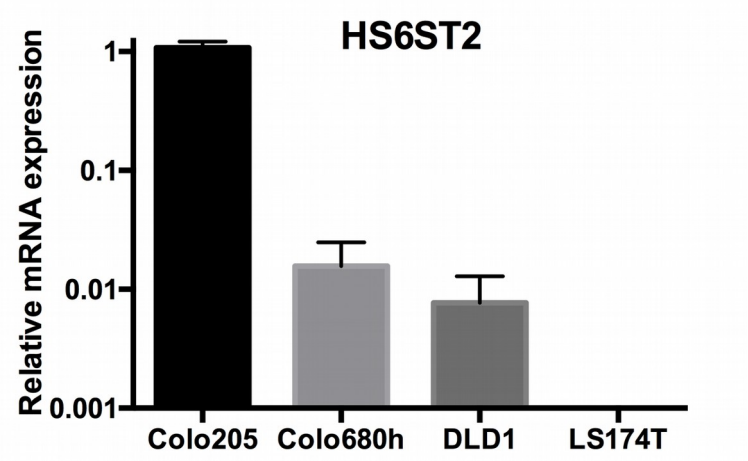

Figure 3.4 Expression of HS6ST2 mRNA in Colo205, Colo680h, DLD1 and LS174T human colorectal cancer cell lines. Relative HS6ST2 mRNA expression levels were determined using quantitative RT-PCR. 18S RNA was used as internal control. HS6ST2 mRNA levels were normalized against 18S mRNA expression levels.

To confirm that PD indeed uses HS as receptor to infect colorectal carcinoma cells, the soluble HS-analogue heparin was used. CVB3 Nancy was used as control. PD and Nancy were incubated with different concentrations of heparin before infection and virus infection was analyzed by plaque assay 48h later. While infection with Nancy was not affected by heparin treatment and the virus titers were similar at each heparin concentration **(Figure 3.5A)**, PD infection was distinctly inhibited by heparin treatment in a dose-dependent manner. The effect was more prominent in Colo205 and Colo680h cell lines than in the cell line DLD1 **(Figure 3.5B)**. Presumably, inhibition of PD infection in DLD1 cell line was lower due to high level of CAR on cell surface.

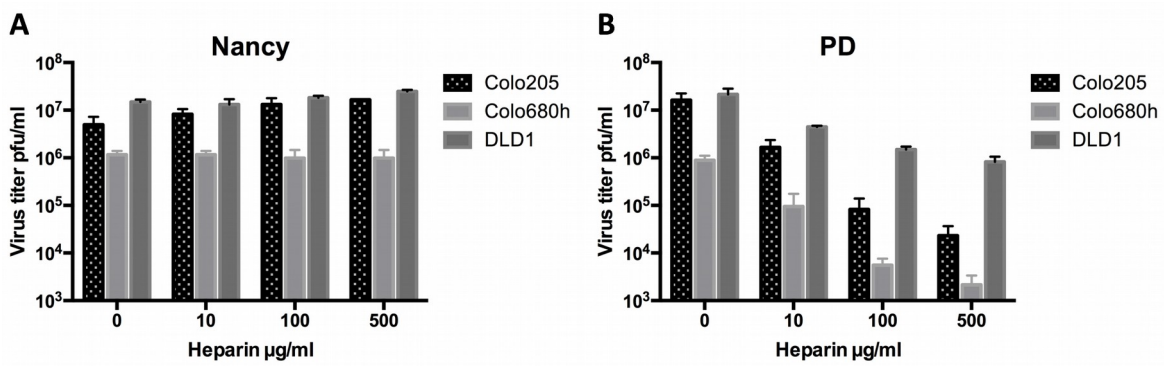

Figure 3.5 Heparan sulfate mediates infection of PD. (**A)** Effect of heparin on infection of colorectal cancer cell lines by Nancy and **(B)** PD. Nancy and PD (MOI of 3) were incubated with heparin for 1 h and added to the cells. Virus infection was analyzed by plaque assay after 48h later. Heparin did not inhibit the infection of cell lines with CVB3 Nancy, whereas PD infection was strongly inhibited in dose-dependent manner.

The HS blocking experiments were repeated and the cytotoxic activity measured by XTT assay. PD induced cell lysis was completely inhibited by 100 μg/ml heparin in Colo205 and Colo680h, whereas inhibition reached about 80 % in DLD1 cell line **(Figure 3.6)**. The lower heparin-induced inhibition of PD infection and cytotoxicity in DLD1 cells compared to Colo205 and Colo680h cells may be due to high level of CAR on cell surface of these cells, as PD can also use CAR for infection [138].

In conclusion, these data demonstrate that PD uses HS to infect colorectal cancer cells, and the interaction with HS is a key factor for the cytolytic activity of PD.

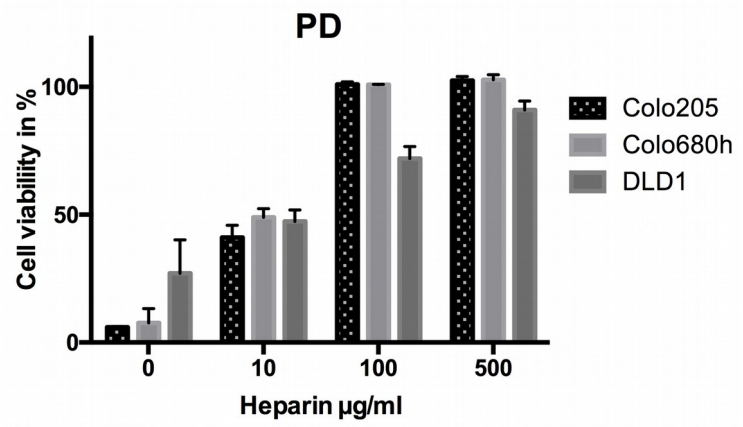

Figure 3.6 Heparin inhibits PD-induced cytotoxicity in human colorectal cancer cell lines. The experiment was performed as described under C. Cell viability was measured 48 h after infection using XTT assay. Strongest inhibition was observed Colo205 and Colo680h. Inhibition in DLD1 cell line was lower at high heparin concentrations (100 µg/ml and 500 µg/ml) probably due to high level of CAR on cell surface, as PD can also use CAR for infection.

3.1.4 CVB3 Strains Have Strong Oncolytic Activity *in vivo*

To evaluate oncolytic efficiency of CVB3 strains *in vivo*, a xenograft BALB/c mouse tumor model was established with the DLD1 human colorectal carcinoma cell line. Tumor cells were inoculated bilaterally into the flanks of the animals and only one of the two tumors was injected with single dose of 3 x 10^6 pfu of CVB3 Nancy, 31-1-93, or PD. Treatment with any of the three viruses resulted in significant suppression of tumor growth of both the virus-injected tumor and the contralateral untreated tumor when compared with uninfected mice tumors (P<0.05; **Figure 3.7A-C**).

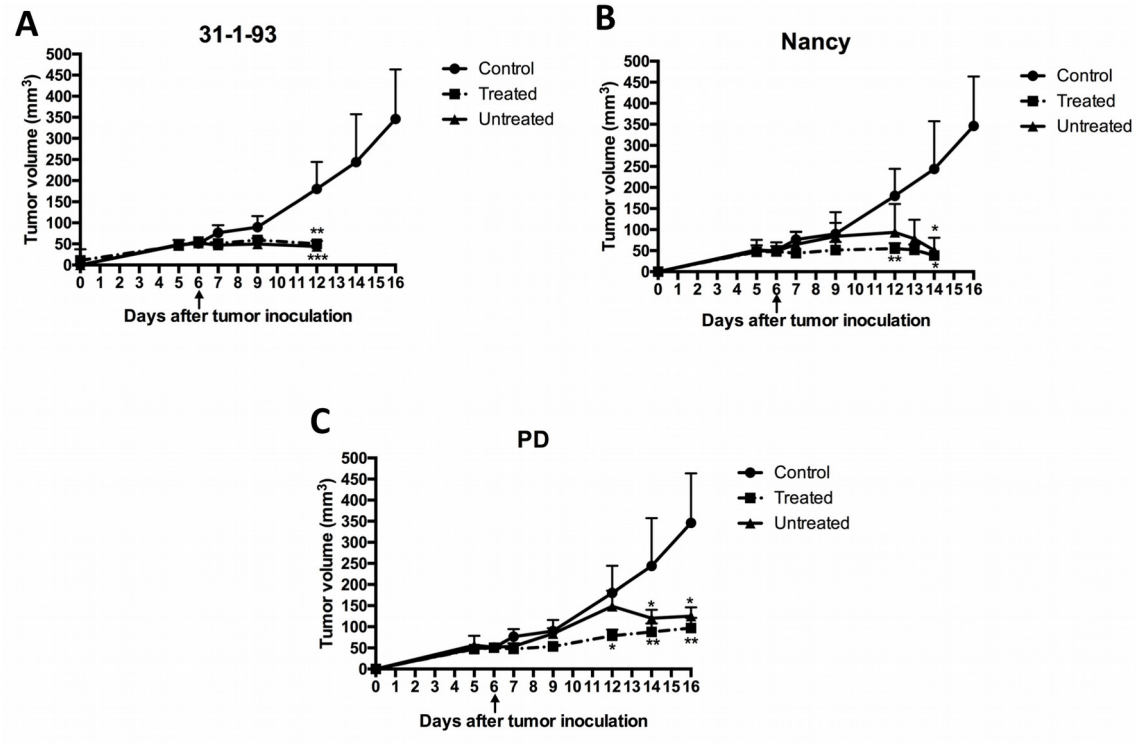

Figure 3.7 *In vivo* oncolytic efficiency of CVB3 strains in DLD1 xenograft mouse model. DLD1 cells were subcutaneously inoculated into the right and left flank of BALB/c nude mice. One of the inoculated tumor was intratumorally injected with single dose (3 x 10^6 pfu) of the CVB3 strains (**A**) 31-1-93 (n = 6), (**B**) Nancy (n = 6) and (**C**) CVB3-PD (n = 6), respectively, at 6 days after tumor inoculation. Control group (n=4) was not treated. Tumor volumes are shown as means ± SEM. CVB3 31-1-93 infected mice died 6 days after viral injection. CVB3 Nancy infected mice were sacrificed at day 8 after viral injection, at which time they were moribund, CVB3 PD and un-injected control mice were sacrificed at day 10 after viral injection. *,P<0.05; **, P<0.01; ***,P<0.001 compared to the control.

Measurement of the amount of infectious virus in the tumors of Nancy and PD infected animals by plaque assay 8 and 10 days after virus injection, respectively, showed that both viruses were detected at similar levels (between 3.2 and 7.2 x 10^6 pfu/g) in the primary injected tumor. Both viruses were also detected in the contralateral, untreated

tumor, but the amount was significantly lower in the PD injected group than in Nancy treated animals (P<0.05; **Figure 3.8A**). Moreover, in two of six investigated animals, PD was not detected in the untreated tumor. *In situ* hybridization confirmed the presence of viral genomic RNA in the tumors of Nancy and PD infected animals, as well as the absence of virus in untreated tumors of two PD infected animals, which also were negative when measured for replicating virus. The investigations also showed that viruses were located in mononuclear immune cells, probably macrophages, within the tumor mass or on the border between the tumor stroma and tumor cells (**Figure 3.8B**). Taken together, these results demonstrate that CVB3 Nancy, 31-1-93, and PD have strong oncolytic activity *in vivo* and this activity is not only restricted to injected tumor but also is seen in non-injected, distant tumors.

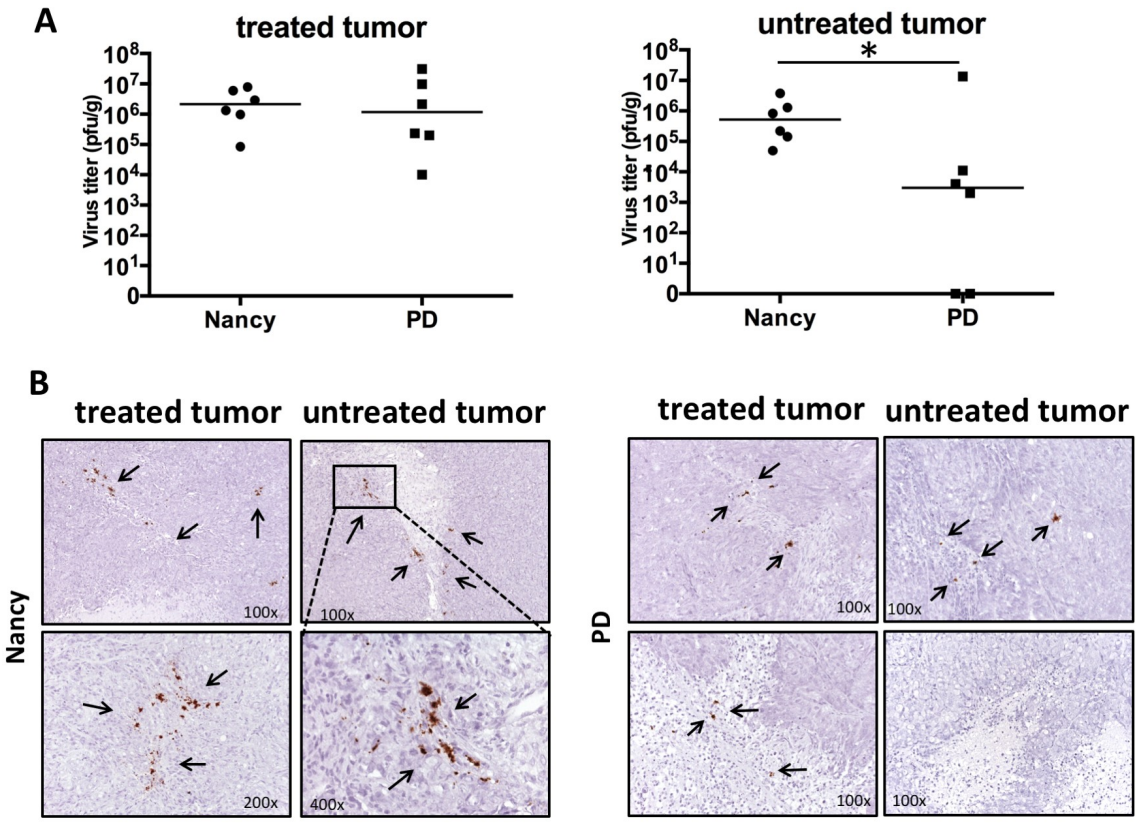

Figure 3.8 Infectivity and replication activity of CVB3 Nancy and PD strain in harvested tumors **A**) *Left*: virus titers in CVB3 Nancy and PD injected tumors. *Right*: virus titers in the un-injected tumor. Horizontal line represents the median value, * P<0.05 **B**) Presence and localization of CVB3-Nancy and PD RNA treated and untreated tumors were determined by *in situ* hybridization using DIG-labeled RNA hybridization probes. Magnification of images: as indicated.

3.1.5 CVB3 Nancy and 31-1-93, but not PD, Induce Severe Side Effects in Treated Mice

Intratumoral injection of CVB3 Nancy and 31-1-93 resulted in severe side effects. All animals which were injected with 31-1-93 died six days after intratumoral virus injection and animals treated with Nancy were moribund six (one animal), seven (one animal) and eight days (four animals) after virus administration and were sacrificed. In contrast, all six animals treated with PD were alive 10 days after intratumoral virus injection and five of them showed a normal physical state, similar to the untreated negative control group. One of the PD infected animals (PD-M1) showed slower movement and lower body weight compared to the other PD-infected animals **(Figure 3.9)**.

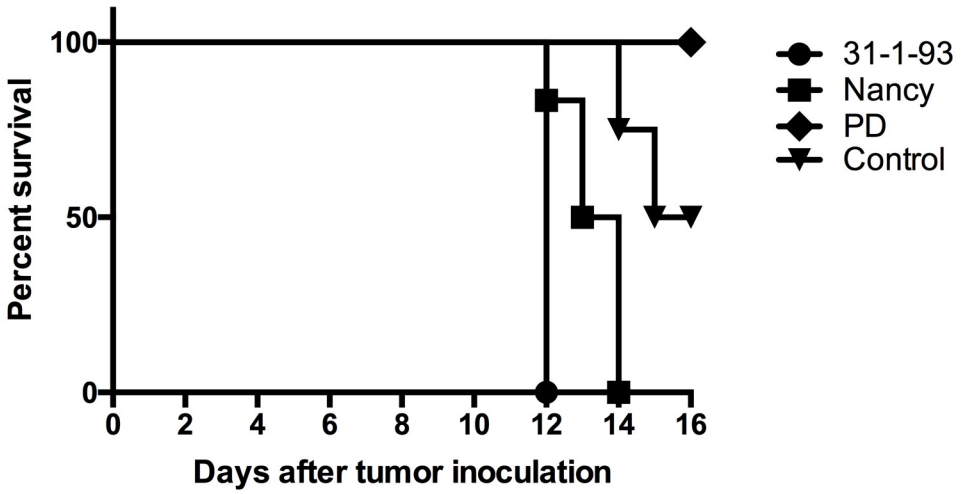

Figure 3.9 Kaplan-Meier survival curves for the CVB3 infected mice. Animals died or were sacrificed as described under Fig. 3.4. The control group mice were sacrificed when the tumor burden reached an upper limit of 500 mm^3 for reasons of animal welfare. P = 0.0006.

To elucidate the cause of the severe side effects, occurrence of Nancy and PD infection were investigated in heart, liver, brain, spleen, kidney and pancreas, respectively, using plaque assays to detect replicating virus. The highest virus titers, of about 10^6 pfu/g, were measured in the heart of the Nancy treated group (**Figure 3.10A**). CVB3 Nancy was also detected in the spleen, kidney and brain, but at distinctly lower levels, whereas it could only sporadically be detected in the liver (one of six animals) at a very low titer (10^2 pfu/g). The pancreas is the most susceptible organ for CVB3 in mice [174]. Examination of the pancreas tissue in Nancy infected animals during dissection showed reduced organ size (not shown) indicating major damage to the tissue. As the destruction of the pancreas by CVB3 made the tissue unsuitable for determination of infectious virus by plaque

assay, real-time RT-PCR was carried out to quantify the virus genomes. High amounts (10^3 to 10^5 copies per µg RNA) of Nancy viral RNA was detected in the pancreas **(Figure 3.10A)**. In sharp contrast, no infectious virus was recovered from any organ of five of the six PD treated animals. Only the animal PD-M1 contained a similar amount of virus as the animals of the Nancy group.

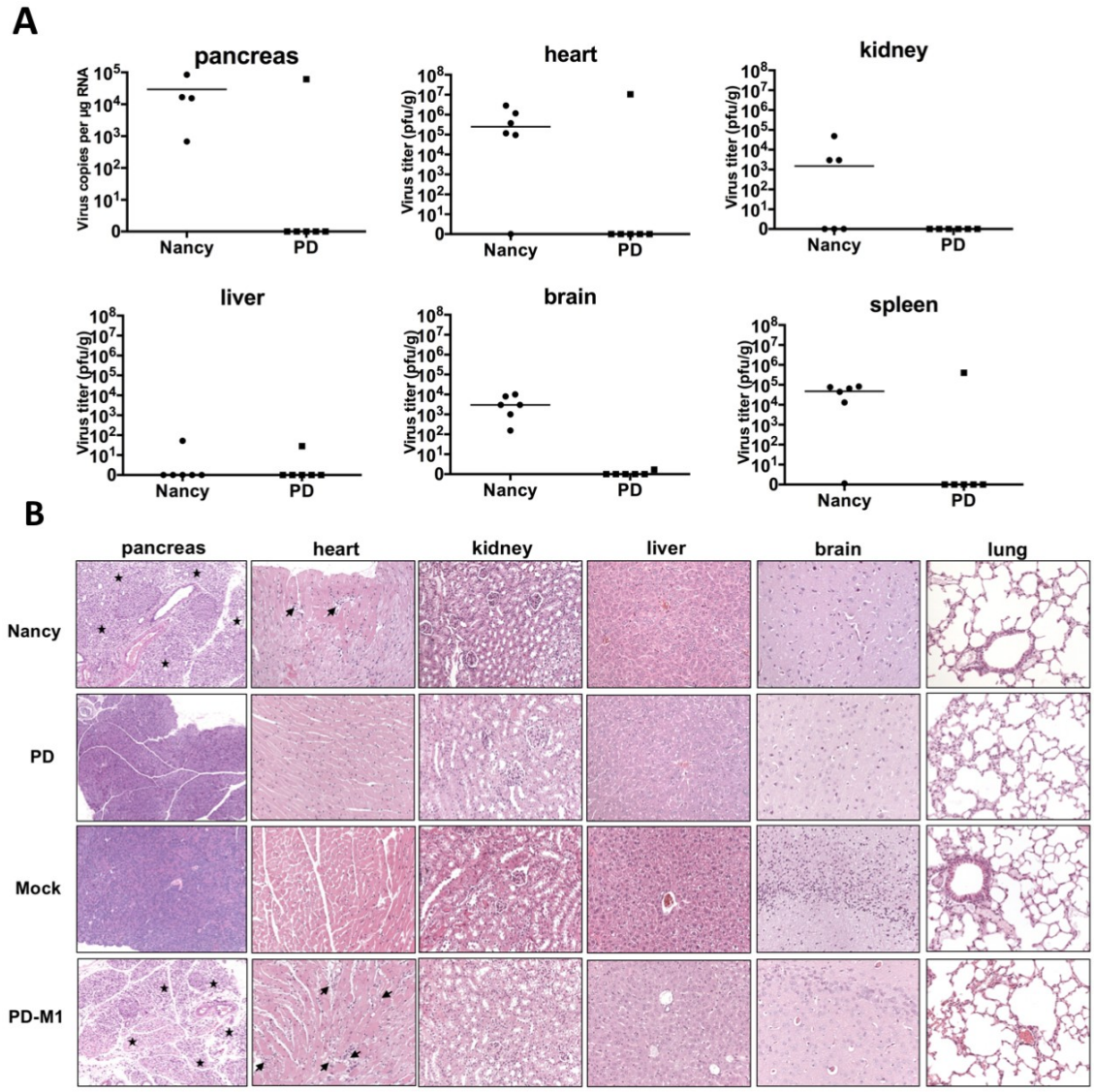

Figure 3.10 Viral load and pathological alterations of CVB3 Nancy and PD in organs of tumor bearing mice. Organs of the animals were harvested at the time points when animals were sacrificed as described under Fig. 3.7. **A**) Amount of replicating virus. Horizontal line represents the median value. **B**) Histological examination of mouse tissues. Organ sections were stained with H & E. All Nancy infected and one of the PD infected animals (referred to as PD-M1) showed complete destruction of the exocrine pancreas (stars represent areas of tissue destruction). Inflammation within the myocardium (arrows) was detected in all Nancy infected animals and in the animal PD-M1. All other PD infected animals were free of pathological alterations in all investigated organs. Mock represent untreated tumor bearing animals. No alteration of tissues was seen in these animals. Shown are representatives of each group.

Histological examination of mouse tissues (pancreas, heart, kidney, liver, brain and lung) confirmed different toxic activity of Nancy and PD. The exocrine pancreas of Nancy infected animals was nearly completely destroyed and heart tissue of these animals showed distinct inflammation. All other organs of Nancy infected animals were unaffected. In contrast, with the exception of the one animal, PD-M1, all organs of PD treated animals did not show any alterations (**Figure 3.10B**). The animal PD-M1 showed organ alterations, which were very similar to those of Nancy infected animals. Furthermore, *in situ* hybridization of the heart of PD-M1 demonstrated that the virus CVB3-PD-M1 infected cardiac myocytes and cardiac endothelial cells (**Figure. 3.11**).

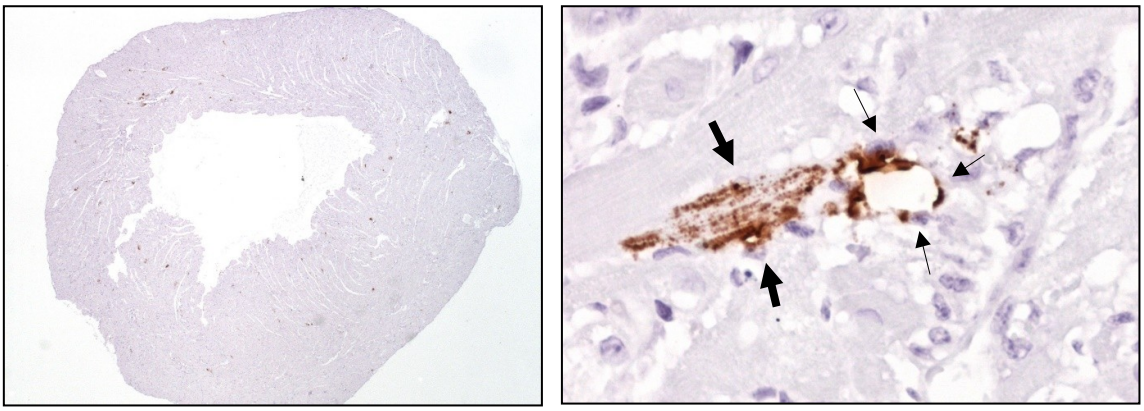

Figure 3.11 Detection of CVB3 PD-M1V virus genomes in the heart. Left: heart section of PD-M1. Brown points represent cells with positive staining of virus PD-M1V RNA (magnification 25-fold). Right: The fat arrows show a virus-infected cardiac myocyte. The thin arrows show the virus-infected endothelial cells of a capillary (magnification 400-fold). Virus RNA was detected by *in situ* hybridization using a DIG-labeled RNA hybridization probe. Slides were co-stained with hematoxylin.

To elucidate the cause of observed PD toxicity in PD-M1, the virus was isolated from the heart tissue of the animals and the genomic region encoding the viral proteins VP4, VP2, VP3 and VP1 which are responsible for cell receptor attachment and internalization of virus, was sequenced. Additionally, 5′ UTR was also sequenced and analyzed as this region has been suggested to have direct role on virus pathogenicity [175]. Sequencing revealed the occurrence of two amino acid substitutions in VP2 (V108I, V245I) and one amino acid substitution in the VP1 region (K230E) relative to the sequence of the starting strain of PD, indicating that the virus (hereafter referred to as CVB3 PD-M1V) isolated from animal PD-M1 was a mutated variant of PD. Particularly, VP2 protein region amino acid mutations was found to be similar to highly cardiotropic H3 strain. In addition, one nucleotide difference was found at position 420 of 5′UTR which does not present in any

other investigated CVB3 strain **(Table 3.2)**. These results revealed that the virus which lead to systemic infection in PD-M1 mouse was genetically different to original PD.

Table 3.2 Nucleotide and amino acid polymorphisms of 5′ UTR and capsid protein region of different CVB3 strains and CVB3 PD-M1V. CVB3 PD is the virus which was used during the study. Mutations of PD-M1V compare to PD are shown in red letters.

Gene	**Position**	**Nucleotide and amino acid and differences among strains**				
		PD	PD-M1V	Nancy	H3	31-1-93
5′ UTR	420nt	C	T	C	C	C
	610nt	T	T	T	T	T
VP2	108aa	V	I	D	I	V
	245aa	V	I	V	I	V
VP3	34aa	M	M	M	M	M
	237aa	Y	Y	F	F	F
VP1	230aa	K	E	K	K	K

3.2 Increasing Tumor Selectivity by microRNA Interference Mechanism

Tumor selectivity of the viruses in oncolytic virotherapy is the most important issue which need to be investigated particularly in pre-clinical studies. As it was shown in the first part of the study, CVB3 strain Nancy, which was described as potent oncolytic virus [121], was able to induce strong side effects in animals. Another strain 31-1-93 was even more virulent in nude mice which caused lethal side effects five days after infection. In addition, relatively safe CVB3 strain PD was found to induce systemic infection in one of six infected animals. These results raised the need of enhancement of CVB3 tumor selectivity before to be used as oncolytic agent.

Therefore, in this part of the study, it was aimed to generate miR-TS equipped recombinant CVB3 ($CVB3_{miR\text{-}TS}$) clones to prevent undesirable virus replication in healthy organs. For that purpose, firstly, miRNAs which are highly expressed in target organs were detected. Afterwards, target sites of selected miRNAs were inserted into the CVB3 genome and subsequently, silencing effect of miRNAs on $CVB3_{miR\text{-}TS}$ replication was investigated *in vitro*. Lastly, oncolytic activity and safety profile of produced different $CVB3_{miR\text{-}TS}$ were analyzed in DLD1 tumor bearing nude mice, *in vivo*.

3.2.1 Analysis of microRNA Expression in CVB3 Target Tissues and CRC Cell Lines

Previous studies revealed that target organs of CVB3 are mainly heart, pancreas and brain [123]. Therefore, two different strategies were planned to prevent virus replication in different healthy organs. First, use of pancreas specific miR-TS sequences to protect the pancreas. Second, utilization of single miR-TS which targets an abundantly expressed miRNA in all target organs but not in colorectal cancer cells. So that, miR-TS equipped virus replication in the cells would be eliminated in presence of cognate miRNA. The binding of miRNA to its complementary sequence induces degradation of viral genome [152].

Analyses which have been carried out by our and other groups revealed a broad spectrum of miRNAs which are expressed selectively and in high copy numbers within the human and mouse tissue that support CVB3 replication [149]. To confirm the previous miRNA expression results and to limit the number of potential suitable miRNAs the expression of several eligible miRNAs was analyzed in mouse heart, pancreas and brain as well as in

colorectal carcinoma cell lines by real-time PCR. For this purpose, firstly, it was focused on expression levels of miR-1, miR-124a and miR-375 which are known to be highly expressed in heart, brain and pancreas, respectively.

It was found that miR-1, miR-124a and miR-375 were highly expressed in mentioned organs (**Figure 3.12**), whereas they were absent or expressed at low levels in the colorectal carcinoma cell lines. In fact, miR-1 was expressed very low in all six colorectal carcinoma cell line (> 1.000 times lower than in the heart) and miR-375 and miR-124a were expressed at low levels in five of six colorectal carcinoma cell lines (>100x lower than in brain) **(Figure 3.13)**. Additionally, these microRNAs were also lack or low expressed in HEK293T and HeLa cell lines, which is important as HEK293T cell line was used for CVB3 production and HeLa cell line was utilized for plaque assay **(Figure 3.13)**.

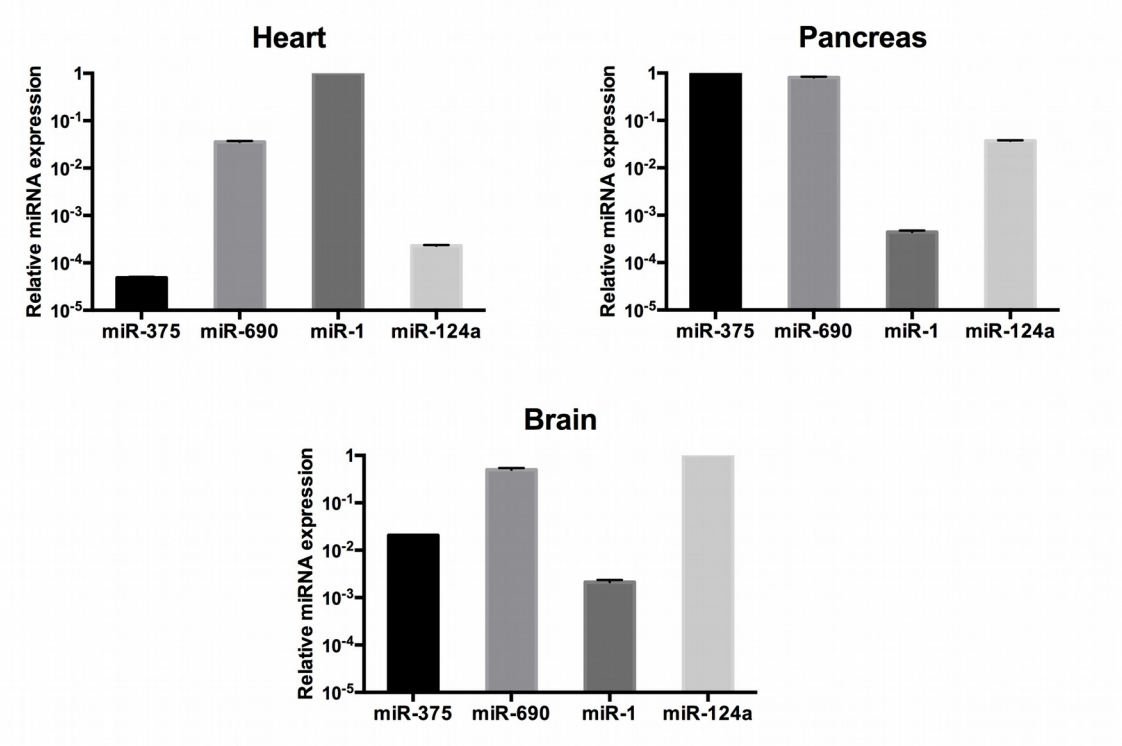

Figure 3.12 Expression level of miR-1, miR-124a and miR-375 in mouse heart, pancreas and brain. The quantification was determined by quantitative RT-qPCR. Each miRNA expression levels were normalized against U6 RNA expression levels. The most abundantly expressed miRNA for each tissue was set at 1.

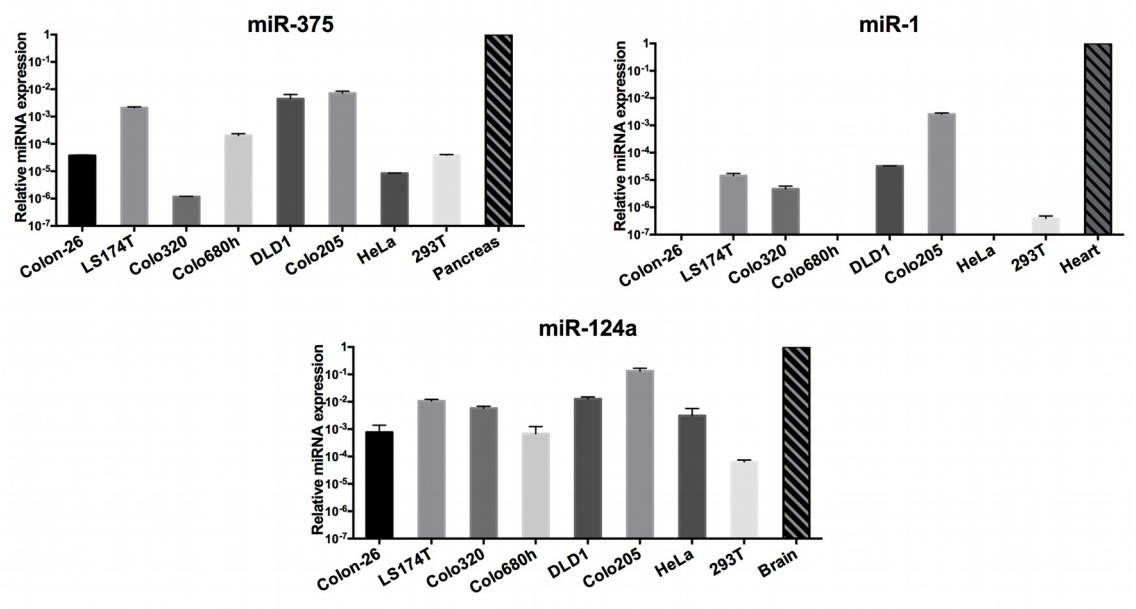

Figure 3.13 Expression level of miR-1, miR-124a and miR-375 in six colorectal carcinoma cell lines (Colon-26, LS174T, Colo320, Colo680h, DLD1, Colo205), HeLa cells, HEK293T cells and related mouse tissues. The quantification was determined by quantitative RT-PCR. Each miRNA expression levels were normalized against U6 RNA expression levels. The most abundantly expressed miRNA for each tissue was set at 1.

Considering second multi-tissue detargeting strategy, ubiquitously expressed miRNAs were investigated. For this purpose, miR-16 and miR-34a were selected for this study as these miRNAs are known to be downregulated in many cancer types while they are ubiquitously expressed in human [176, 177]. In addition, miR-690 was also selected as it was found to be expressed at high levels in heart brain and pancreas **(Figure 3.12)**. However, quantitative RT-PCR analysis revealed that miR-34a and miR-16 were highly expressed in target organs but also in CRC cell lines. Importantly, miR-690 was only expressed at high levels in target tissues, but at very low levels of all human colorectal carcinoma lines (>10.000x) **(Figure 3.14)**.

These findings indicate that miR-1, miR124a and miR-375 seem to be proper candidates for detargeting the virus from heart, brain and pancreas, respectively. In addition, ubiquitous expression levels of miR-690 make it an additional candidate for multi-tissue detargeting as a second strategy.

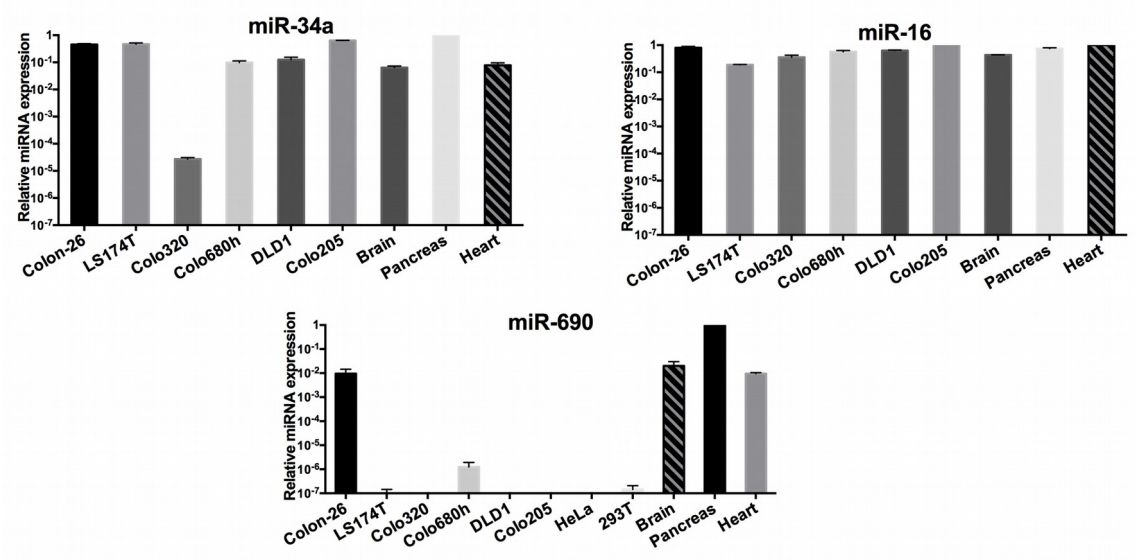

Figure 3.14 Expression level of miR-34a, miR-16 and miR-690 in five human colorectal carcinoma cell lines (LS174T, Colo320, Colo680h, DLD1, Colo205), one mouse CRC cell line (Colon-26) and mouse target tissues. The quantification was determined by quantitative real time RT-PCR. Each miRNA expression levels were normalized against U6 RNA expression levels. The most abundantly expressed miRNA for each tissue was set at 1.

3.2.2 Effect of Low Level miRNA on Repression of Transgene Expression

According to microRNA expression analysis, it was found that in colorectal carcinoma cells miR-1, miR-124a and miR-375 expressions were from 10^2-fold to 10^8-fold lower compared to the relevant primary target organs of CVB3 (**Figure 3.13**). To confirm that low miRNA expression in colorectal carcinoma cells is insufficient to inhibit replication of miR-TS containing CVB3, an initial experiment was performed using a luciferase reporter assay. In this assay, a plasmid containing three copies of the miR-TS of the miR-375 (miR-375TS(3x)) located in the 3′ UTR of a luciferase reporter was transfected into LS174T colorectal carcinoma cells. This cell line was chosen as it expresses the miR-375 approximately 100-fold lower compared to pancreas (**Figure 3.13**). In parallel, LS174T cells were co-transfected with the luciferase reporter plasmid and a plasmid expressing the miR-375 or they were transfected with a control plasmid containing 3 copies miR-TS of miR-39 (miR-39TS(3x)) which is not expressed in mammals, in the 3′UTR of the luciferase reporter. Analysis of luciferase expression 48h later revealed a reduced luciferase activity only in cells co-transfected with miR-375TS and miR-375 expression plasmid, but not in cells transfected only with the miR-375TS or miR-39TS incorporated plasmid (**Figure 3.15**). This indicates that low microRNA expression levels are not sufficient to induce silencing of miR-TS bearing transgenes. Hence, low expression of

miR-1, miR-375, miR-124a and miR-690 in colorectal carcinoma cell lines should not lead to inhibition of CVB3 equipped with the corresponding miR-TS. Moreover, results demonstrate that presence of miR-375TS reduced luciferase expression about 80% when miR-375 is abundantly expressed.

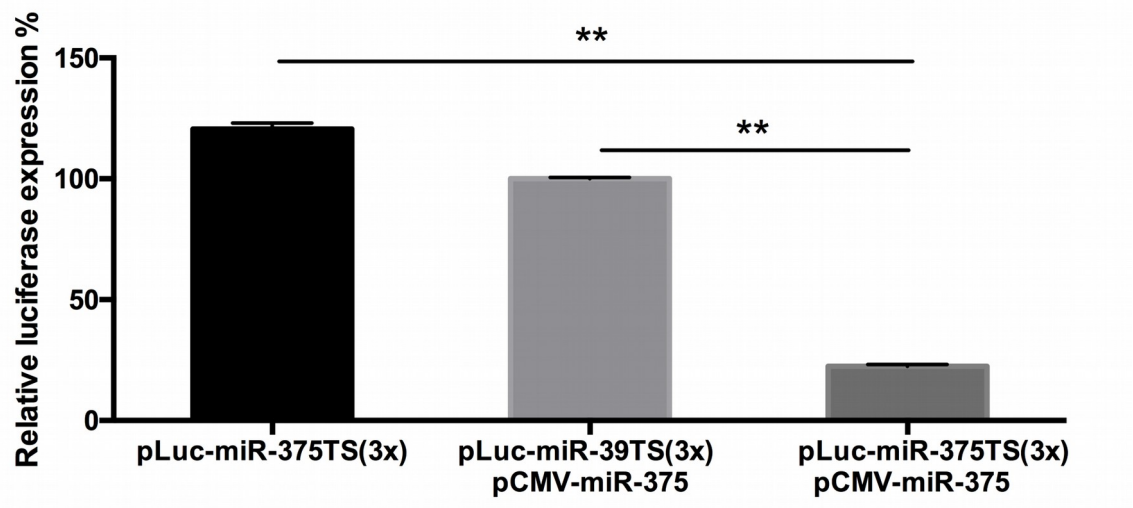

Figure 3.15 Luciferase reporter assay to test miR-375TS functionality. LS174T cells were transfected with a plasmid containing 3 copies of miR-375TS in the 3 ′UTR of a luciferase reporter (column 1) or co-transfected with this reporter plasmid and an miR-375 expression plasmid (column 3). As a control, cells were co-transfected with luciferase expression plasmid containing 3 copies of miR-39TS and miR-375 expression plasmid (column 2). **, $P<0.01$

3.2.3 Production of miR-TS Incorporated CVB3 Clones

Following detection miRNA levels and that 100x fold lower miRNA expression in CRC cells does not inhibit transgene expression, miR-TS cognate to miR-375, miR-690 and miR39 were inserted into the pMKS1 plasmid which contains infectious clone of CVB3. Target sites were inserted into the 3′ UTR of the CVB3 genome and the plasmids were transfected into the HEK293T cell line for virus production. Afterwards, $CVB3_{miR\text{-}TS}$ was purified from cell culture supernatant and viruses were titrated by plaque assay. It was planned to insert at least three tandem copies of each selected miR-TS, as in a previous study was described that three copies of miR-TS sequence are sufficient to reduce transgene expression strongly in cells expressing the cognate microRNA [153].

The produced miR-TS incorporated recombinant CVB3 variants are shown in **Figure 3.16**.

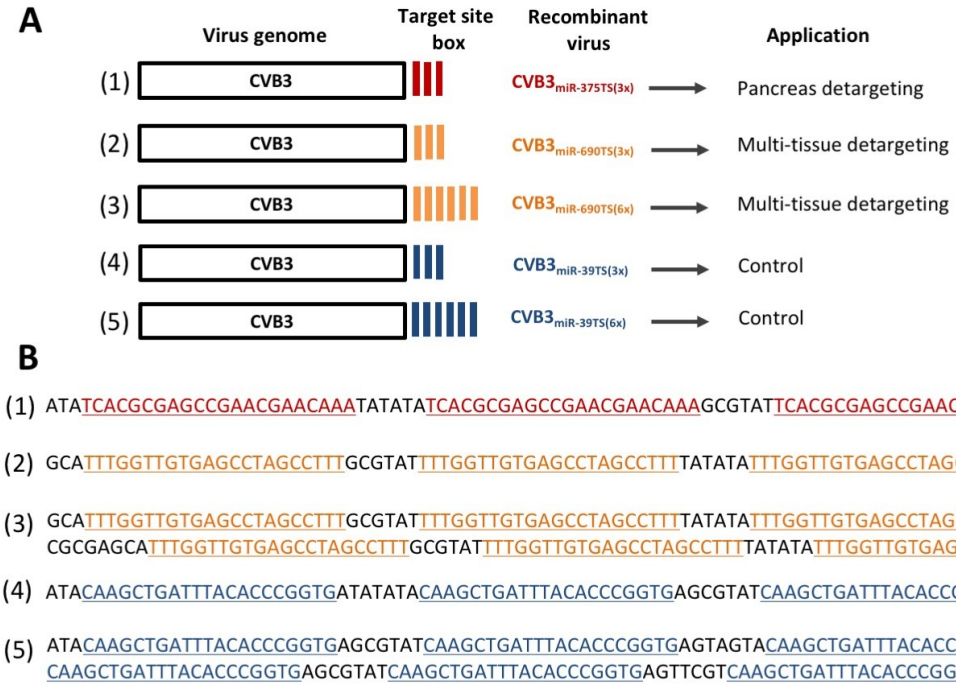

Figure 3.16 Structure of miR-TS in equipped recombinant CVB3 variants. **A)** Illustration of inserted target site box inserts and the purpose of production. miR-TS sequences were inserted into the 3′ UTR region of CVB3. Five different $CVB3_{miR\text{-}TS}$ variants produced for pancreas detargeting ($CVB3_{miR\text{-}375TS(3x)}$) or multi-tissue detargeting ($CVB3_{miR\text{-}690TS(3x)}$ and $CVB3_{miR\text{-}690TS(6x)}$) or to be used as control ($CVB3_{miR\text{-}39TS(3x)}$ and $CVB3_{miR\text{-}39TS(6x)}$). **B)** Inserted miR-TS box consists of three or six complete complementary to the cognate miRNA. Six nucleotides were inserted as spacer to increase miRNA binding. Each miR-TS is shown in different color corresponding to cognate miRNA; red, miR-375; orange, miR690; blue, miR-39.

For pancreas detargeting, three tandem copies of miR-375TS incorporated CVB3 ($CVB3_{miR\text{-}375TS(3x)}$) clone was produced. The pancreas is the most vulnerable organ for CVB3 infection. It has been shown that prevention of the pancreas from CVB3 infection result in protection of mice from virus-induced pancreatitis and myocarditis [124]. In addition, it has been also described that all cardiovirulent CVB3 strains need to cause pancreatitis, however, pancreovirulent CVB3 strains do need to cause myocarditis [134]. Therefore, it was planned to evaluate whether only prevention of pancreas should be sufficient to inhibit systemic virus infection in mice.

In addition, it was also aimed to compare the silencing potential of miR-690 as it was found to be expressed ubiquitously in healthy organs but not in colorectal carcinoma cells. Therefore, three and six copies of miR-690TS were used separately to evaluate down regulation effect with respect to number of TS. Lastly, control CVB3 clones with miR-39TS were generated.

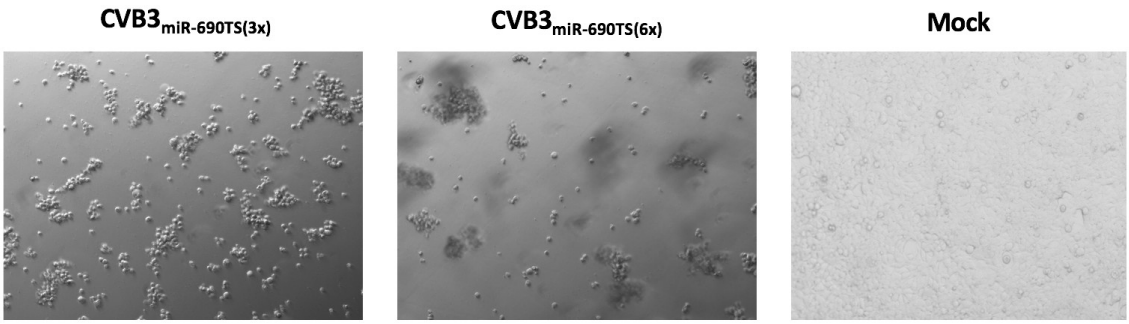

Figure 3.17 Production of miR-TS incorporated recombinant CVB3 variants. HEK293T cells were transfected with target sites inserted pMKS1 plasmid. 48h post transfection complete cell lysis was observed and viruses were harvested for virus titer determination. $CVB3_{miR\text{-}690TS(3x)}$ and $CVB3_{miR\text{-}690TS(6x)}$ indicate virus clones containing with 3 and 6 repeats of miR-690TS, respectively.

Constructed recombinant virus clones were transfected to HEK293T cells for virus production. Transfected cells were incubated till the observation of complete cell lysis, approximately 48h after transfection as shown for $CVB3_{miR\text{-}690TS(3x)}$ and $CVB3_{miR\text{-}690TS(6x}$ **(Figure 3.17)**.

In conclusion, different $CVB3_{miR\text{-}TS}$ clones were successfully produced for further investigations.

3.2.4 MicroRNA-Mediated Regulation of CVB3 replication *in vitro*

Down regulation of $CVB3_{miR\text{-}TS}$ replication by cognate miRNAs was analyzed. For this purpose, CVB3 susceptible HEK293T cell line was transfected with miR-690 or miR-375 expression plasmids which also encode GFP. Following transfection, cells were monitored under fluorescence microscope to observe the transfection efficiency. After 24 hours, when expression of miRNA reached high levels, cells were infected with $CVB3_{miR\text{-}375TS(3x)}$, $CVB3_{miR\text{-}690TS(3x)}$, $CVB3_{miR\text{-}690TS(6x)}$ or control viruses ($CVB3_{miR\text{-}39TS(3x)}$ and $CVB3_{miR\text{-}39TS(6x)}$).

The first experiment was carried out with 3x or 6x miR-690TS encoding CVB3 clones. Two questions were aimed to be addressed, (i) functionally of inserted miR-TS on miRNA-mediated virus regulation, (ii) whether the higher number of inserted TS repeats would increase the down regulation of virus replication.

HEK293T cells were transfected with miR-690 expression plasmid and after 24h, cells were infected with $CVB3_{miR\text{-}690TS(3x)}$ or $CVB3_{miR\text{-}690TS(6x)}$ at an MOI of 0.1. As control, $CVB3_{miR\text{-}39TS(3x)}$ and $CVB3_{miR\text{-}39TS(6x)}$ were used. Following 24h incubation time, cells were subjected to freeze-thawing cycles for three times and supernatant was collected for viral

titration. MiR-690 was able to down regulate virus replication around 1-$\log_{10}$ step for both $CVB3_{miR\text{-}690TS(3x)}$ and $CVB3_{miR\text{-}690TS(6x)}$ compare to control viruses. In addition, down regulation levels of 3x or 6x miR-TS encoding CVB3 clones were similar which showed that insertion 6 copies of TS did not increase inhibition compare to 3 copies TS encoding CVB3 **(Figure 3.18)**.

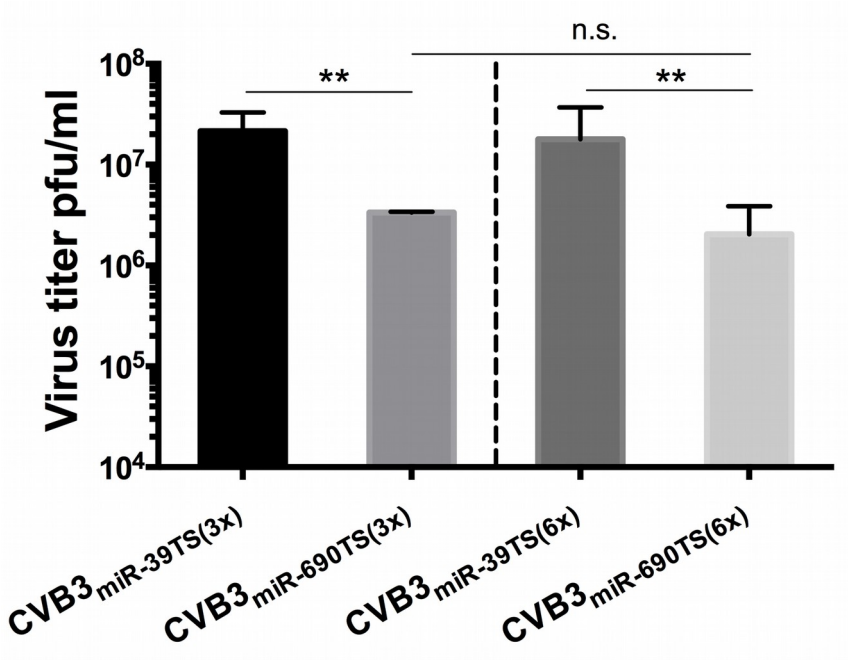

Figure 3.18 microRNA regulate 3x and 6x miR-TS encoding CVB3 replication. HEK293T cells were transfected with miR-690 expression plasmid (pCMV-miR-690) and after 24h, cells were infected with viruses at MOI of 0.1. Viral titers in the supernatants of the treated cells were determined at 18 h post infection by plaque assay. $CVB3_{miR\text{-}39TS(3x)}$ or $CVB3_{miR\text{-}39TS(6x)}$ were used as control as miR-39 is not expressed in mammalian cells. **, $P<0.01$. n.s., not significant.

Pancreas is the main target organ of the CVB3, therefore it was thought that preventing virus replication alone from pancreas would be sufficient to eliminate side effect of CVB3 [124].To investigate the possibility of this hypothesis, pancreas specific miR-375TS were incorporated into the CVB3 genome. Firstly, to test functionality of the miR-375 *in vitro* investigations were carried out. HEK293T cells were transfected with miR-375 expression plasmid (pCMV-miR375) and 24 h later cells were infected with $CVB3_{miR\text{-}375TS(3x)}$. After another 18h incubation period, cells were administered to three freeze/thaw cycles and the supernatant was collected to determine virus titer by plaque assay. Similar to inhibition of $CVB3_{miR\text{-}690TS(3x)}$ inhibition results, miR-375 inhibited $CVB3_{miR\text{-}375TS(3x)}$ replication (>10x) in HEK293T cells significantly **(Figure 3.19)**.

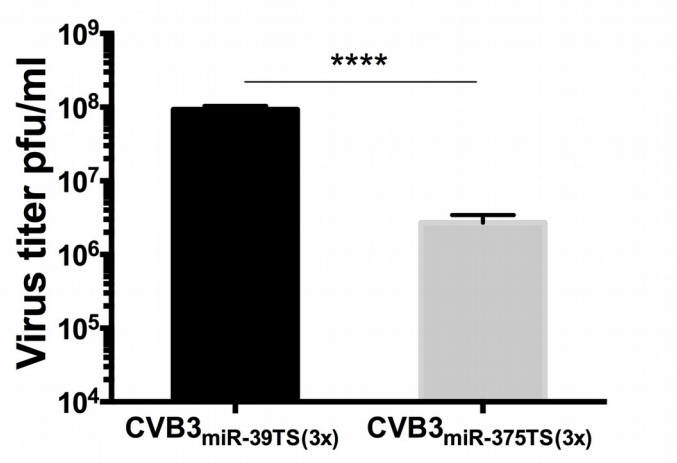

Figure 3.19 miR-375 inhibits $CVB3_{miR\text{-}375TS(3x)}$ replication[1]. HEK293T cells were transfected with miR-375 expression plasmid (pCMV-miR-375) and after 24h, cells were infected with viruses at MOI of 0.1. Viral titers in the supernatants of the treated cells were determined at 18 h post infection by plaque assay. ****, $P<0.0001$.

Taken together, these results show that CVB3 replication can be inhibited by exploiting miR-375 and miR-690 via incorporating the virus genome with miR-375TS and miR-690TS, respectively.

3.2.5 Effect of Endogenous Low Level miR-375 expression on $CVB3_{miR\text{-}375TS(3x)}$ Replication

Following *in vitro* investigations, it was aimed to analyze oncolytic efficiency and safety of miR-TS incorporated CVB3 clones in human CRC bearing nude mouse model. Similar to first part of the study, it was aimed to establish DLD1 tumors on mice. According to miRNA expression data **(Figure 3.13**, **3.14)**, while miR-690 was not expressed in DLD1, there was a low level endogenous miR-375 expression. Therefore, to reveal that $CVB3_{miR\text{-}375TS(3x)}$ still retain to replicate in DLD1 without perturbation by miR-375, cell viability assay was performed.

DLD1 cells were infected with $CVB3_{miR\text{-}39TS(3x)}$ or $CVB3_{miR\text{-}375TS(3x)}$ at MOI of 1, 10 and 100. Cell viability was analyzed 24h, 48h and 72h post infection. Both viruses were not able to lyse the cells at an MOI of 1. However, using MOI of 10 and 100 both viruses lysed the cells efficiently 72h post infection. There was only around 20% difference in cell killing efficiency, which was not significant, between miR-39TS and miR-375TS containing CVB3 clones at 72h post infection. **(Figure 3.20)**.

[1] This experiment was performed by M. Pryshliak.

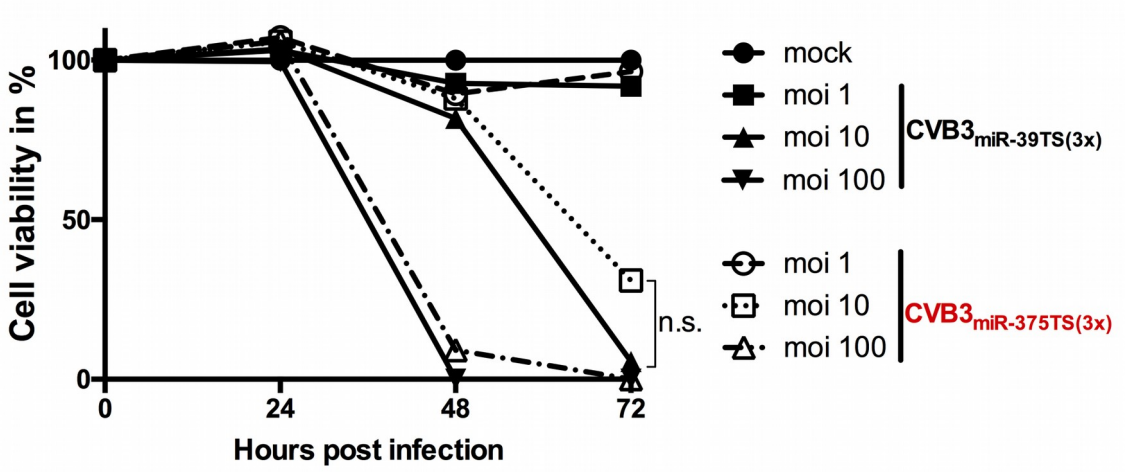

Figure 3.20 Cell viability assay for DLD1 colorectal carcinoma cell line. Cells were infected with $CVB3_{miR\text{-}375TS(3x)}$ and $CVB3_{miR\text{-}39TS(3x)}$ at MOI of 1, 10 and 100. Cytotoxicity was evaluated 24h, 48h and 72h post infection by XTT assay. Both viruses caused complete cell lysis at MOI of 100. The cell viability difference between $CVB3_{miR\text{-}375TS(3x)}$ and $CVB3_{miR\text{-}39TS(3x)}$ infected cells were not significant at MOI of 10. Both viruses did not lyse the cells at MOI of 1. n.s., not significant.

These results indicate that $CVB3_{miR\text{-}375TS(3x)}$ is still able to lyse DLD1 human CRC cell line which express endogenously low level of miR-375.

3.2.6 *In vivo* Studies with miR-TS Encoding CVB3

In vitro analysis revealed significant inbition of virus replication by inserting miR-TS into the virus genome. Therefore, next, it was aimed to investigate oncolytic activity and safety profile of $CVB3_{miR\text{-}375TS(3x)}$ and $CVB3_{miR\text{-}690TS(3x)}$, *in vivo*. In addition, it was also planned to compare pancreas-detargeted ($CVB3_{miR\text{-}375TS(3x)}$) and multi-tissue detargeted ($CVB3_{miR\text{-}690TS(3x)}$) viruses with respect to their attenuation levels in mice.

As previous experiment, xenograft BALB/c mouse tumor model was established with DLD1 human colorectal carcinoma cells. Tumor cells were inoculated bilaterally into the flanks of the mice and once the tumor size reached 50 mm^3, only one of the selected tumor burden was administered with single intratumoral dose of $3x10^6$ pfu $CVB3_{miR\text{-}375TS(3x)}$ or $CVB3_{miR\text{-}690TS(3x)}$. The third group was injected similarly with miR-39TS containing viruses and the fourth group was not treated with any virus to observe normal tumor growth.

As expected, $CVB3_{miR\text{-}39TS(3x)}$ infected animals showed strong side effects which led to mortality of the animals 4 days post virus infection. In contrast, $CVB3_{miR\text{-}375TS(3x)}$ was not recovered from the pancreas. Even if low amount of virus (around 10 pfu/g) was detected

in the heart, it did not let to myocarditis **(Figure 3.21)**. It is important to note that there were no any visible side effects on animals compare to uninfected mice and all of these animals survived till end of the experiments with a normal physical state indicating that miR-375 targeting was adequate to inhibit systemic viral infection. A surprising result was the inadequate attenuation of miR-690TS encoding CVB3 clone which lead the systemic viral infection result in lethal side effects five days post virus administration. High level of viral particles was recovered from heart and pancreas of $CVB3_{miR\text{-}690TS(3x)}$ infected animals indicated that miR-690 targeting was not sufficient to protect mice from unintended virus replication in healthy organs.

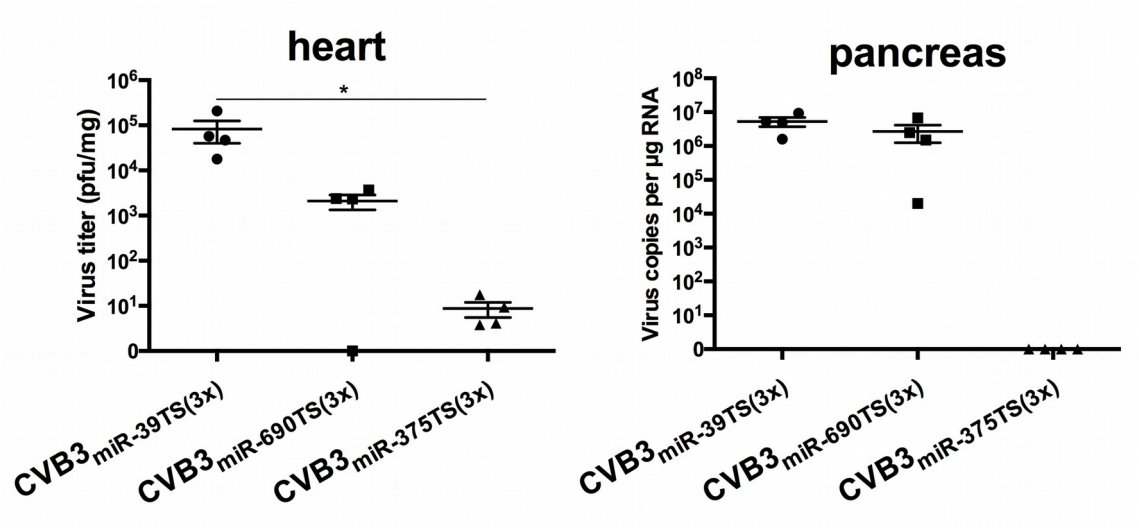

Figure 3.21 Viral load of $CVB3_{miR\text{-}39TS(3x)}$, $CVB3_{miR\text{-}690TS(3x)}$ and $CVB3_{miR\text{-}375TS(3x)}$ in heart and pancreas of tumor bearing mice. Control animals were not infected with any virus. miR-690TS was not sufficient to prevent virus replication both in heart and pancreas. In contrast, miR-375TS insertion let to total protection of pancreas from virus. *, $P<0.05$.

Next, virus replication in inoculated tumor burdens was evaluated. In both treated and untreated tumors, high level of replicating virus was detected **(Fig. 3.22A, B)**. These analyses were performed at different days as $CVB3_{miR\text{-}39TS(3x)}$ infected animals were moribund 4 days post infection and had to be sacrificed that day. Similarly, $CVB3_{miR\text{-}690TS(3x)}$ infected animals were sacrificed 5 days post infection due to moribund condition. Only $CVB3_{miR\text{-}375TS(3x)}$ treated animals were healthy till end of the experiment (10 days post infection). Strong tumor regression was observed in both treated and untreated contralateral distant tumor of $CVB3_{miR\text{-}375TS(3x)}$ infected animals indicating that the virus retained its strong oncolytic activity **(Fig. 3.22B)**.

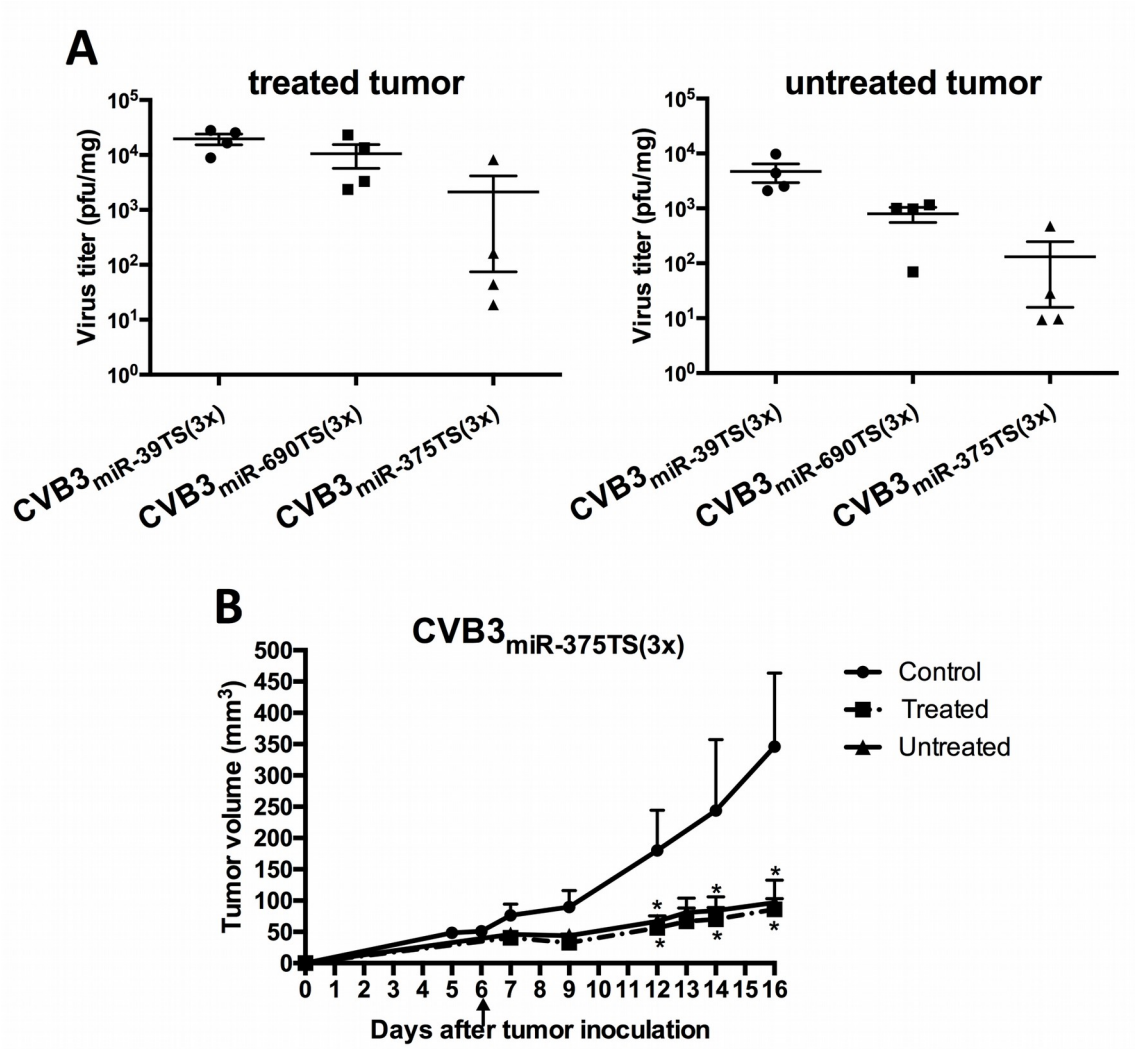

Figure 3.22 Infectivity and replication activity of $CVB3_{miR\text{-}39TS(3x)}$, $CVB3_{miR\text{-}690TS(3x)}$ and $CVB3_{miR\text{-}375TS(3x)}$ in inoculated tumours. Plaque assay was performed from harvested treated tumor and untreated tumor. **A**) *Left*: virus titers in $CVB3_{miR\text{-}39TS(3x)}$, $CVB3_{miR\text{-}690TS(3x)}$ and $CVB3_{miR\text{-}375TS(3x)}$ injected tumors. *Right*: virus titers in the un-injected tumor **B)** Tumor volumes are shown as means ± SEM. of $CVB3_{miR\text{-}39TS(3x)}$ infected mice were moribund and sacrificed 4 days after viral injection. $CVB3_{miR\text{-}690TS(3x)}$ infected mice were sacrificed at day 5 after viral injection due to moribund condition. $CVB3_{miR\text{-}375TS(3x)}$ and un-injected control mice were sacrificed at day 10 after viral injection. *, $P<0.05$

In conclusion, these results revealed that insertion of pancreas specific miR-375TS into the CVB3 genome led to modulate tissue tropism and protection of unintended virus replication in healthy organs, *in vivo*. Importantly, modification of virus genome did not cause attenuation in oncolytic activity of CVB3 so that tumor growth was inhibited significantly in infected animals of both treated and untreated contralateral tumor.

3.3 Enhancing the Viral Fitness and Generation of Tumor Cell Adapted $CVB3_{miR\text{-}TS}$ Mutants

CVB3 showed different viral fitness to different colorectal tumor cells. While some colorectal cells were permissive to CVB3 infection, some of them were resistant to infection **(Figure 3.3)**. *In vivo* experiments also revealed that CVB3 infection of the tumors may not be sufficient to completely destroy tumor burden **(Figure 3.7, 3.22B)**. Therefore, it was aimed to analyse whether efficiency can be increased by developing more efficient and tumor cell adapted CVB3 by using directed evolution strategy. For this investigation, oncolytic $CVB3_{miR\text{-}690TS(6x)}$ was selected as a prototype. The reason of miR-TS containing virus selection was to evaluate genomic stability of TS insert following serial passages. $CVB3_{miR\text{-}690TS(6x)}$ was passaged in DLD1 colorectal carcinoma cell line and viral fitness was analysed after passage 5 with XTT, plaque and cell killing assays.

Determination of required time to reach a plateau viral titer is critical step in virus adaptation. Therefore, firstly, single step growth curve analysis was done to evaluate growth kinetics of viruses in DLD1 cell line. Control virus and $CVB3_{miR\text{-}690TS(6x)}$ replication reached to plateau 72h post infection **(Fig. 3.23)**. So that during passaging, cells were incubated for 72h after infection and each time plaque assay was performed to infect the cells with the same titer (MOI:0.1).

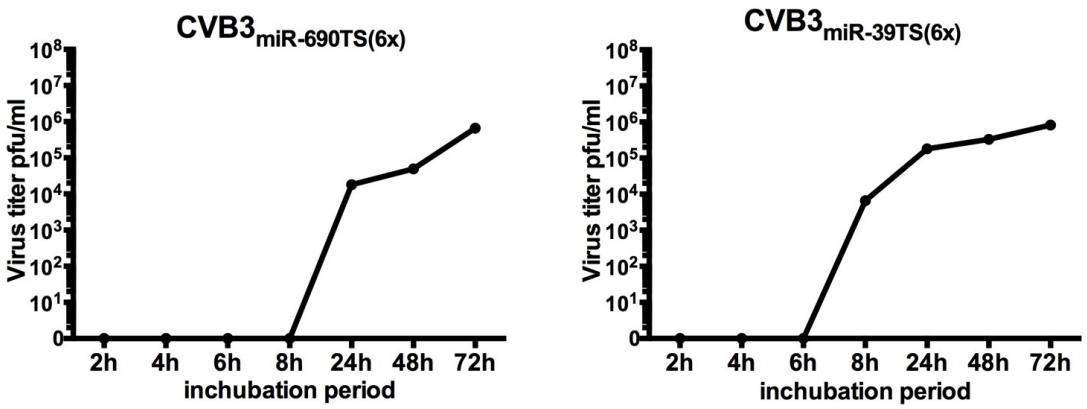

Figure 3.23 Single step growth curve for $CVB3_{miR\text{-}690TS(6x)}$ and $CVB3_{miR\text{-}39TS(6x)}$. DLD1 cell line were infected with viruses at MOI of 0.1. Plaque assay was performed for detection of virus titer in indicated time points from collected supernatant.

Passaging was performed up to 5 times as in the fifth passage, complete cell lysis was observed more rapid than before, after 48h post infection. Following passage five, adapted virus ($CVB3_{(6x)TS}$-pas5) was collected from supernatant and cytopathic effect

(CPE) was evaluated by performing cell killing assay. The $CVB3_{(6x)TS}$-pas5 showed significantly higher CPE compare to primary virus ($CVB3_{(6x)TS}$). Even at an MOI of 1, $CVB3_{(6x)TS}$-pas5 induced cytolysis against DLD1 cell lines at 144h post infection, whereas $CVB3_{(6x)TS}$ could able to lyse the cells at an MOI of 100 **(Figure 3.24A).** Therefore, adapted virus was about 100-fold more efficient than parental virus. Additionally, the results of XTT assay confirmed that new adapted virus mutant was able to lyse all cells from high dose (MOI:100) to low dose (MOI:1) at 144hpi. In contrast, primary $CVB3_{(6x)TS}$ were only able to lyse around 70% of cells at MOI of 100 at 144hpi **(Figure 3.24B)**.

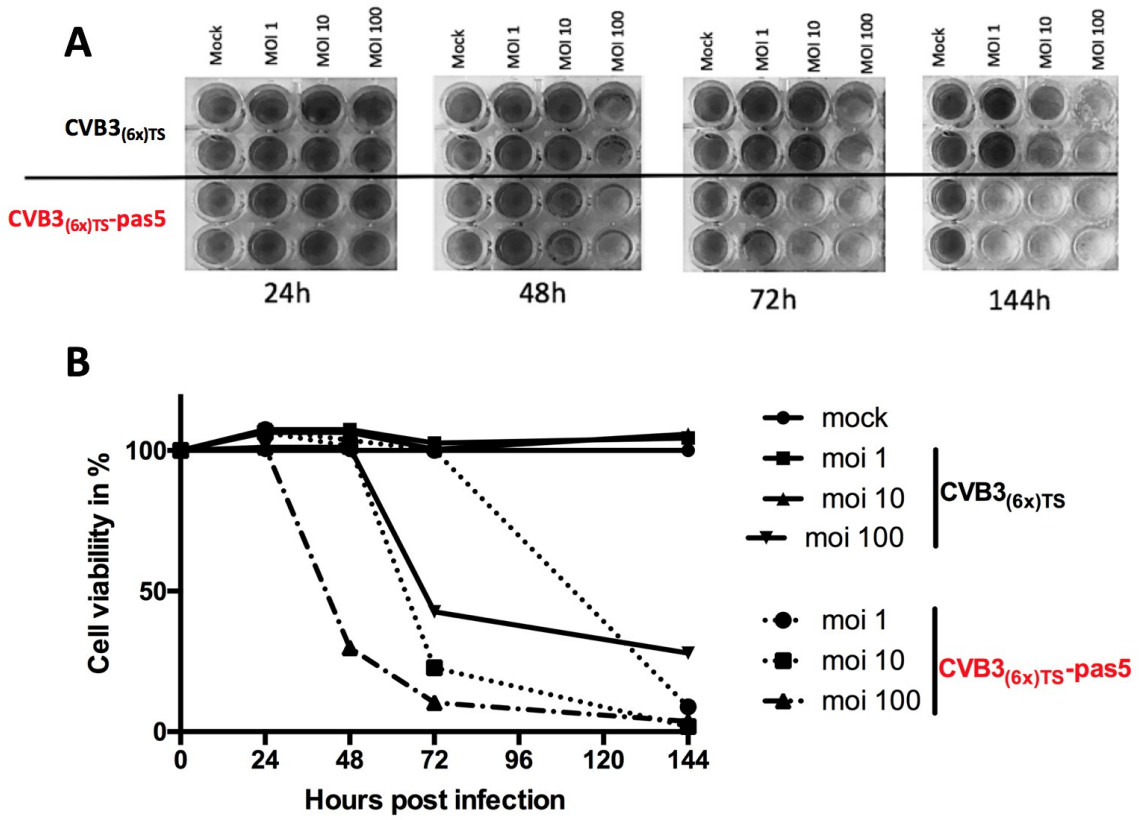

Figure 3.24 Adaptation of CVB3 mutant to DLD1 cell line. **A)** Cell viability assessed by crystal violet staining shows increased oncolytic activity of CVB3 even in low MOI 144h post infection. Stained wells indicate existence of living cells. **B)** XTT assay was performed to evaluate cell viability indicating higher viral fitness of adapted virus.

Next, replication of $CVB3_{TS(6x)}$-pas5 and $CVB3_{TS(6x)}$ was compared at different time points by plaque assay. DLD1 cells were infected with the viruses at low dose (MOI of 1) and supernatant was collected 24h, 48h and 72h post infection. As expected, $CVB3_{6xTS}$-pas5 showed higher replication in all three different time points. The replication rate difference increased overtime reaching its maximum level after 72h. At this time point it was 35x times higher pfu/ml **(Figure 3.25A)**. Another important aspect of higher viral fitness for

the adapted virus compare to parental virus was the plaque size. Interestingly, adapted virus showed larger plaque compared to parental non-adapted CVB3 **(Figure 3.25B)**

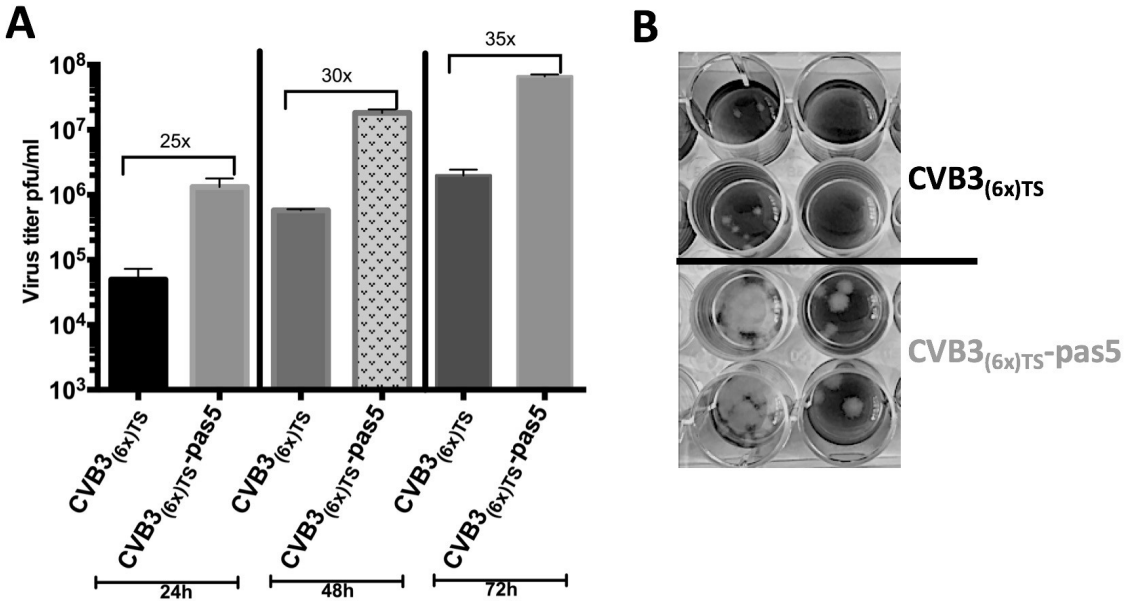

Figure 3.25 Difference in viral replication between $CVB3_{TS(6x)}$ and $CVB3_{TS(6x)}$-pas5. **A)** Viral fitness of adapted virus was increased 25 to 35 times compared to parental virus. **B)** Adapted virus generates bigger plaque sizes.

Lastly, to compare stability of inserted miR-TS in adapted and primary virus genomes, sequencing analysis was performed. Adapted virus $CVB3_{6xTS}$-pas5, parental virus $CVB3_{6xTS}$ and plasmid clone of $CVB3_{miR\text{-}690TS(6x)}$ was evaluated. Interestingly, following virus production in HEK293T cells, some point mutations were already detected in first copy of miR-690TS sequence. Moreover, after 5 passages of the virus, mutations were found in the first three miR-TS sequence. The mutations occurred from 5′ side of sequences therefore last three miR-TS near 3′ UTR were stable **(Figure 3.26)**.

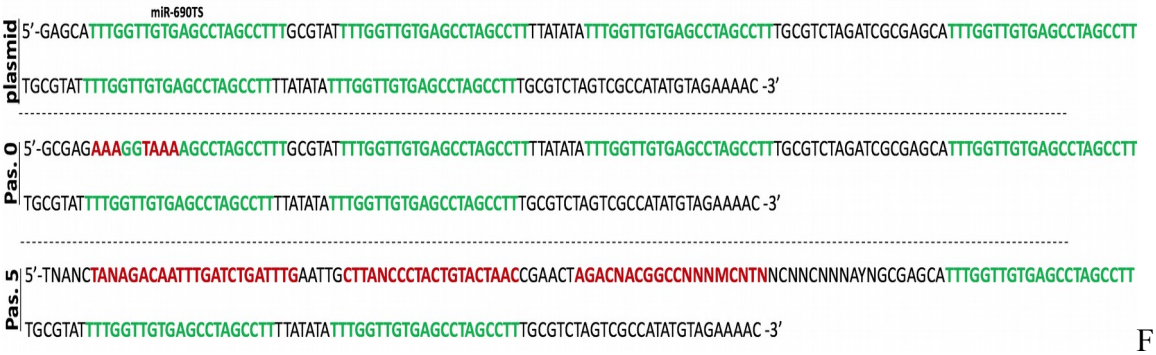

Figure 3.26 Genetic stability of inserted miRNA target sequences during virus adaptation. Plasmid: $CVB3_{miR6xTS}$ containing plasmid. Pas. 0: Primary $CVB3_{miR6xTS}$, Pas. 5: Adapted $CVB3_{miR6xTS}$-pas5

In conclusion, these results confirmed that successfully adapted CVB3 with higher viral fitness against DLD1 cell line was generated by using directed evolution strategy even after 5 passages. New virus was able to lyse DLD1 cell line in low doses while primary non-adapted virus could only show the same effect with 100x times higher doses. In addition, three of six miR-TS copies were mutated, however in the end of the experiment 3x TS was still inside virus genome which is enough number for detargeting of the virus as it was proved in previous inhibition experiments.

3.4 Evaluation of Oncolytic Efficiency of CVB3 Strains against Murine CRC cell line for Immunocompetent Mouse Models

Efficiency of CVB3 strains was shown in human CRC cell line bearing immunocompromised mouse model **(Figure 3.4)**. Even if for oncolytic virotherapy studies application of these mouse models are very common and relatively sufficient, using immunocompetent mouse models together with murine cancer cells gives more detailed information for the effect of the viruses. Therefore, an initial experiment was performed to evaluate oncolytic activity of CVB3 strains in Colon-26 murine colorectal cancer cell line. Interestingly, in this experiment also CVB3 PD was the most efficient strain. Between four different CVB3 strains, only CVB3 PD was able to lyse Colon-26 cell line even in very low MOI at 24h and 72h post infection (**Figure 3.27)**.

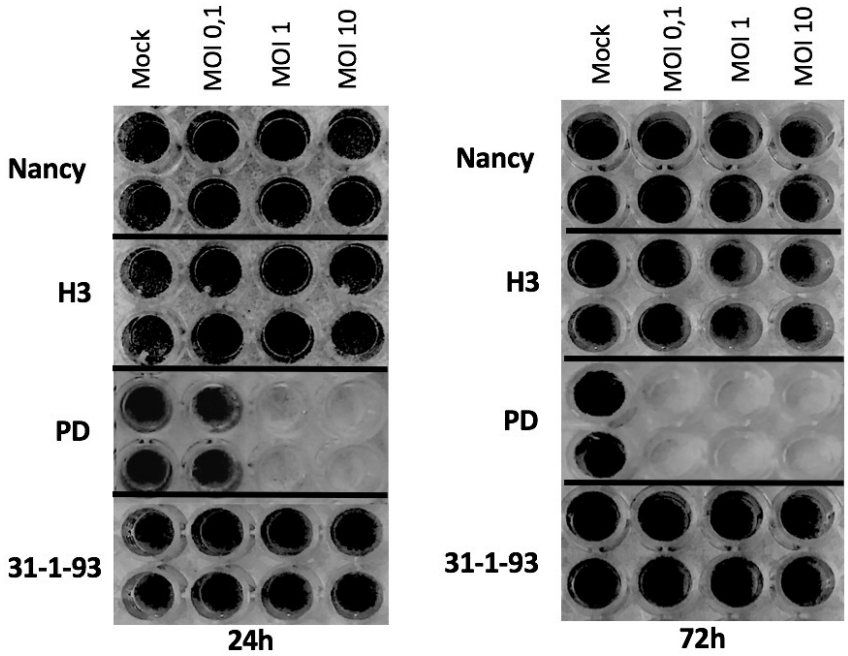

Figure 3.27 Oncolytic activity of CVB3 Nancy, H3, 31-1-93 and PD in Colon-26 cell line.

CHAPTER 4

DISCUSSION

4.1 CVB3 as an Oncolytic Virus

Colorectal cancer has a poor prognosis in advanced stages. Between 25% of newly diagnosed cases present metastatic disease and 50% of early stage patients will develop a metastasis. While surgical resection for patients with localized tumors (stage I and II) results in 90% 5-year survival rate, this percentage decrease dramatically up to 10% for CRC patients with the distant metastasis cases (stage IV) [46]. Surgery is not an applicable option for most patients (75-90%) with advanced stages due to tumor or patient specific reasons and unfortunately, current conventional treatment strategies have only palliative effect by delaying the disease progression, increasing the survival and maintain life quality [178]. Chemotherapy and targeted therapy are approved non-surgical therapeutics for late stage of colorectal cancer. However, the most widely used fluoropyrimidine based chemotherapy regimens have a modest positive impact together with severe side effects on patients [179]. Besides, 85% of patients with advanced CRC tumors show resistance to 5-FU first line therapy [180]. Despite significant improvement in overall survival with the help of monoclonal antibodies that target VEFG and EFGR, complex heterogeneity of the disease cause to limit number of patients who benefit from these therapeutics [66]. Therefore, there is an urgent need for new therapies.

4.2 Evaluation of CVB3 Strains as Oncolytic Agent for Treatment of CRC

Oncolytic virotherapy is a new method for treatment of the cancer [181, 182]. Oncolytic viruses have tumor selectivity which make them special in cancer treatment. The selectivity is achieved by several circumstances [156]. The host receptor-virus interaction is the most important factor as all viruses need to enter cells to replicate.

Usually the virus host cell receptors are overexpressed on tumor cells [183]. In this way, wild-type viruses can easily infect and lyse the malignant cells without any modifications in virus genome. On the other hand, oncolytic viruses can be also engineered to retarget them to tumor specific receptor [184].

Additionally, tumor causing mutations, such mutations leading to activated RAS pathway, are known to increase the tumor selectivity of the oncolytic viruses [185, 186, 187]. Importantly, deficiencies in antiviral defence system of cancers cells, such as absent of interferon, provide a favourable environment for virus replication [188].

Enteroviruses represent one group of viruses containing members with oncolytic activity. Major advantages of the enteroviruses are their fast replication cycle, the production of high amounts of virus progeny, the small virus size, which enables better tumor penetration, and their ability to stimulate a powerful anti-tumor immune response [108, 113, 189]. CVA21 and PVS-RIPO are the best investigated oncolytic enteroviruses. Both are currently under clinical investigation for treatment of cancer [117]. More recently, Miyamoto et al. [121] showed that another enterovirus, CVB3 (strain Nancy), has oncolytic activity and was highly effective at destroying lung carcinoma *in vivo*. However, the pre-clinical study of Miyamoto et al. also revealed that the Nancy strain induced hepatic and cardiac injury in tumor bearing mice.

There are several clinical and laboratory strains of CVB3, which are characterized by different tissue tropism, pathogenicity and course of infections [129]. Thus, it was hypothesized that there might be CVB3 strains with oncolytic activity but with an improved safety profile. Accordingly, in this study it was aimed to compare a panel of different CVB3 strains for their anti-tumor efficiency and safety *in vitro* and *in vivo* using colorectal carcinoma cells as target. It is shown that the CVB3 strain PD is a potent and efficient oncolytic virus against colorectal carcinomas *in vitro* and *in vivo* and more importantly PD does not regularly induce side effects *in vivo*. No systemic infection with PD was detected and virus could not be recovered from the organs of treated animals. In contrast, two other analyzed CVB3 strains, Nancy and 31-1-93, although showing similar anti-tumor efficiency as PD, induced severe systemic infection in mice, leading to the death of the animals.

Expression of virus receptors on the cell surface is an important prerequisite for virus infection. CVB3 Nancy and 31-1-93 utilize CAR and DAF for infection [135, 136]. It was found that the majority of colorectal carcinoma cells lines express moderate to high

levels of CAR and DAF, but, with exception of the DLD1 cell line they were more or less resistant against both strains. It may be possible that the receptor expression was not high enough to mediate efficient virus infection, but lack of a clear correlation between CAR/DAF expression and virus induced cell lysis as well as individual differences between Nancy and 31-1-93 suggests that additional, receptor-independent mechanisms may be responsible for this result. The CVB3 strain PD lysed colorectal carcinoma cell lines more efficiently than the strains Nancy and 31-1-93. A major difference of PD towards Nancy and 31-1-93 is the receptor tropism. PD uses N- and 6-*O*-sulfated HS for infection of cells [171].

HS are linear polysaccharides consisting of repeating disaccharides, which are linked to the cell surface by core proteins. Although nearly ubiquitously expressed, HS vary in sulfate content and charge among different tissues and cells [138, 190]. In cancer, HS plays a complex role in tumor progression, metastasis, and invasion [191]. N- and 6-*O*-sulfated HS are highly abundant in many cancer cells, including colorectal carcinoma cells [173]. The latter was confirmed in this study by detection of HS6TS2, which is involved in synthesis of N- and 6-O-sulfated HS. Furthermore, our results reveal the importance of HS for the oncolytic activity of PD in colorectal carcinoma cells. In this regard, it was found a clear correlation between cellular HS6TS2 expression levels and sensitivity of colorectal carcinoma cell lines to PD, as well as was demonstrated that blocking the binding of PD to HS strongly inhibits lytic activity of the virus.

In contrast to recently described oncolytic CVB3 [121] and other CVB3 strains investigated in this study, PD was completely attenuated in vivo. This is in accordance with other studies showing the HS-binding viruses are in general attenuated in vivo [192]. Even though the mechanism is not fully understood, attenuation seems to be caused by a reduced ability of the virus to spread from the site of inoculation via the blood stream and by a more rapid clearing from the circulation [192]. However, in the case of PD, the specific tropism of PD to N- and 6-O-sulfated HS may provide an additional explanation. The pancreas, heart, spleen and brain are major target organs of CVB3 strains using CAR and DAF for infection. However, these organs do not express HS6ST2 [173]. Thus, resistance of these organs against PD as found in this study may be directly related to the lack of the specific PD receptor.

It was found that intratumoral injection of tumor bearing nude mice with PD, but also Nancy and 31-1-93 led to significant inhibition of growth of the injected tumor. Even more importantly, a similar growth suppression was also observed for the distant, non-injected subcutaneous tumor. Whereas these results demonstrate *per se* the efficacy of CVB3 mediated oncovirotherapy in colorectal cancer, suppression of the untreated

tumor reveals the potential of PD to treat metastatic or disseminated colorectal cancer. It is important to mention that the DLD1 cell line, which was used to establish subcutaneous tumors is highly resistant to radiation [193] and against 5-fluorouracil which is used as chemotherapeutic agent for the treatment of colorectal cancer [194]. PD may therefore be an alternative treatment modality for colorectal cancers, which are resistant to conventional therapies. Interestingly, CVB3 Nancy and PD RNA was detected in immune cells within the tumor masses and on the tumor-stroma border zone, whereas tumor cells seemed not to be infected. The failure of virus detection in tumor cells was a surprising result, as virus-induced cell lysis is thought to be a major mechanism of anti-tumor efficiency of oncolytic viruses [195]. The reasons for this are currently not clear. However, the detection of CVB3 genomes in immune cells and their specific localization in the tumors suggests that virus-induced immune-mediated mechanisms may be involved in anti-tumor efficiency of the CVB3 strains. This conclusion is supported by a previous study demonstrating that CVB3 replication in lung carcinoma results in recruitment of natural killer cells and granulocytes into the tumor tissue [121]. Furthermore, our data indicate that immune cells may be carriers and probably a reservoir for hematogenous distribution of the viruses within a tumor or to distant tumors.

One (animal PD-M1) of the six PD treated animals became ill and showed high levels of viral progeny in its heart, spleen and pancreas. Sequence analysis of viral RNA which was isolated from heart revealed two amino acid substitutions in VP2 and one amino acid substitution in VP1 in the virus compared to PD indicating that the isolated virus, CVB3-PD-M1V, was a mutant of PD. Even though further functional investigation was not carried out, the detected mutation may be responsible for different phenotype of PD-M1V compared to PD. In fact, the amino acid substitutions found in VP2 of PD-M1 are linked to be specific for some CVB3 strains inducing myocarditis [127, 175]. Two mechanisms, which are closely related to the life cycle of CVB3, the high mutation rate and the quasispecies dynamics [196], may be responsible for the occurrence of CVB3-PD-M1V. The rapid genomic evolution result in generation of minor populations with different mutations compare to parental virus. Especially, during *in vitro* propagation of the virus this risk increases. There may be different ways to overcome development of undesirable PD mutants. For example, the use of cDNA clones [127, 165] may reduce the strong heterogenicity of quasispecies and reduce the risk of the emergence of mutants. Production of cDNA clone let to obtain pure and genetically homogeneous virus stocks [197]. MicroRNA-mediated virus detargeting

strategies, which have successfully been used for several oncolytic viruses, including members of enteroviruses, represents another way to prevent toxicity [118, 198, 199, 200].

In summary, these findings demonstrate strong oncolytic efficiency of different CVB3 strains in colorectal carcinoma, but only the strain PD achieved a significant safety profile. Thus, PD represents a promising CVB3 strain for treatment of colorectal carcinoma. Nevertheless, to minimize the risks for application of the virus in human patient further improvement of the safety are still needed. Therefore, as a second part of the study microRNA-regulated recombinant CVB3 was produced and investigated under *in vitro* and *in vivo* conditions.

4.3 Elimination of Virus Pathogenicity via microRNA-Dependent Targeting

Tumor selectivity is the one of the most important concern in oncolytic virotherapy field. A potent oncolytic virus should selectively replicate and destroy tumor cells but not replicate in non-transformed healthy cells. On the other hand, Group B coxsackieviruses have been identified as human pathogen which can cause myocarditis, pancreatitis and aseptic meningitis [123, 139]. Importantly, most of these cases have been reported in young children and infants, therefore the reason of the systemic CVB3 infection is associated with non-developed or poor immune system. Enterovirus surveillance studies which were conducted in USA, China and Spain indicate that 90% of all CVB3 infection cases occur in infants and children [201, 202, 203]. In addition to poor immune system it is described that in young children the expression of CAR in myocardium is higher than in adults which means upregulation of receptor during embryonic and neonatal period plays an important role in high sensitivity to disease [204]. However, the main causative reason of the CVB3 virulence is still unclear. Therefore, even if the infants known to be under high risk of CVB3 infection, pathogenicity of CVB3 still need to be limited before to be used as oncolytic agent to minimize all potential risks which can be caused by viral infection, especially for particular CVB3 strains.

Many different genetic engineering strategies have been developed and evaluated to enhance selectivity of viruses for virotherapy. Among these approaches, it is shown that miRNA-mediated detargeting of the virus is one of the most affordable and useful strategy [149, 154]. Consistently, in the present study, pathogenesis of infectious CVB3

strain H3 [127] was successfully eliminated without damaging oncolytic activity by incorporating the virus genome with tissue specific miR-TS.

In the light of the findings obtained from the first part of the study, CVB3 strains have different virulence levels on mice. However, under all circumstances, the main target organs of CVB3 are pancreas, heart and brain. Therefore, it was aimed to prevent virus replication in these organs by incorporating the virus genome with miRNA target sequences cognate tissue specific miRNAs. In this context, two different strategies were evaluated. In the first approach, a ubiquitously expressed miRNA was planned to be exploited for CVB3 detargeting. Similar strategy was successfully used by other research groups, especially by exploiting let-7 family miRNAs which are ubiquitously expressed tumor suppressors [205]. As let-7 miRNAs are downregulated in cancer cells, let-7 target sequences were inserted to many type of oncolytic viruses such as VSV, poliovirus and adenovirus [206, 207, 208]. For this purpose, in the present study, expression levels of miR-34, miR-16 and miR-690 were analyzed. The miR-34 and miR-16 were shown to be highly conservative in human cells and additionally, their expression is downregulated in many cancer types due to their active role in cell proliferation, cell cycle and apoptosis [176, 177, 209, 210]. In line with these observations, miR-34a and miR-16 were found to be enriched in pancreas, brain and heart, however, high expression levels were also observed in colorectal cancer cells which hinders the use of any of in these miRNAs. Distinctly, miR-690 found to be enriched in mentioned healthy organs but not in colorectal cancer cells. miR-690 is linked to several independent functions such as wound repair, fibroblast shift, regulation of testosterone in the liver and activation of myeloid derived suppressor cells [211]. However, there is no study which was directly investigated its role in carcinogenesis or its expression pattern in cancer types. Even so, due to its different features and particularly its high expression pattern in target organs, it was selected for present study to investigate the efficacy of multi-tissue detargeting of CVB3 by ubiquitously expressed miRNA.

Target site of miR-690 was inserted into the 3′ UTR of CVB3 genome in 3 or 6 repeated copies. Subsequently, the functionality of miR-690TS was tested *in vitro*, in the presence of high miR-690 expression. Significant inhibition of virus replication was observed in produced miR-690TS encoding CVB3 clones between one to two log steps compared to control miR-TS bearing CVB3. These results were in agreement with

another study which conducted with recombinant CVB3 containing muscle specific miRNA targets [212]. They showed that attenuation levels around 100-fold found to be sufficient to protect mice from lethal side effects. In addition, in previous studies, it has been described that the number of miR-TS has a direct impact on miRNA mediated detargeting and insertion of three miR-TS repeats was suggested for an optimum regulation level and to avoid risk of saturation [213]. Consistent with these observations, no significant difference was observed in down regulation of $CVB3_{miR\text{-}690TS(3x)}$ and $CVB3_{miR\text{-}690TS(6x)}$.

Pancreas detargeting was evaluated as second approach to eliminate CVB3 pathogenicity by exploiting pancreas specific miR-TS. Pancreas is the most vulnerable organ for CVB3 infection and it has been shown that CVB-induced myocarditis takes place only as a consequence of pancreatitis development [134]. Therefore, it was aimed to analyze whether prevention of pancreas from virus invasion would be sufficient to protect the mice from systemic CVB3 infection and therefore, side effects. The miR-375 has been shown to be enriched in pancreas [214, 215] which was consistent with the results obtained in this study. Additionally, the level miR-375 expression was found to be at least two log steps lower in human CRC cell lines compared to pancreas tissue. Dual luciferase reporter assay confirmed that these expression levels did not perturb transgene expression by the plasmid containing cognate miR-TS. This result was in agreement with other studies shows that repression of miR-TS equipped transcript can be only achieved in relatively high expression levels [216]. Hence, three copies of miR-375TS was inserted into the 3′ UTR of CVB3 genome and $CVB3_{miR\text{-}375TS(3x)}$ was successfully produced. The functionality of the inserts was tested in the presence of miR-375 expression, *in vitro*. Consequently, miR-375TS and miR-690TS containing viruses were selected for *in vivo* investigation, (i) to evaluate the essential role of pancreas in CVB3 pathogenicity (ii) to compare targeting specificity of ubiquitously expressed miR-690 versus pancreas specific miR-375. For better comparison, $CVB3_{miR\text{-}690TS(3x)}$ was selected instead of $CVB3_{miR\text{-}690TS(6x)}$ due to equal number of TS inserts as $CVB3_{miR\text{-}375TS(3x)}$.

In vivo investigation of therapeutic efficiency and safety profile of genetically engineered miR-TS containing CVB3 revealed that insertion of miR-TS for pancreas enriched miR-375 significantly attenuated the virus and caused no systemic virus infection. Importantly, oncolytic activity of recombinant $CVB3_{miR\text{-}375TS(3x)}$ retained as the

tumor growth remarkably diminished. Similar to previous *in vivo* experiment of this study, DLD1 cell line was used for the establishment of human tumors on mice. Very close oncolytic activity was observed, in a such way that $CVB3_{miR\text{-}375TS(3x)}$ was not only able to regress the growth of the injected tumor but also the distant non-injected tumor size was suppressed. This observation was confirmed by measuring the viral progeny in both tumors. High viral titers were observed in non-injected and injected tumors of each mouse, indicates that miRNA regulated recombinant CVB3 is still efficient to replicate and kill the tumor as its wild-type variant. Safety profile of the $CVB3_{miR\text{-}375TS(3x)}$ was further analyzed in collected pancreas and heart tissues. No virus progeny was detected in pancreas of $CVB3_{miR\text{-}375TS(3x)}$ treated animals indicates that miR-375 successfully blocked undesirable virus replication in pancreas and animals were protected from systemic virus infection. Even if very small amount of viral progeny was recovered from heart tissue of $CVB3_{miR\text{-}375TS(3x)}$ treated mice, no inflammation or any visible side effects were observed on animals.

$CVB3_{miR\text{-}39TS(3x)}$ was used to monitor pathogenicity of the virus as miR-39 is not expressed in mammalian cells [217, 218]. As expected, $CVB3_{miR\text{-}39TS(3x)}$ caused severe side effects on mice four days post virus infection and led to mortality of the animals. Unexpectedly, in contrast to $CVB3_{miR\text{-}375TS(3x)}$, $CVB3_{miR\text{-}690TS(3x)}$ was not attenuated and showed similar lethal effects on mice as $CVB3_{miR\text{-}39TS(3x)}$. The miR-690 targeting had only a minimal protection response. All mice of this study group had to be sacrificed five days after virus injection. Very high virus progeny was recovered in the pancreas tissues of $CVB3_{miR\text{-}39TS(3x)}$ and $CVB3_{miR\text{-}690TS(3x)}$ injected mice, almost at similar levels. The reason of non-sufficient inhibition of virus replication in presence of miR-690 is unclear. It has been suggested that the consideration of absolute levels of miRNAs may give a more precise sight to understand the silencing potential of respective miRNA [216]. In this study miRNA expression analysis was demonstrated in relative quantification levels. While miR-375 is a well identified and tissue enrichment specificity is well described miRNA [215], there is limited data about miR-690 expression pattern. On the other hand, it is also described that highly expressed miRNAs are not always adequate to inhibit gene regulation, which means abundant expression level of miRNA is not the only factor, so that additional determinants might be included such as the concentration of other miRNA response elements for respective miRNA or the type of Argonaute proteins presenting in the cell environment [219].

Lastly, it was clearly identified that CVB3 replicate in exocrine pancreas rather than endocrine pancreas which contains alpha and beta cells [220]. Even if the level of miR-690 or miR-375 in endocrine and exocrine pancreas was not evaluated in present study, Tang et al. showed that miR-690 is overexpressed in beta cells and interestingly downregulated in case of high glucose levels while miR-375 found to be consistent during glucose treatment [221]. Therefore, expression site of the miR-690 or the inconsistent expression profile regarding glucose level may a reason of the insufficient detargeting of $CVB3_{miR\text{-}690TS(3x)}$. It is also important to consider the shifts in the host miRNA expression profile. For example, it was shown that expression of miR-375 is increased in the case of pancreas injury which may also influence the strong detargeting of the virus [222].

As mentioned above, low amount of virus was detected in $CVB3_{miR\text{-}375TS(3x)}$ treated mice without visible side effects on animals. A general concern for the use of miRNA regulated RNA viruses as therapeutic agent is the possibility of existence of escape mutants due to high mutation rate which has been discussed in other reports [198], [199, 223]. Additionally, a minor virus population, which has no miR-TS in their genome, may also occurred during propagation of virus to reach required titer for the experiment (viral quasispecies is discussed in other parts). On the other hand, some viruses may also directly have replicated in the heart before pancreas. However, it was not possible to isolate viral RNA from heart due to low virus titer to check whether the miR-375TS were mutated or deleted. Besides, another explanation could be that the detected viruses were not originated from myocytes but were originated from infected immune cells which circulate in the bloodstream. No matter how, this small amount of undesired heart infection did not cause a visible side effects on animals in comparison to miR-39TS containing CVB3 infected animals. In addition, all *in vivo* experiments carried out using immunodeficient mice model which lack humoral immune system that clear virus from bloodstream [224]. Avirulent CVB3 strains such as CVB3/GA or CVB3/0 are known to make infection in heart and pancreas without inducing pancreatitis or myocarditis that result in lethal side effects, due to rapid clearance of the virus from the animals by day seven post virus infection [134, 225, 226]. To eliminate all possibility of CVB3 induced heart infection, less cardiovirulent or avirulent strains' cDNA can be used to incorporate with miR-TS, e.g. CVB3 PD. Additionally, 5 ′UTR region of CVB3 genome has been described as determinant of cardiotropism [227, 228]. Particularly, a single mutation in

234. position nt. from U to C was found to attenuate cardiovirulence in animals [229]. Together with miR-375TS insertion, making such a single mutation in virus genome or directly constructing a hybrid virus which contains 5′ UTR of avirulent strain might let to obtain highly tumor selective recombinant virus.

In conclusion, the findings of this part of the study show that miRNA-mediated detargeting can be used to increase tumor selectivity of CVB3 without loss in oncolytic capacity. The miR-TS containing virulent CVB3 strain is able to kill the infected tumor and distant uninfected tumor which shows the virus circulation in blood do not cause pancreatitis or myocarditis.

4.4 Enhancing Viral Fitness of CVB3 in Resistant Cell Lines and Investigation of miR-TS Stability

As mentioned before colorectal cancer is a very heterogeneous disease, more than ten different mutations can be involved in tumor formation, therefore, resistance to treatment is not only encountered in chemotherapy or targeted therapy but also in oncolytic virotherapy. In the first part of the study, different susceptibility of CRC cell lines against CVB3 strains is shown. Resistance of the cells against virus infection may arise due to various reasons including cell receptor expression, different intracellular abnormalities of tumor cells or host cell antiviral responses [230]. Two main approaches have been exploited to overcome the resistance of the cells against oncolytic viruses, which are the manipulation of viral genome by genetic engineering and natural adaptation of the viruses to the resistant tumor environment.

Capability of viruses to adapt cancer cells for better replication was discovered in the very early times of oncolytic virotherapy, resulting from absence of genetic engineering techniques [100]. Moore et. al. adapted the Russian encephalitis virus to sarcoma cells to make the virus more tumor specific and they reported the increase of oncolytic activity following adaptation compared to parental virus [231]. However, since the rapid development in genetic engineering, researchers' interest shifted to use of new techniques due to extensive opportunities come together. Even if great advances in manipulation of viral genome by gene techniques, most of the viruses attenuated following changes in viral genome [163]. Besides, the complex context of virus-host interaction due to tumor cell differences within the patients is another limiting factor to obtain an efficient oncolytic virus through genetic engineering [155]. Directed evolution

brings an alternative tool without bias to any molecular mechanisms [232]. Therefore, it is also suggested that combination of directed evolution and genetic engineering approaches is more effective to obtain a potent, efficient and safe new oncolytic virus [233].

Within this scope, in this study it was aimed to improve viral fitness of miR-TS encoding CVB3 for primary resistant CRC cell line by using a directed evolution strategy. It was found that after five serial passaging of CVB3 in DLD1 cell line, which was relatively resistant to parental CVB3, cytolytic efficiency was enhanced significantly. The result was confirmed by cell killing assay, XTT assay and plaque assays. These experiments revealed that adapted CVB3 showed higher efficiency compare to the parental non-adapted virus. Even if there is no other study focused on increasing CVB3 oncolytic efficiency by using directed evolution strategy, there are many other studies which used this approach to change virus tropism. In several studies, it has been shown that CVB3 can adapt to new environment due to its high mutation rates, short life cycle and large population size. For instance, CVB3 Nancy was adapted to low CAR but high DAF expressing rhabdoymosarcoma cells. As a consequence CVB3 RD was generated which can use DAF to infect the cells without need to CAR [136]. The CVB3 strain PD, which was used in this thesis, was also generated by serial passaging of CVB3 P in HuFi H cell line. Consequently, CVB3 PD obtained a unique receptor tropism by capability of utilizing HS in addition to CAR and DAF [166]. More importantly it was observed that adaptation of virus to a specific cell line resulted in attenuation of its virulence which is a key desirable characteristic for oncolytic virotherapy. In this context, a study compared the virulence of six different CVB3 strains. Results revealed that CVB3 PD was the only strain which did not induce myocarditis or pancreatitis [130]. On the other hand, the most cardiotropic strains such as CVB3 31-1-93 and CVB3 H3 were generated by adaptation of CVB3 to heart tissue for investigation of virus induced myocarditis [128, 234]. In all these studies, it was underlined that the main genomic changes during CVB3 adaptation occurred on capsid protein region which indicates virus evolve to utilize available cell receptors more efficiently to infect the target host cells. A recent study by Borderia et al., further investigated the adaptive process of CVB3 adaptation to highly permissive and less permissive environment [126]. CVB3 (Nancy strain) was serially passaged to highly permissive HeLa cell and less permissive A549 cell line. Their findings indicated that

CVB3 adapted differently to two cell lines with respect to receptor and co-receptor availability. However, a dominant mutation was also observed which was not relate to any receptor footprint showed evolution is not only a receptor dependent process.

Replication rate of viruses is an accepted sign of viral fitness which means more rapidly replicating virus is assumed to have greater fitness compare to slower growing variants [235]. Virus infection of a cell monolayer results in plaque formation due to necrosis or apoptosis. Therefore, the size of plaque is assumed to be a representative measure of viral fitness [236]. Correspondingly, in the present study it also shown that the plaque size of adapted CVB3 was markedly larger than original virus. All results indicated that CVB3 evolved to infect DLD1 cell line more efficiently after five serial passages. Evaluation of genomic changes which resulted in increase of the viral fitness was not performed in this study. However, it is assumed that like other CVB3 adaptation studies, virus evolved to use available host receptors more efficiently [237]. In the first part of the study it was shown that DLD1 cells express abundant level of CAR and DAF on cell surface similar to highly permissive HeLa cell line. Nevertheless, as DLD1 cells display polarity when develop confluency in cell culture, the location of CAR is suggested to be on tight junction area of cellular membrane which is difficult for virus to reach and attach [238]. Indeed, many studies were performed to investigate the role of the different tight junction proteins on carcinogenesis by using DLD1 cell line [239, 240, 241]. Moreover, the used CVB3 for adaptation contains VP2-138D amino acid residue rather than DAF specific binding footprint VP2-138N, indicates that this clone had low DAF affinity [242]. Hence, presumably the virus evolved to bind more efficiently to DAF which mediates the transfer of CVB3 to the CAR sequestered in tight junction of the DLD1 cells [137, 243].

Adapted CVB3 contained six copies miR-690TS ($CVB3_{miR\text{-}690TS(6x)}$), therefore, genetic stability of miR-TS was evaluated following serial passaging. Interestingly, the first three miR-690TS copies were found to be mutated. It is assumed that two main reasons may cause to this observation. Firstly, existence of high population size with random mutations on target site sequence may resulted in complications to make precise genome sequencing via sanger method. To obtain more detailed and accurate results deep sequencing should be used to find out the real situation. Secondly, negative effect of foreign inserts on replication kinetics may induced deletion of miR-TS. CVB3 has been also investigated as vector for gene therapy [117]. For different reasons,

recombinant CVB3 clones expressing molecular markers, cytokines or proteins have been generated. The stability of many of these constructs have been proven, however loss of insertion can occur in virus population after serial passaging [139]. For instance, it was described that after several passaging of recombinant CVB3 in HeLa cells, an inserted eGFP cDNA was deleted [244]. It is suggested that loss of the insert come out depends on the passage number, the type of the gene, its nucleotide sequence, the length of the insert and the passage condition [139]. It is important to note that 3′ UTR secondary structures are involved in viral RNA replication and a disruption in this region may cause a stress to virus which result in deletion or mutation of foreign insert [245]. He et al. [212] showed that insertion of miR-TS at the end of 3′ UTR of the CVB3 genome was not tolerable even at the position where the foreign sequence could not disrupt the dominant hairpin structure. On the other hand, they found that insertion of TS for muscle specific miRNAs before KOZAK like sequence resulted in production of safe and stable recombinant CVB3. Of note, in present study, miR-690TS was inserted to the CVB3 genome right after the stop codon, however due to long insert size, the hairpin structure may be disturbed which resulted in the mutation of inserted sequence. Therefore, finding a more stable site for TS insertion may prevent the unintended loss of miR-TS sequence even after several passages. For example, Zeng et al. [246] found that insertion of GFP at the junction between VP1 and 2A genes significantly increased the functional stability. Animal experiments showed that the expression was observed in the heart and pancreas of the infected mice 6 days post infection. More importantly, GFP expression was observed even after ten passages in cell culture. According to study, which was mentioned above, when the GFP was inserted in the VP4 region following the start codon, genomic deletions started to be detected after 2 passages in cell culture [244]. Different insertion sites for miR-TS can be used to obtain more stable recombinant virus during adaptation process. Therefore, further experiments need to be performed to find an optimal site for TS insertion without distraction of viral translation. It is important to note that sequencing results of the adapted the $CVB3_{miR\text{-}690TS(6x)}$ genome revealed that the mutations occurred at the first three TS, however last miR-TS was still stable. Therefore, insertion of several repeats of desired miR-TS may help to overcome this handicap as it was shown in this study, there was no significant differences in downregulation level of $CVB3_{miR\text{-}690TS(6x)}$ over $CVB3_{miR\text{-}690TS(3x)}$.

In summary, in this study it was aimed to investigate oncolytic efficiency of different CVB3 variants against human CRC which is one of the most death causing cancer, eliminating the possible side effect of the CVB3 and finally enhancing the viral fitness by adapting virus to resistant tumors for a personalized therapy. For these purposes, firstly, different CVB3 variants were evaluated based on their different receptor tropism, *in vitro* and *in vivo*. There are limited number of wild-type oncolytic RNA viruses under clinical trials but they are considered to be the most potent candidates. Natural tumor selectivity, fast replication cycles, production of large number of progeny, small size for more efficient infusion to solid tumors are important advantages of RNA viruses. In this study, heparan sulfate binding CVB3 strain PD was demonstrated as a safe and potent oncolytic virus. Specific receptor tropism and non-toxic tumor selective feature of the virus, even in athymic mice, underline the potential of PD as an oncolytic agent. However, it is very inevitable to analyse the oncolytic capacity of PD in the immunocompetent animal models. Particularly, during last years "virotherapy" is also preferred to be named as "immunovirotherapy" due to direct effect of oncolytic viruses on awakening of immune cells against tumor. Other evaluated CVB3 strains induced strong side effects on animals and only one PD treated mouse showed systemic viral infection. Therefore, parallel to investigation of oncolytic efficiency of different CVB3 strains, to further enhance the safety profile, one of the most recent and effective approach, miRNA mediated detargeting strategy was adapted to CVB3. Highly cardiotropic CVB3 strain was attenuated significantly by incorporating the pancreas specific miR-TS into the virus genome. Importantly, all treated animals were protected from side effects without perturbing the oncolytic activity of the engineered virus. These promising findings with highly virulent strain give a strong opinion about the eligibility of this approach to further increase the tumor selectivity when needed. Finally, one of the main limiting criteria, not only for oncolytic virotherapy but also for all other cancer therapy methods, was aimed to be achieved by adaptation strategy. Many methods have been developed to inhibit the resistance of the cancers against therapeutic agents. CVB3 was adapted to resistant tumor cells by several passing which consequently, resulted in significant enhancement of viral fitness. New adapted virus was able the lyse the cancer cells that the parental virus not able to infect. Even if these *in vitro* results are promising, many steps are needed to be taken for deeper understanding of eligibility of this approach in the clinic. Especially, recent developments in 3D cell culture and stem cell organoid engineering may help to adapt

the viruses to more realistic tumor environments. Although in vitro passaged viruses are considered as 1st generation oncolytic viruses and the focus of the scientists has shifted to the 2nd (genetically engineered) and 3rd generation (transgene armed) oncolytic viruses, adaptation strategy can be combined with these new technologies. In this respect, this study presents a combination of two approaches from past to present.

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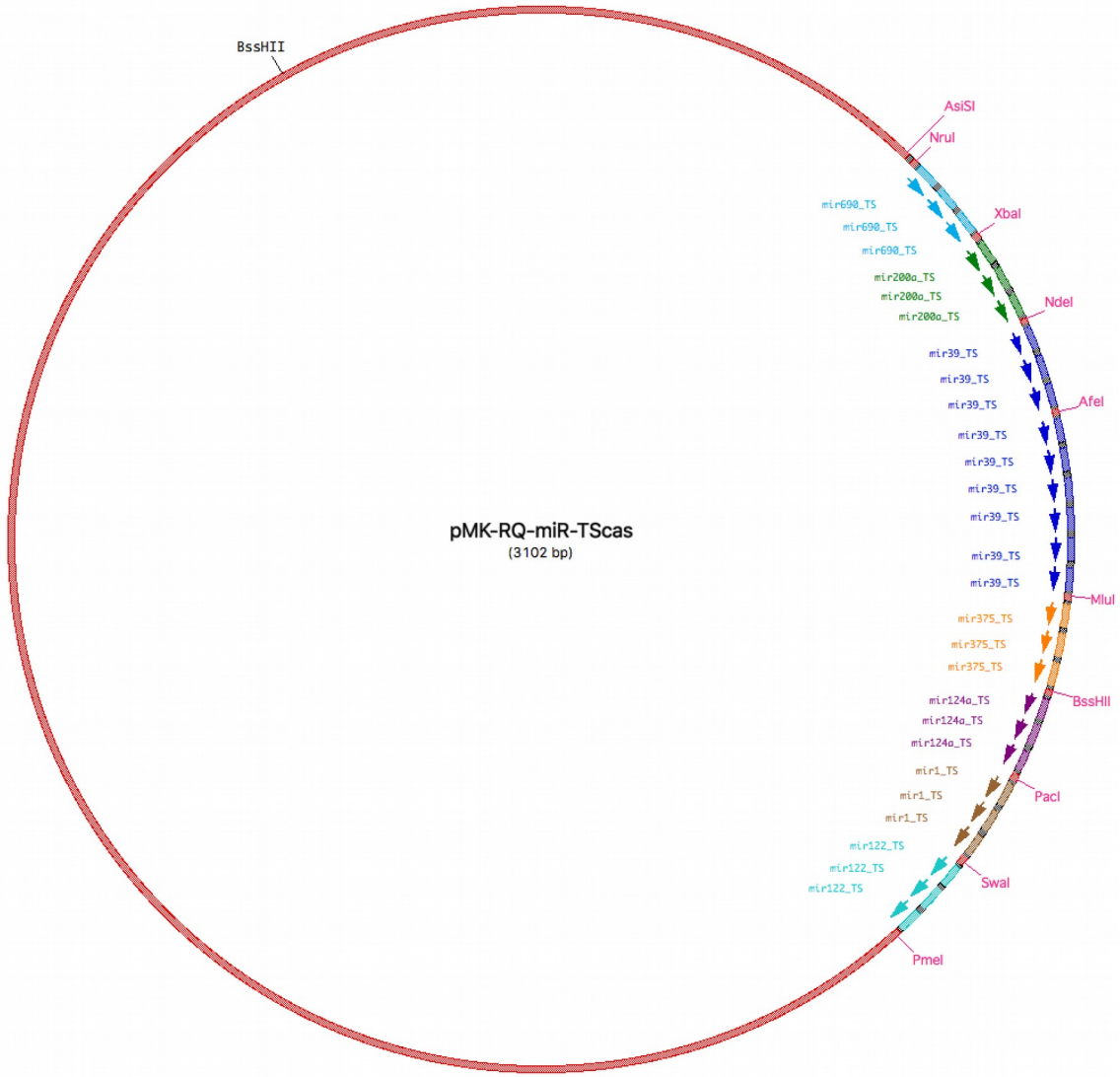
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mir39_TS
MluI
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mir375_TS
mir375_TS
BssHII
mir124a_TS
mir124a_TS
mir124a_TS
mir1_TS
mir1_TS
mir1_TS
PacI
mir122_TS
mir122_TS
mir122_TS
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PmeI
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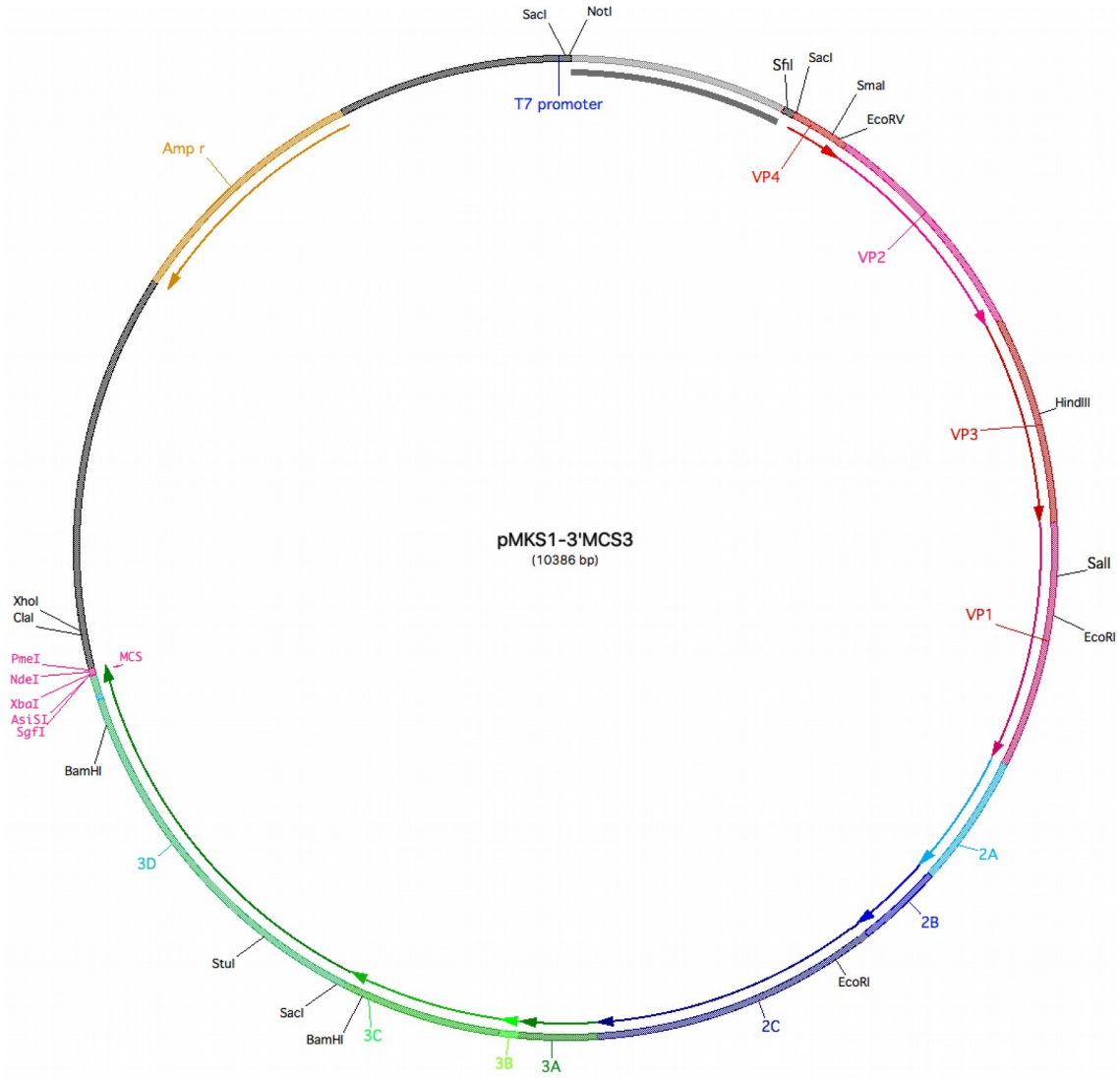
(3102 bp)

Appendix 1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SmaI
EcoRV
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VP2
HindIII
VP3
SalI
VP1
EcoRI
2A
2B
EcoRI
2C
3A
3B
3C
BamHI
SacI
StuI
3D
BamHI
SgfI
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PmeI
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ClaI
XhoI
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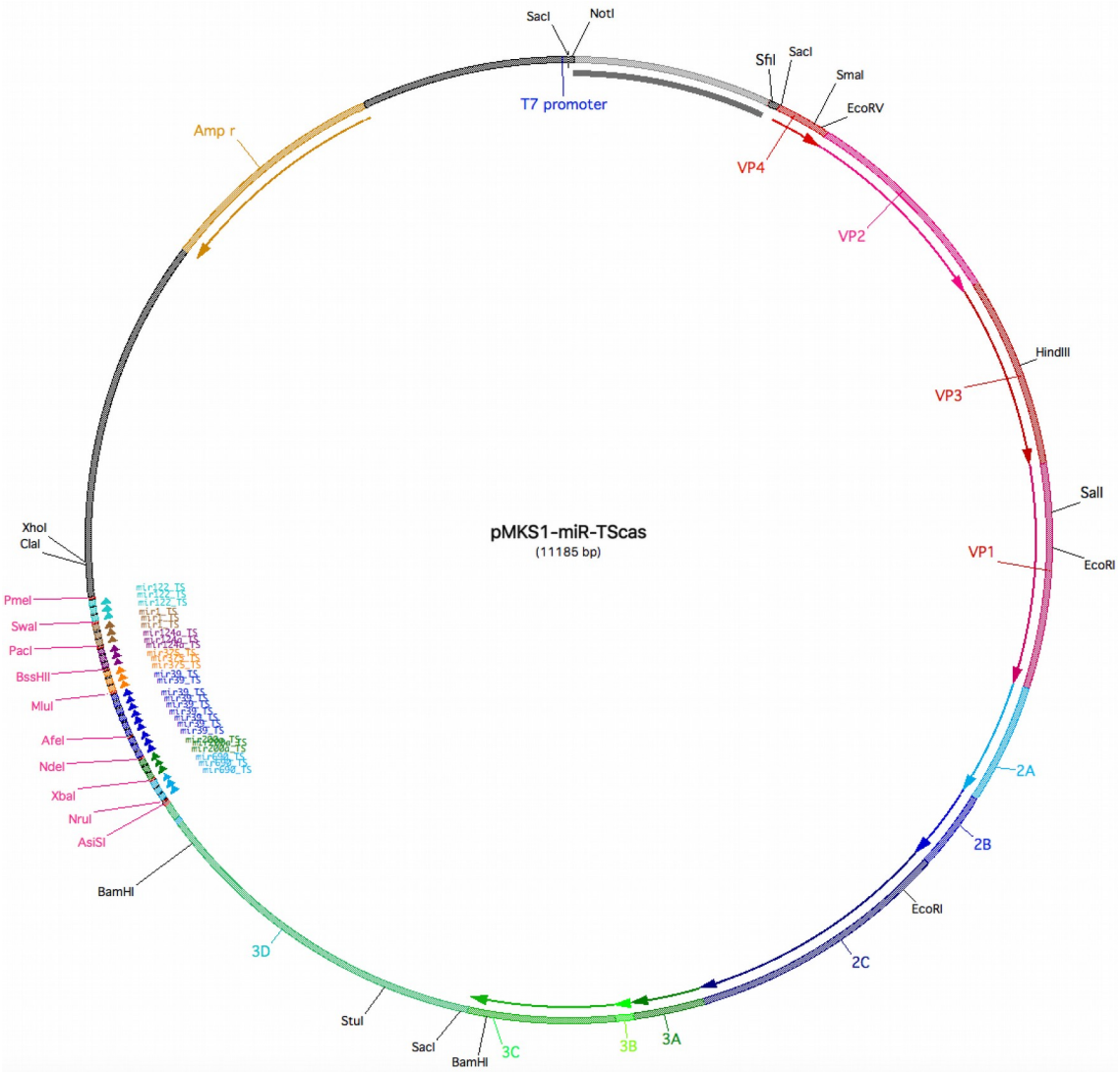
(10386 bp)

Appendix 2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HindIII
VP3
SalI
VP1
EcoRI
2A
2B
EcoRI
2C
3A
3B
3C
BamHI
SacI
StuI
3D
BamHI
AsiSI
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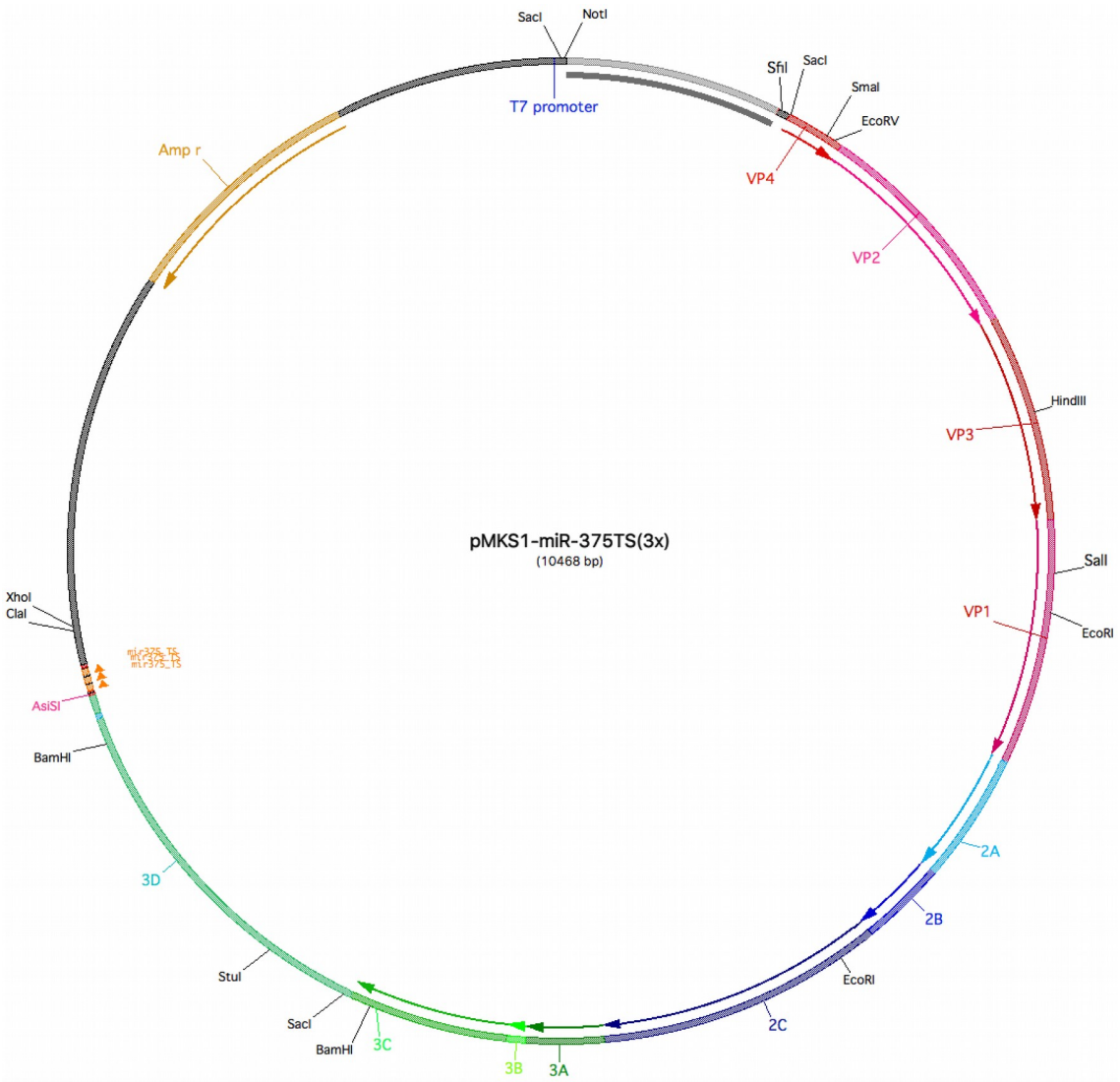
PmeI
XhoI
ClaI
Amp r

Appendix 3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SalI
VP1
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2A
2B
EcoRI
2C
3A
3B
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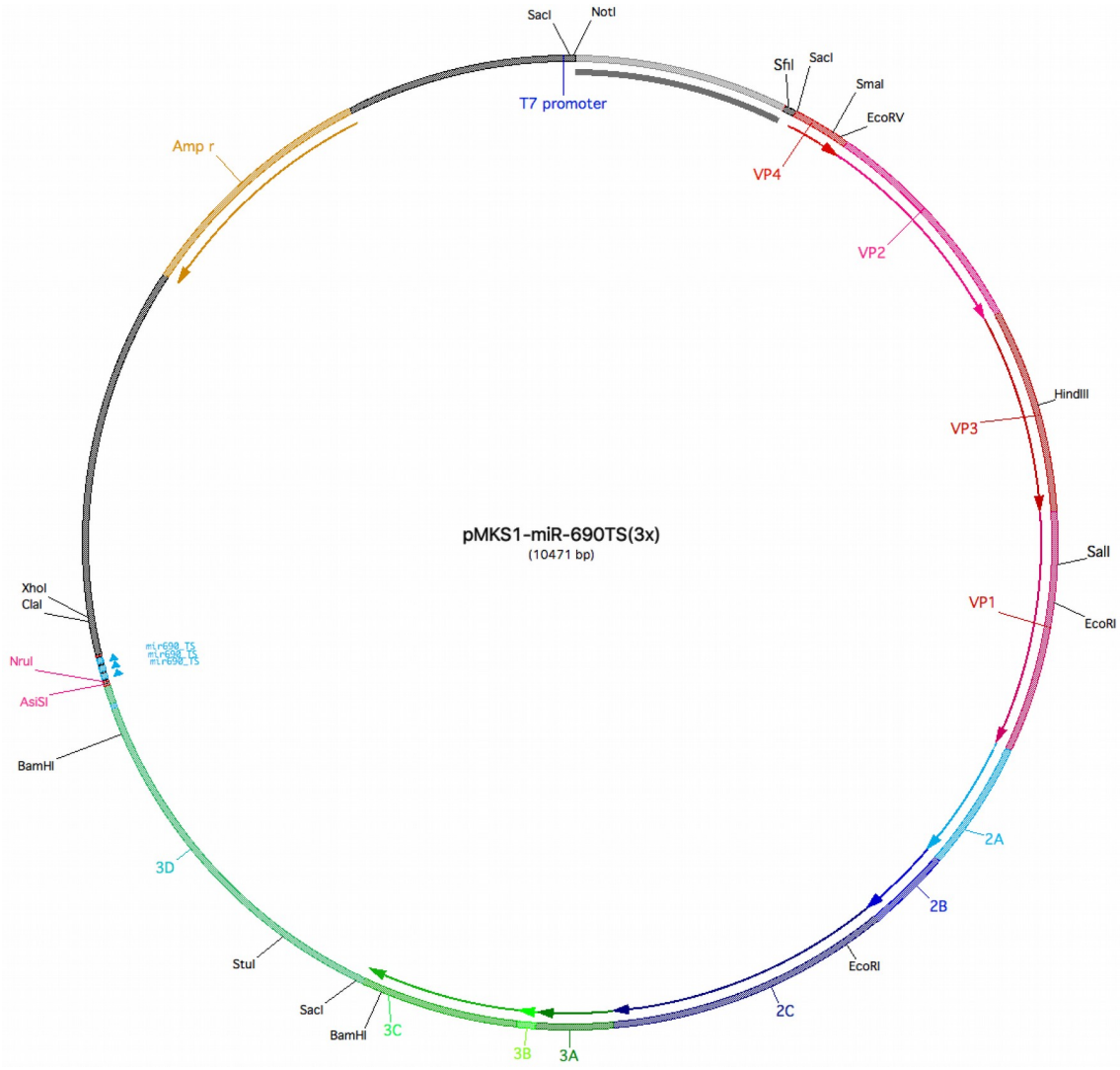
3D
AsiSI
BamHI
XhoI
ClaI
Amp r

Appendix 4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VP2
HindIII
VP3
SalI
VP1
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2A
2B
EcoRI
2C
3A
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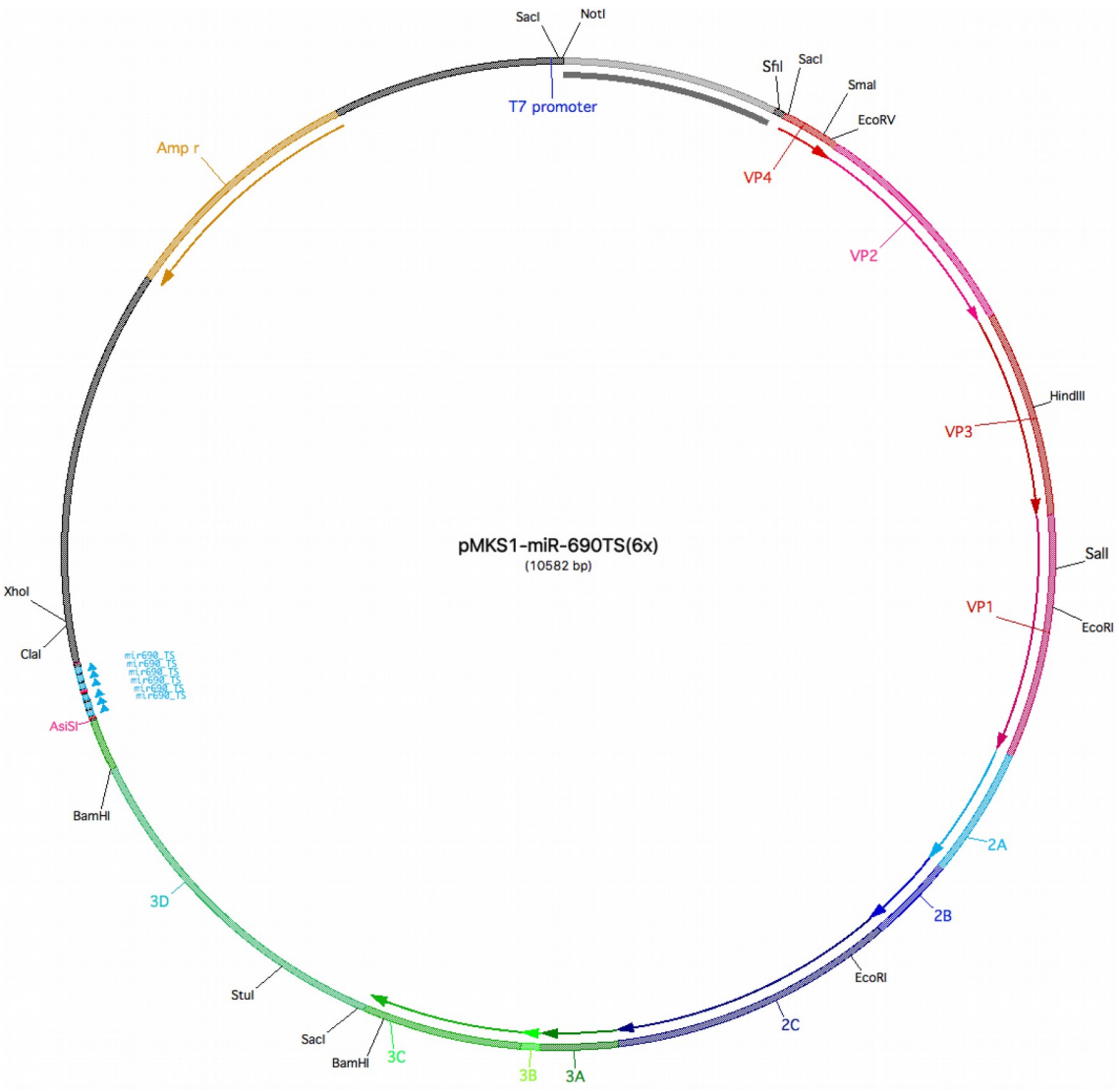
NruI
XhoI
ClaI
Amp r

Appendix 5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VP2
HindIII
VP3
SalI
VP1
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2A
2B
EcoRI
2C
3A
3B
3C
SacI
BamHI
StuI
3D
BamHI
AsiSI
ClaI
XhoI
Amp r
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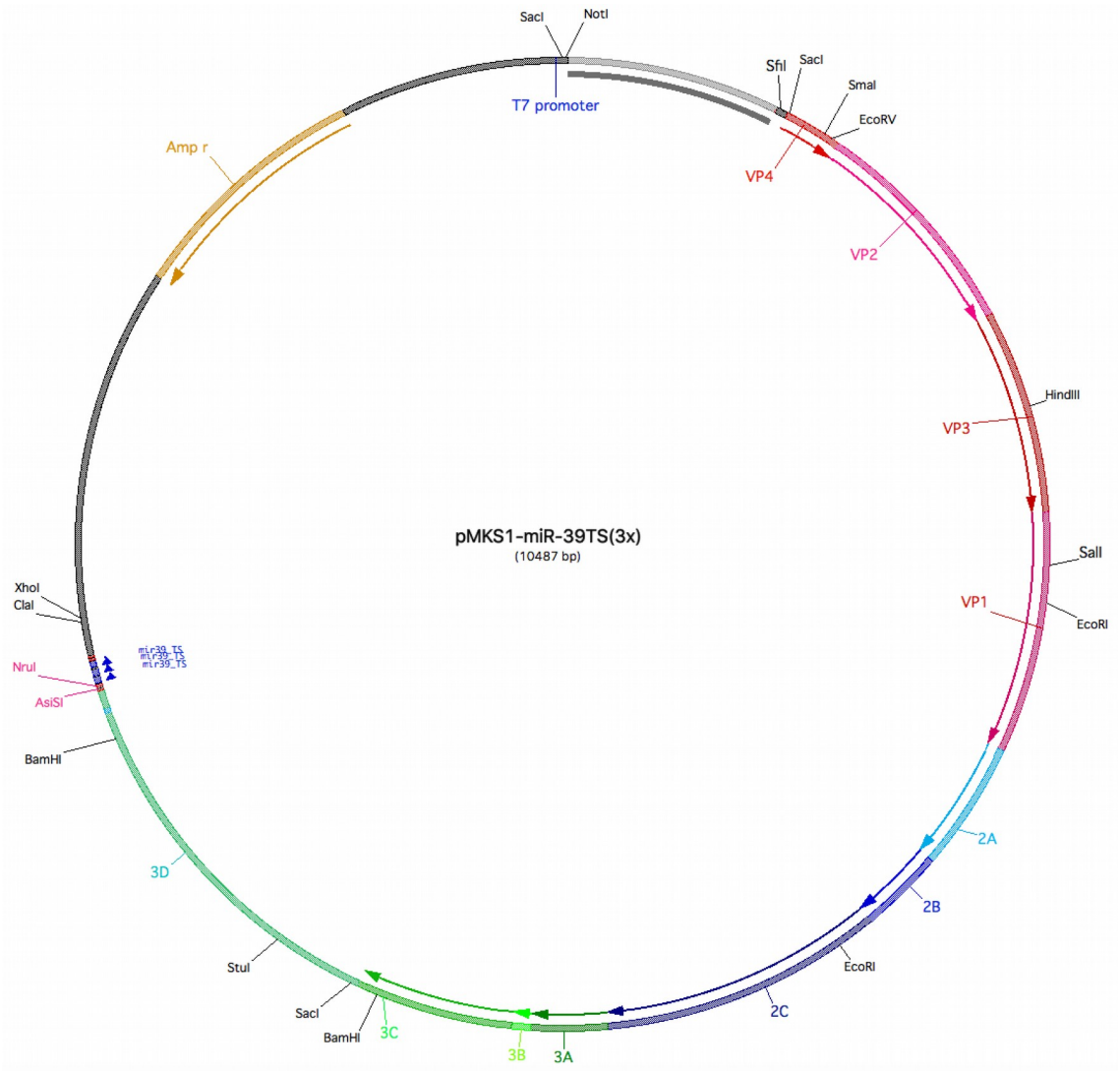
(10582 bp)

Appendix 6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VP3
SalI
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2B
EcoRI
2C
3A
3B
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3D
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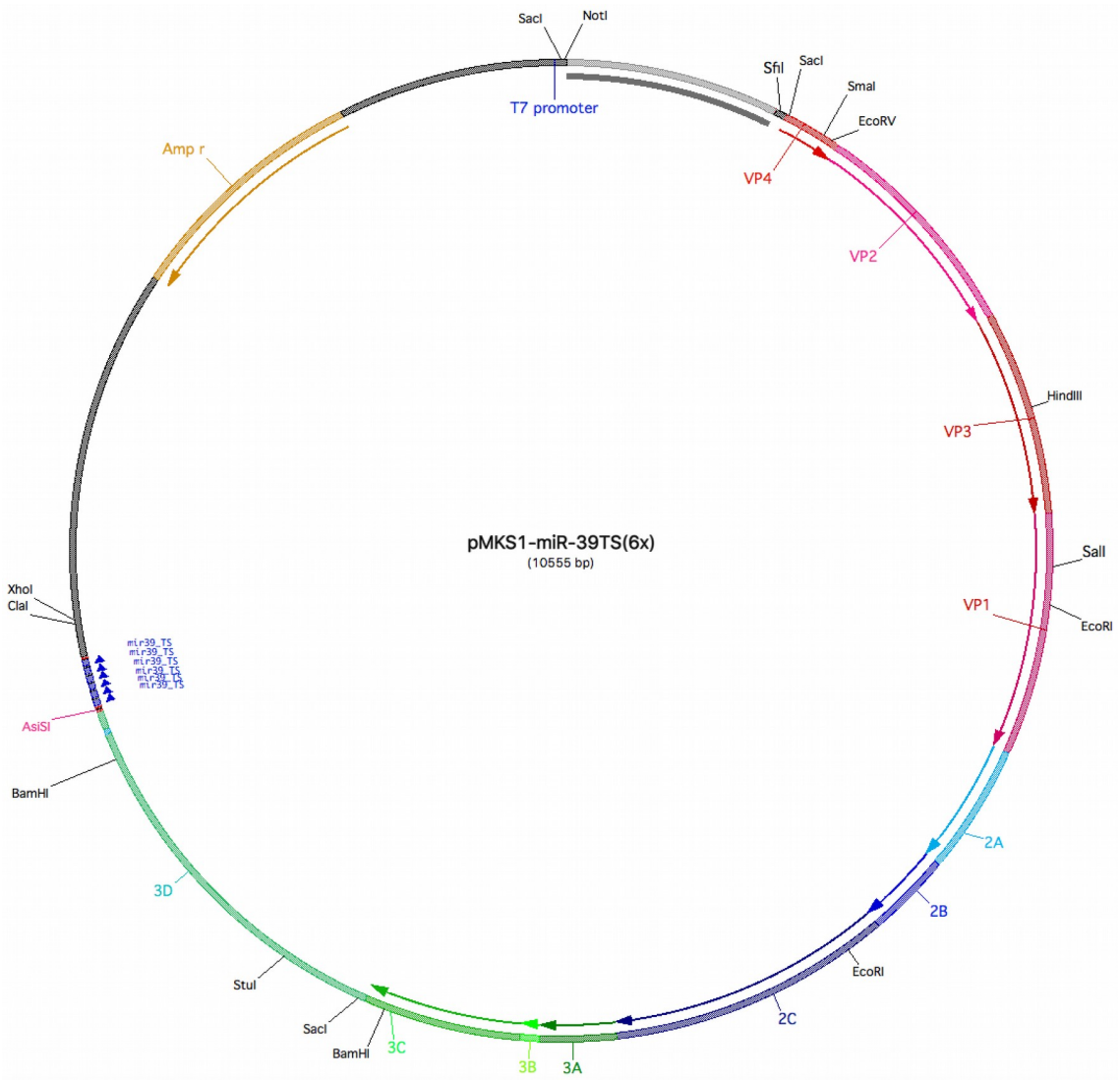
AsiSI
NruI
XhoI
ClaI
Amp r

Appendix 7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VP2
HindIII
VP3
SalI
VP1
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2A
2B
EcoRI
2C
3A
3B
3C
BamHI
SacI
StuI
3D
BamHI
AsiSI
XhoI
ClaI
Amp r
pMKS1-miR-39TS(6x)
(10555 bp)

Appendix 8

PUBLICATIONS

[1] **Hazini, A**. Pryshliak, M. Bruckner, V. Klingel, K. Sauter, M. Pinkert, S. Kurreck, J. and Fechner, H., (2018). "Heparan Sulfate Binding Coxsackievirus B3 Strain PD: A Novel Avirulent Oncolytic Agent Against Human Colorectal Carcinoma", Hum Gene Ther. https://doi.org/10.1089/hum.2018.036

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