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born in: Landau, Germany
Oral-examination:

**Specific transfer of oncolytic adenoviruses
by mesenchymal stem cells for the
elimination of pancreatic tumour stem
cells**

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DECLARATION BY THE CANDIDATE

I hereby declare that this thesis is my own work and effort.
Where other sources of information have been used,
they have been indicated or acknowledged

Signature:

Date:

To my parents

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Abstract

Pancreatic adenocarcinoma has a very poor prognosis with currently existing therapies prolonging patient life for only a few weeks. Therefore novel therapy options are urgently needed. Present theories maintain that only a small fraction of tumour cells (the cancer stem cells (CSC)) are responsible for the highly aggressive behaviour of pancreatic cancer. These cells show a stem cell like phenotype and a high resistance to chemotherapy.

Oncolytic viruses are promising candidates for therapeutic agents. Besides being replicated inside of host cells they can be attenuated to malignant cells and armed with therapeutic genes that will be translated by infected cells. The viruses used in this project were provided by our cooperation partner Dr Nettelbeck. The group of Dr Nettelbeck examined infection parameters and oncolytic activity of the viruses and optimized them for replication and release in mesenchymal stem cells (MSC). Additionally I could demonstrate an efficient elimination of pancreatic CSC *in vitro*.

A major issue limiting the efficiency of virus therapies so far is their delivery. Systemic injected viruses are cleared from the blood by the liver and inactivated by the immune system. To overcome this disadvantage MSC isolated from the bone marrow were used in the present project to enhance delivery and shield the viruses from the host's immune system. MSC exhibit a strong homing ability towards tumour tissue. Migration assays *in vitro* ascertained that homing is still present after infection with the oncolytic adenoviruses.

As a model for *in vivo* experiments xenografts transplanted to fertilized chicken eggs were used which grow as stroma-enriched tumours. The invasion of tumour transplants *in vivo* was successfully demonstrated after injection of infected MSC into blood vessels. In this model I could show that infection with an oncolytic adenovirus markedly reduced tumour growth. The infected tumours exhibited a strong cytopathic effect with altered morphology.

The potency of tumour growth reduction strongly depended on the applied adenovirus after injection of the infected MSC. Viruses with enhanced lytic or anti-tumourigenic activity showed a superior performance, while an unmodified virus did not reduce tumour growth. The strongest anti-tumourigenic effect was found for a TRAIL expressing virus. This was confirmed by a reduction of proliferation and CSC marker expression and elevation of apoptosis. Therefore, the application of oncolytic

adenoviruses using MSC as cell carriers seems to be a promising strategy in combating pancreatic cancer, especially when viruses with enhanced anti-tumour effects are used.

Zusammenfassung

Das Adenokarzinom der Bauchspeicheldrüse weist eine sehr schlechte Prognose auf. Eine Chemotherapie verlängert das Überleben der Patienten meist nur um wenige Wochen. Daher besteht eine hohe Priorität in der Entwicklung neuer, effektiver Wirkstoffe. Laut neuer Theorien ist vor allem ein kleiner Teil der Tumorzellen, die sog. Krebsstammzellen (CSC) für Therapieresistenz und Rezivität verantwortlich. Diese Zellen weisen einen stammzellähnlichen Phänotyp auf und werden nur schlecht von herkömmlichen Therapien bekämpft.

Ein vielversprechender neuer Wirkstoff sind onkolytische Adenoviren. Diese können modifiziert werden, um maligne Zellen effizient zu infizieren und zu eliminieren. Außerdem können Gene für andere Wirkstoffe in die Viren eingefügt werden und ihre Wirksamkeit so weiter gesteigert werden. Die in dieser Arbeit verwendeten Viren wurden von der Gruppe von Dr. Nettelbeck konstruiert. Diese Arbeit wurde in Kooperation mit der AG Nettelbeck durchgeführt. Seine Gruppe untersuchte die Infektionseffizienz und onkolytische Aktivität in unterschiedlichen Zelllinien und optimierte die Viren für Replikation und Freisetzung in mesenchymalen Stammzellen (MSC). Darüberhinaus konnte ich eine effiziente Eliminierung pankreatischer CSC *in vitro* zeigen.

Ein limitierender Faktor bei Virustherapien ist der Transport der Viren. Diese werden in der Leber abgebaut und vom Immunsystem inaktiviert. In dieser Arbeit wurde die Effizienz des Transports durch den Einsatz von MSC als Virusträger untersucht. Diese Zellen, die aus dem Knochenmark isoliert werden, wandern in Tumorgewebe ein, wie in mehreren Publikationen gezeigt wurde. Mittels eines Migrationsassays wurde hierbei sichergestellt, dass eine Infektion die Invasionsfähigkeit der MSC nicht beeinträchtigt.

Darüberhinaus konnte die Einwanderung virusinfizierter MSC *in vivo* gezeigt werden. Hierbei wurden Tumorzelltransplantate auf Hühnerbruteiern als *in vivo* Model verwendet. Die wachsenden Tumore werden von Hühnerblutgefäßen durchwachsen und entwickeln Tumore, deren Morphologie dem von Patiententumoren ähnelt. Eine Infektion durch einen onkolytischen Adenovirus verringerte in diesem Model das Tumorwachstum beträchtlich. Die infizierten Tumore zeigten darüberhinaus eine stark veränderte Morphologie.

Bei Injektion infizierter MSC, in zum Tumor führende Blutgefäße, zeigten verschiedene Adenoviren unterschiedliche Wirkungen. Während ein Virus ohne erhöhte anti-tumorigene Wirkung unwirksam war, reduzierten Viren mit erhöhten anti-tumor Eigenschaften das Wachstum der Tumore. Insbesondere ein TRAIL exprimierender Virus erwies sich als effektiv. Diese Ergebnisse zeigen, dass mittels MSC transportierte onkolytische Adenoviren vielversprechende Wirkstoffe einer neuen Therapie des Bauchspeicheldrüsenkrebses darstellen.

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1. Abbreviations

5-FU	5-fluorouracil
ABC	ATP-binding cassette transporters
AEC	Sodium POE 10 fatty alcohol ether carboxylate
Ad	Adenovirus
ALDH	Aldehyde dehydrogenase
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
CAM	Chorioallantoic membrane
CAR	Coxsackie-Adenovirus receptor
CD	Cluster of differentiation
c-Met	Met proto-oncogene (hepatocyte growth factor receptor)
CNS	Central nervous system
CSC	Cancer stem cell
CXCR	CXC chemokine receptor
DMEM	Dulbecco's modified eagle medium
DNA	Desoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
DMSO	Dimethylsulphoxid
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EMT	Epithelial-mesenchymal transition
ESA	Epithelial cell adhesion molecule
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
GFP	Green fluorescent protein
HEPES	2-hydroxyethyl-piperazinyl-2-ethansulfonicacid
IFN- β	Interferon type I
IgG	Immunoglobulin G
K-FSM	Keratinocyte-serum free medium

Ki67	Antigen identified by monoclonal antibody Ki-67
MDR	Multidrug resistance
MSC	Mesenchymal stem cells
NBT	Nitro blue tetrazolium chloride
PBS	Phosphate buffered saline
PDAC	Pancreatic ductal adenocarcinoma
PFA	Paraformaldehyde
RT	Room temperature
SD	Standard deviation
TBS	Tris-buffered saline
TCID ₅₀	50% Tissue Culture Infective Dose
TRAIL	TNF-related apoptosis-inducing ligand

Units

Å	angstrom
°C	degree Celsius
d	days
g	gram
g	acceleration relative to free-fall (standard gravity)
h	hours
IU	international unit
kB	kilo-base pair
l	litre
m	metre
M	Molar concentration (molarity)
min	minutes
rpm	rounds per minute
s	seconds
U	enzyme unit

2. Introduction

2.1 Pancreatic cancer

While recent decades have seen an improvement in the treatment and survival rates of many cancer varieties this is not true for pancreatic adenocarcinoma, the most common malignancy of the pancreas. The overall 5-year survival rate for this malignant disease is less than 4% and treatment with the most common chemotherapy agent gemcitabine results in only a moderate improvement of survival [1]. The growth of pancreatic cancer is asymptomatic until late stages. Thus the disease is often diagnosed in an advanced state when metastases are already present in most cases. Therefore surgery, which is the only curative option available, is not possible in a great majority of patients [2]. Even with surgical removing of the tumour the 5-year survival rate is only 20-25% with recurrence being common [3]. Although a few risk factors for pancreatic cancer have been identified, data remains inconclusive in many cases [4]. The best-established risk factor remains tobacco smoking [5], which increases risk for pancreatic cancer significantly, even long after quitting [6]. Another known risk factor is the presence of chronic inflammatory pancreatitis [7].

In all cases when the tumour is inoperable or recurrent only palliative therapies exist at the moment. Although the most popular chemotherapy agent gemcitabine offers a significant increase in survival, the overall survival remains under one year [8]. This remains true even when gemcitabine is combined with other agents like Nab-paclitaxel or oxaliplatin. These combined therapies show only slightly improved performance and due to stronger side effects can only be used with patients in good physical condition. Additionally response rates remain poor for all therapies used. Drug delivery to pancreatic tumours has been demonstrated to be low. PDAC contains extensive stromal tissue that is poorly vascularized and perfused shielding the tumour from harmful compounds [9]. The therapy protocol with the biggest improvement in overall survival, the FOLFIRINOX regimen consisting of a combination of three agents shows strong side effects limiting patient quality of life and its usefulness [10].

With the poor performance of today's therapies the development of novel effective therapy agents remains an important task.

2.2 Pancreatic cancer stem cells

The high presence of cancer stem cells (CSC) in pancreatic adenocarcinoma could be a major reason for the high resistance to conventional therapies this malignancy exhibits. CSC were identified in various tumour types including CNS, breast, prostate, melanoma and pancreas [11]. Solid tumours are heterogeneous, with many different kinds of cells present in the tumour tissue. The great bulk of malignant cells in a tumour are non-CSC cells. The CSC constitute only a small fraction of tumour cells. However these cells exhibit a much stronger tumour initiating potential than the more differentiated tumour cells. They have a high self-renewal capacity and are thought to be mostly responsible for tumour progression and metastasis [12, 13].

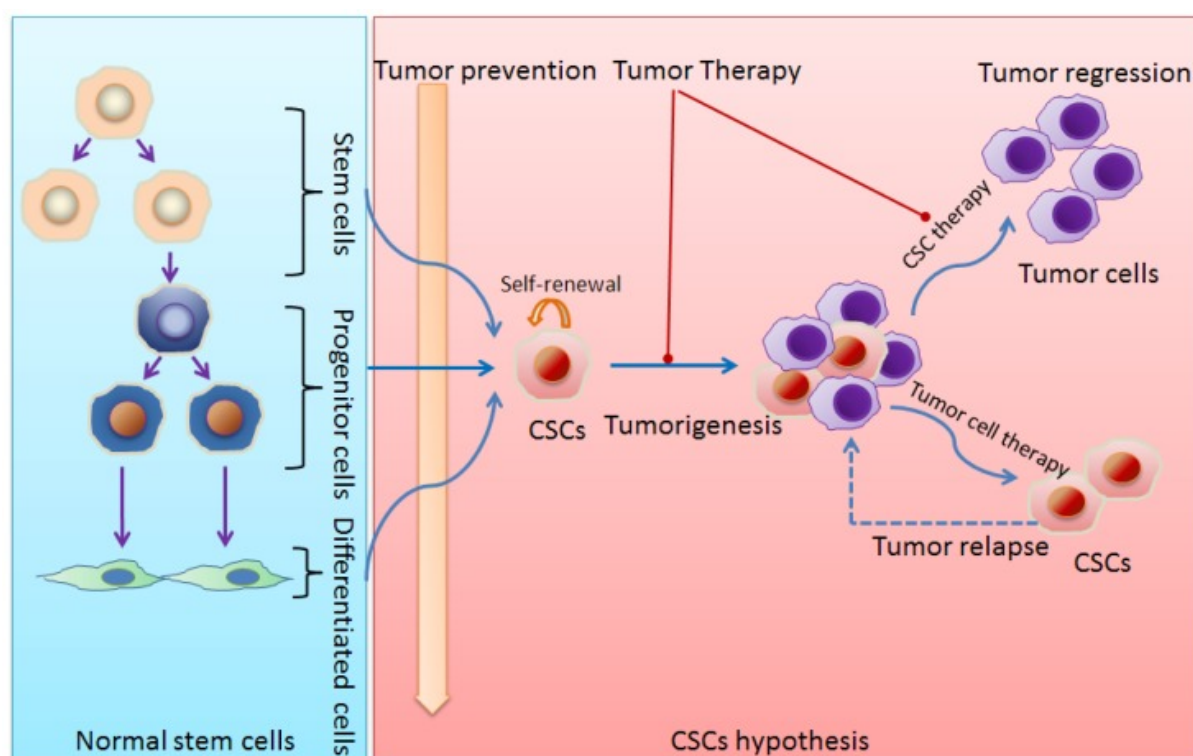


Figure 1: Role of CSC in tumorigenesis and cancer therapy (adapted from [14])

Besides their tumour-initiating abilities CSC are also highly resistant towards chemotherapy [15]. They express a high level of genes coding for ABC transporters and ALDH enzymes. This enables them to expel and inactivate cytotoxic substances [16]. This characteristic is shared with normal stem cells. Further characteristics

shared with stem cell include the expression of many stem cell markers. Many of these marker genes are engaged in self-renewal. CSC self-renewal and their uncommitted state are maintained by signal pathways also involved in maintenance and regulation of normal stem cells like the Wnt/ β -catenin, Hedgehog or Notch pathways [17, 18]. Like in the case of stem cells a specific microenvironment is involved in the regulation of CSC behaviour. Signals from nearby non-malignant stromal cells can support either an uncommitted state or differentiation of the CSC, thus forming a specific CSC niche [19]. The origin of CSC is debated. Theories include development from aberrant normal stem cells or from differentiated cells through EMT processes [20, 21]. Both mechanisms are likely to be occurring in different tumours. The acquisition of CSC characteristics by differentiated tumour cells under certain circumstances was demonstrated [22]. The varying origins of CSC show that this cell population is not entirely homogenous in itself. In accordance to this markers for CSC differ between tumour types and even between CSC subpopulations in one tumour. The data for the relevance of many markers are therefore controversial [23]. The most commonly used markers for pancreatic CSC are CD24 and CD44. CSC are hereby defined as cells exhibiting a CD24⁺/CD44⁺/ESA⁺ phenotype [24]. Other markers used for identification of pancreatic CSC include ALDH-1 activity, or the expression of nestin, CD133 or CXCR-4 [25, 26]. A recently discovered marker for pancreatic CSC is the tyrosine kinase receptor c-Met [27]. Cells exhibiting high levels of c-Met expression showed also strong tumorigenic potential. Moreover a high c-Met expression in PDAC is correlated with a poor prognosis and with invasion [28].

2.3 Oncolytic viruses in cancer therapy

Viruses infect and destroy animal and human cells with great efficiency. This ability can be exploited for the elimination of malignant cells in patients. Although an anti-tumour effect of certain virus strains was observed a century ago efforts to use viruses for cancer therapy met with poor success [29]. The therapies showed low efficiency and risked severe side effects. But with the advent of genetic manipulation capabilities it is now possible to create viruses optimized for infection and lysis of tumour cells.

Currently viruses from many different strains are used for establishing virotherapies, some of which have proceeded to clinical trials. Viruses employed include

adenovirus, herpes virus, parvovirus and others [30]. One of the most commonly applied viruses is the herpes simplex virus. It has been used in clinical trials for different tumours, e.g. melanoma or glioma [31]. In a trial with metastatic melanoma complete regression was observed in a few cases. Another commonly used virus is the measles virus from the family *Paramyxoviridae*. It shows natural oncolytic activity and targets preferentially malignant cells [32]. In contrast other strains utilized in virotherapy like adenovirus or herpes virus do not exhibit a preference for cancer cells and show no natural oncolytic activity. However, they can be attenuated to malignant cells. One potential method is to modify the virus to recognize and attach to a different receptor protein for cell entry. Proteins that are expressed preferentially by malignant cells are excellent candidates for such modifications. Other possibilities are regulation of virus replication by promoters only being active in malignant cells or modification of immune-evasion proteins [33]. Such modifications restrict efficient replication and/or virus entry to transformed cells and protect normal cells. Besides targeting cancer cells their efficient elimination by the oncolytic virus is crucial for therapy response. One problem in the development of oncolytic viruses described is the narrow species range of the viruses, making comparison between animal models and patients difficult. So far therapy results in clinical trials have been promising, although efficacy has still to be increased for clinical use [34]. The studies demonstrated also the safety of virotherapy as only minor toxicity from the used virus strains has been reported. To enhance antitumor efficiency armed viruses have been developed. In this viruses additional therapeutic genes have been cloned which are expressed in host cells and released upon cell lysis. With such a Bystander-effect also cells not directly infected can be eliminated. Additionally genes affecting the tumour microenvironment like proteases can be used [35]. With this enhanced virus spread or a modified tumour angiogenesis can be achieved. Besides the direct killing of malignant cells oncolytic viruses induce an immune response against the tumour tissue, making them also promising agents for cancer immunotherapy and adding a further target for enhancing antitumor activity [36]. A protein used for arming oncolytic viruses is the tumour necrosis factor (TNF)-related apoptosis-inducing ligand TRAIL. TRAIL activates death receptors and induces apoptosis in malignant cells due to activation of the extrinsic apoptotic pathway. While targeting malignant cells it shows no significant cytotoxicity in normal cells [37]. Oncolytic adenovirus constructs

expressing TRAIL showed enhanced anti-tumour activity in malignant glioma and hepatoma when compared to the unarmed virus [38, 39]

A modified adenovirus is frequently employed as the oncolytic agent in virotherapies. There have been clinical trials for different malignancies utilizing this virus [40-42]. Adenovirus causes most often infections of the respiratory tract, most commonly in children. Infections for the most part produce symptoms similar to the common cold, although symptoms of adenovirus infection can vary. More severe cases have been described, especially in people with suppressed immune system like organ transplant recipients [43]. Adenovirus is a member of the family *Adenoviridae*. It is a highly variable virus including over 60 described serotypes in humans divided among seven species (named A – G) [44]. The most therapeutically applied serotype is Ad serotype 5 (Ad5), which is used almost exclusively for virotherapy [45]. Adenoviruses are large (ca. 950 Å) non-enveloped viruses. Their genome consists of dsDNA. It is contained in an icosahedral protein capsid. The capsid consists of two different subunits: hexons and pentons. The pentons form the vertices and are the basis of the fibre domains, which comprise a shaft ending in a knob-domain [46].

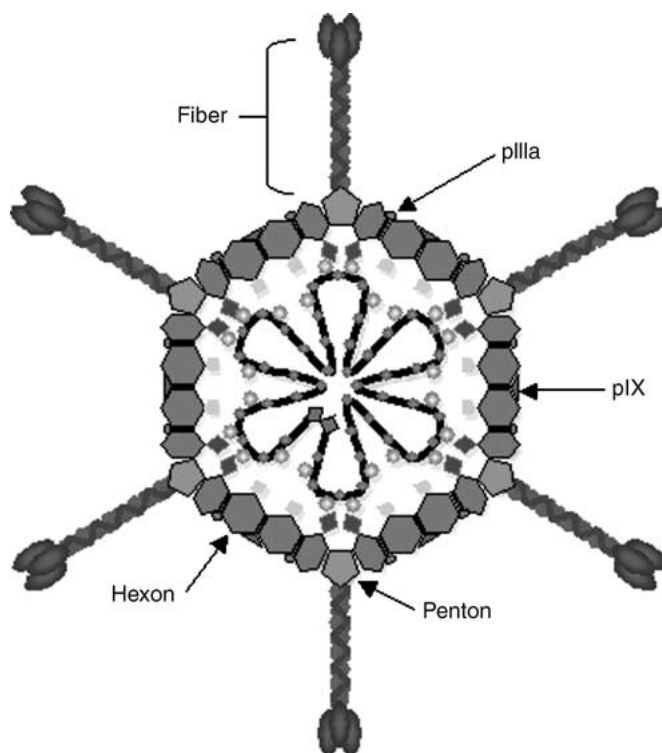


Figure 2: Structure of an adenovirus (adapted from [47])

The fibre knob domain is highly involved in adhesion to host cells. In most Ad varieties it binds with strong affinity to the Coxsackie Adenovirus Receptor (CAR) initializing virus entry. Exceptions are members of adenovirus species B that use

CD46 for binding to cells. CD46 is a receptor present on the surface of all nucleated cells, shielding them from autologous immune reactions. Other known molecules used by some Ad serotypes alternatively for cell entry include integrins, sialic acid and desmoglein 2 [48]. Unfortunately, CAR is down regulated in many tumours reducing Ad affinity. CAR expression however varies strongly between different tumour types [49]. The loss of CAR expression and alternations in its subcellular distribution have been correlated with tumour progression in some cancers, e.g. in colon cancer [50]. While absence of CAR limits infection this could be overcome in CAR deficient melanoma cells by switching the fibre domain of Ad5 with that of Ad3. As a member of species B Ad3 does not use CAR for adhesion. The chimeric Ad showed efficient infection and oncolysis of the melanoma cells independent of CAR [51].

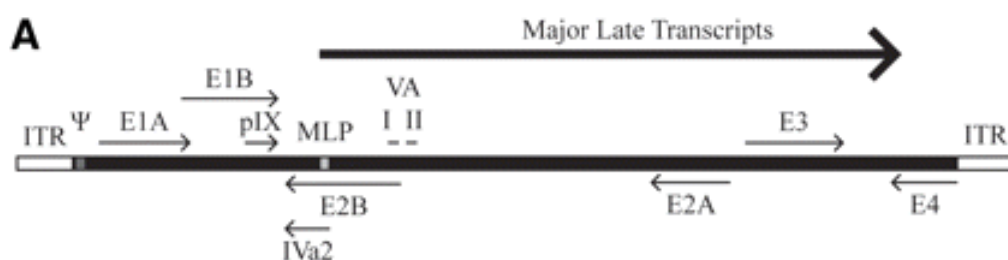


Figure 3: Simplified Ad5 genome (adapted from [52])

The adenoviral genome is about 30 – 40 kB long and about 36kB in Ad5. It codes for approximately 39 genes. The genes can be divided into early genes and late genes, depending on the time point of their expression. The early genes are expressed before DNA replication and are responsible for modifying the host cell, activating other virus genes or evading immune response. The late genes encode mostly for virion proteins and are expressed after DNA replication [52]. An example of host cell modification is repression of p53 activity by a product of early gene region E1B, the E1B55K protein. It also plays a role in protection of viral replication from inhibition by interferon type1 [53]. Apoptosis is prevented by another product of this early gene, E1B19K, in infected cells [54]. Another proteins, the E1A proteins, deregulate the cell cycle by interaction with Retinoblastoma protein and its target transcription factor [55]. They induce DNA replication in quiescent cells with strongly altered replication kinetics [56]. Modification of early genes offers another path of virus attenuation. An oncolytic adenovirus with mutations in the E1A region and in E1B19K has been

shown to efficiently replicate and eliminate several malignant cell types [57]. In contrast its replication in non-malignant cells was strongly reduced. As cell cycle and apoptosis pathways are deregulated in malignant cells viral proteins acting on these pathways in normal cells are often redundant in this case.

In addition to the problems mentioned above which reduce the efficiency of oncoviral therapies another obstacle which has to be overcome is an inefficient delivery of the viruses. Systemic application of oncolytic viruses results in only a small fraction of them reaching the tumour [58]. Circulating viruses are attacked and inactivated by the host's immune system. Additionally viruses are cleared from the blood by the Kupffer cells (liver macrophages). Also, other organs like lung or spleen can accumulate virus particles. In the case of adenovirus more than 90% of virus is accumulated in the liver. The high level of virus particles can lead to liver toxicity. Some strategies to improve delivery and reduce liver tropism have been investigated. The pre-treatment with the anticoagulant warfarin combined with depletion of Kupffer cells reduced virus levels in the liver and enhanced antitumor activity [59]. Another strategy being used is modifying the virus capsid. Certain chimeric constructs have shown better tumour infection and lower liver toxicity [60]. Evading the immune system is the other essential for effective tumour delivery. Options for preventing virus destruction include the use of agents binding and neutralizing antiviral antibodies [61]. Direct coating of the capsid with polymer complexes can also be used to mask the virus from the immune system [62]. Apart from direct modification of the oncolytic viruses a further promising approach is the utilization of cells exhibiting tumour tropism as virus carriers. Cell carriers that deliver the virus to the tumour shield it from the immune system and additionally should prevent excessive liver accumulation and toxicity. One cell type feasible for use as a carrier are cells of the immune system, like T-cells and dendritic cells [63]. Besides infecting the tumour these cells demonstrate antitumor activity themselves. Other promising carriers are adult stem cells. Neural stem cells were shown to deliver oncolytic adenoviruses efficiently in an animal model of glioma and prolong survival [64]. Bone marrow derived mesenchymal stem cells (MSC) have also been studied for their usability as carriers for oncolytic viruses [65].

2.4 Mesenchymal stem cells

Mesenchymal stem cells are multipotent cells, which can be isolated from most organs in post-natal vertebrates [66]. They are defined by their ability to differentiate into multiple cell types *in vitro*, like osteoblasts, adipocytes and chondrocytes. *In vitro* they exhibit a fibroblast like morphology and exhibit a characteristic adhesion to plastic surfaces under cell culture conditions. Markers expressed by MSC on the cell surface include CD105, CD73, CD44, CD90, and CD71. In contrast they do not express any hematopoietic markers like CD45, CD14 or CD34 [67]. Being originally described to differentiate into various mesenchymal lineages [68], subsequent studies could also demonstrate the induction of commitment to non-mesenchymal fates. For example, a neural differentiation has been described for MSC [69]. The ability to form miscellaneous cell types makes them highly interesting for tissue engineering. Currently MSC therapies for various conditions are investigated, including bone and cartilage repair, vascular diseases or neurological disorders [70]. Additionally the most commonly applied MSC isolating methods, like the isolation from the bone marrow of the iliac crest, are minimal invasive and can be performed under ambulant conditions. MSC play also a role in cancer progression. They integrate into the tumour microenvironment after migrating there. Their migration is promoted by secretion of inflammatory signals by the tumour. MSC can both develop an pro- or anti-tumour supportive phenotype in the tumour stroma, depending on the microenvironment [71]. Additionally MSC modulate the immune reaction to the tumour, as they exhibit immunosuppressive effects on various cells of the immune system [72]. While their exact role in the tumour is not fully understood, MSC have been demonstrated to enhance EMT and CSC stemness in pancreatic cancer [73].

2.5 MSC in tumour therapy

The migration and integration of MSC into the tumour microenvironment makes them excellent candidates for therapy vectors targeting malignant tissues. Combined with their immunoprivileged status and their ability to expand inside the tumour they are highly promising candidates [74]. As MSC can have anti-oncogenic properties, this could be used for interfering with the growth of the cancer. But as the signals leading to an anti-oncogenic behaviour in MSC are poorly understood this strategy is still in a very early phase [75]. Another option is the use of genetically modified MSC expressing anticancer agents. Engineered MSC expressing different therapeutic

substances, including Interleukins, IFN- β or TRAIL, have been used in *in vivo* studies against various malignancies [76]. MSC expressing TRAIL, as an example, have been used as a second agent for co-treatment of metastatic renal cell carcinoma or malignant glioma greatly enhancing therapy efficacy [77, 78]. Another approach is the use of MSC as cell carriers for oncolytic virus delivery, as mentioned previously. A Phase I clinical trial with MSC transporting oncolytic measles virus to ovarian cancer showed promising results. Survival of patients treated with infected MSC was significantly increased. This was not true for patients treated with the virus alone [79]. A study using MSC for delivery of a conditionally replicative adenovirus demonstrated enhanced survival in a mouse model [80]. Furthermore MSC were able to deliver oncolytic adenovirus to intracranial glioma xenografts [81]. These findings demonstrate the ability of MSC as virus carriers, even reaching poorly accessible tumour sites, like in the case of glioma.

3. Materials and Methods

3.1 Materials

3.1.1 Equipment and consumables

96 well suspension culture plates	Greiner bio-one, Frickenhausen
Accu-jet pro	BRAND, Wertheim
Biosafety cabinet, Napflow 1200	Thermo Fisher Scientific, Waltham, USA
Camera N°241956	Visitron systems, Puchheim
Cell culture flasks	TPP, Trasadingen, Switzerland
Cell culture plates, 24 well	TPP, Trasadingen
Cell culture plates, 6 well	Greiner bio-one, Frickenhausen
CO ₂ incubator, Sanyo	MS Laborgeräte, Wiesloch
Centrifuge, Biofuge pico	Heraeus, Hanau
Centrifuge, Multifuge 3	Heraeus, Hanau
Chicken eggs, fertilized	Hockenberger, Eppingen
Cryotubes	Nunc, Wiesbaden
Decloaking chamber	Biocare Medical, Concord, USA
Egg incubator	Bruja, Hammelburg
Falcon tubes	Greiner Bio-one, Frickenhausen
Gloves latex powder free	Hartmann, Heidenheim
Gloves nitril powder free	Ansell, Munich
Leukosilk S	BSN, Hamburg
Microlance 3 needles	BD, Heidelberg
Microscope Leica DMRB	Leica, Wetzlar
Microscope BIOREVO BZ-9000	Keyence, Neu-Isenburg
Microtome	Leica, Wetzlar
Neubauer haemocytometer	BRAND, Wertheim
Pasteur pipettes	Budenberg, Mannheim
Pipettes, Discovery comfort	HTL, Warsaw, Poland
Pipette tips	Greiner bio-one, Frickenhausen
Serological pipettes 25ml, 10ml, 5ml	Sarstedt, Nümbrecht

Serological pipettes 2ml	Greiner bio-one, Frickenhausen
Syringe 5ml	BD, Heidelberg
Syringe, Omnican F 1ml	BBraun, Melsungen
Thermanox cover slips	Nunc, Wiesbaden

3.1.2 Cell culture media and supplements

Advanced DMEM/F12	Life Technologies, Darmstadt
Amphotericin B	Lonza, Verviers, Belgium
B27 supplement	Life Technologies, Darmstadt
Biocoll	Biochrom, Berlin
bFGF	Tebu Biotech, Offenbach
Collagenase Typ IV	Worthington Biochemical, Lakewood, USA
Defined K-FSM	Life Technologies, Darmstadt
Dexamethasone	Sigma-Aldrich, Taufkirchen
DMEM (with 4.5 mg/l glucose)	Sigma-Aldrich, Taufkirchen
DPBS	Sigma-Aldrich, Taufkirchen
DMSO, 99%	Applichem, Darmstadt
FBS	Sigma-Aldrich, Taufkirchen
Fibronectin	Sigma-Aldrich, Taufkirchen
hEGF	R&D Systems, Wiesbaden-Nordenstadt
HEPES	Sigma-Aldrich, Taufkirchen
Insulin	Sigma-Aldrich, Taufkirchen
MSC Expansion Media	Miltenyi, Bergisch Gladbach
NH Adipodiff Medium	Miltenyi, Bergisch Gladbach
NH Chondrodifff Medium	Milltenyi, Bergisch Gladbach
NH Osteodiff Medium	Miltenyi, Bergisch Gladbach
Penicillin-Streptomycin	Life Technologies, Karlsruhe
Trypan blue	Biozol, Eching
Trypsin-EDTA (1x), 0,05%	Life Technologies, Darmstadt

3.1.3 Chemical agents and kits

Acetic acid, glacial	VWR, Briare, France
Avidin-Biotin blocking kit	Linaris, Wertheim-Bettingen
AEC single solution	StemCell Technologies, Köln
BCIP/NBT	Sigma-Aldrich, Taufkirchen
Bull's eye decloaker	Biocare Medical
Chloroform	Sigma-Aldrich, Taufkirchen
Collagen, PureCol	INAMED, Fremont, USA
DNase	Peqlab, Erlangen
Ethanol	Carl Roth, Karlsruhe
Entellan	Merck, Darmstadt
Fast Green (FCF)	Sigma-Aldrich, Steinheim
Fluoromount G	Biozol, Eching
Goat serum	Alexis, Karlsruhe
Haematoxylin solution, Mayers	Sigma-Aldrich, Taufkirchen
Hydrogen peroxide 30%	AppliChem, Darmstadt
Hydrochloric acid	J.T.Baker, Deventer, Netherlands
Isopropanol	Carl Roth, Karlsruhe
Matrigel	BD Bioscience, Heidelberg
Methanol	Carl Roth, Karlsruhe
Methylcellulose	Sigma-Aldrich, Steinheim
Oil Red O	Sigma-Aldrich, Steinheim
Paraffin (Paraplast)	Sigma-Aldrich, Taufkirchen
Paraformaldehyde	Merck, Darmstadt
Potassium chloride	Riedel-de Haen, Seelze
Potassium dihydrogen phosphate	Merck, Darmstadt
PKH26 mini kit	Sigma-Aldrich, Steinheim
ProTags Mount Aqua	BIOCYC, Luckenwalde
Roti-Histol	Carl Roth, Karlsruhe
Safranin O	Sigma-Aldrich, Steinheim
Sodium chloride	Riedel-de Haen, Seelze
Sodium dihydrogen phosphate	Merck, Darmstadt
Tween-20	Sigma-Aldrich, Taufkirchen

Weigert's Iron Hematoxylin Set
Xylene

Sigma-Aldrich, St. Gallen, Switzerland
Linaris, Wertheim-Bettingen

3.1.4 Buffers and solutions

10x PBS

2g KCL
2g KH_2PO_4
14.41g $\text{Na}_2\text{HPO}_4 \times 2\text{H}_2\text{O}$
80 g NaCl
Added ddH₂O up to 1 L
pH 7.4

10 x TBS

87.6 g 1.5 M NaCl
12.1 g 100 mM Tris
1 g NaN_3
Added ddH₂O up to 1 L
pH 7.5

Single Cell Isolation Medium

500 ml Advanced DMEM/F 12
10 ml 1 x B27 supplement
20 ng/ml EGF
20 ng/ml basic FGF
0.5 µg/ml dexamethasone
5 µg/ml insulin
5ml Pen/Strep (1:100)

3.1.5.1 Antibodies

Anti-Adenovirus, mouse
(monoclonal, 1:200)

Merck Millipore, Darmstadt

Anti-CD24 SWA11, mouse
(monoclonal, 1:100)

Kindly provided by Prof. P.Altevogt

Anti-Cytokeratin 19, mouse
(monoclonal, 1:100)

Abcam, Cambridge, UK

Anti-Caspase 3, rabbit (polyclonal, 1:50)	R&D Systems, Minneapolis, USA
Anti-c-Met, rabbit (polyclonal, 1:50)	Enzo Life Sciences, Lörrach
Anti-Ki67, rabbit (monoclonal, 1:100)	Abcam, Cambridge, UK
Anti-mouse IgG (H+L), biotenyated (goat, polyclonal)	Vector Laboratories, Burlington, Canada
Anti-rabbit IgG (H+L), biotenyated (goat, polyclonal)	Vector Laboratories, Burlington, Canada
Anti-rabbit IgG, AlexaFluor 488- conjugated (goat, polyclonal)	Molecular Probes, Karlsruhe
Anti-mouse IgG, AlexaFluor 594- conjugated (goat, polyclonal)	Molecular Probes, Karlsruhe

3.1.5.2 FITC-conjugated monoclonal antibodies

Anti-CD34, mouse	AdD Serotec, Puchheim, Germany
Anti-CD44, rat	Novus Biologicals, Cambridge, UK
Anti-CD45, mouse	AbD Serotec, Puchheim
Anti-CD90, mouse	AbD Serotec, Puchheim
Anti-CD105, mouse	AbD Serotec, Puchheim
Anti-CD166, mouse	AbD Serotec, Puchheim

3.1.6 Oncolytic adenovirus constructs

All used oncolytic adenoviruses were provided by our cooperation partner Dr Dirk Nettelbeck (DKFZ, Heidelberg, Germany). The viral capsid contains a fibre chimera. It has the shaft of serotype 5 and the knob domain of serotype 3. All used strains contain a gene for GFP, which is expressed by infected cells allowing their identification. Oncolytic activity of the different viruses in various pancreatic cell lines was examined by our cooperation partner, as were infection rates and virus release in MSC. The following virus constructs were used in the present work:

Virus	Description
Ad-SA GFP	Control virus [82]
Ad-CMV	Replication incompetent virus [82]
Ad-IL	Control virus; expresses luciferase [83]
Ad-19K-	E1B19K deletion; enhanced cell lysis [83]
Ad-TRAIL	Expresses soluble TRAIL
Ad-FCU-1	Expresses the suicide gene FCU1 turning a prodrug to 5-FU [84]

3.1.7 Cell lines

Established pancreatic cancer cell lines MIA-PaCa2 and Panc-1 were obtained from the American Type Culture Collection (ATCC).

The primary human pancreatic adenocarcinoma cell line PaCaDD-183 was kindly provided by Dr Felix Rückert (University Hospital Mannheim).

3.2 Methods

3.2.1 Cell culture methods

3.2.1.1 Cell culture conditions

Cells were cultivated in a CO₂ incubator at 37°C and 5% CO₂. Media were supplemented with 10% heat-inactivated FCS and 10mM HEPES, unless stated otherwise. Media, DPBS and trypsin solutions were pre-warmed to 37°C prior to use.

For determining cell number cells were mixed in a 1:1 ratio with trypan blue solution (0.125%) immediately after trypsinisation. Only unstained viable cells were counted in an improved Neubauer counting chamber. Cells were seeded in cell culture flasks and cultivated until they reached 80-90% confluence.

3.2.1.2 Freezing and thawing of cells

Cells were trypsinised and cell number was determined. Aliquots of 5×10^6 – 1×10^7 cells were resuspended in medium with 10% (v/v) DMSO. Cryotubes were put in an isopropanol-filled cryocontainer and placed in a -80°C freezer. After 24h cells were relocated to a -140°C freezer for long-term storage.

Frozen cells were thawed at 37°C in a water bath. Immediately after thawing cells were resuspended in 10 ml of medium and centrifuged. The supernatant was resuspended in appropriate medium and seeded in culture flasks. After 24 h medium was changed to remove any traces of DMSO.

3.2.1.3 Culture of MIA-PaCa2 and Panc-1

MIA-PaCa2 cells were cultured in cell culture flasks as adherent cells. DMEM medium with 4.5 $\mu\text{g/l}$ glucose and l-glutamine was used for growth of these cells. When the cells reached 90% confluence they were trypsinized. After detachment the reaction was stopped with serum containing medium. The cells were centrifuged and seeded 1:10 in fresh medium into new flasks.

3.2.1.4 Culture of PaCaDD cells

PaCaDD cells were cultured as adherent cells in cell culture flasks. For culture DMEM medium containing 10% heat-inactivated FCS and 10 mM HEPES was mixed 3:1 with complete K-FSM medium. Cells were passaged when the monolayer reached 90% confluence as described under 3.2.1.3

3.2.1.3 Isolation and culture of MSC

Mesenchymal stem cells were isolated from the bone marrow of healthy donors after approval by the ethical board of the University of Heidelberg. Written informed consent was obtained from all donors. Bone marrow was obtained from the Iliac crest and collected in syringes containing heparin (10,000 IU). The BM solution was diluted 1:3 with DPBS and filtered through a 100 μm cell strainer. MSC were separated from

other components by density gradient centrifugation. 30ml of the BM solution were layered on top of 15 ml Biocoll and centrifuged for 40 min at 480 g without brake. After centrifugation the MSC-containing red interphase layer was removed and resuspended in 50 ml of PBS and centrifuged for 7min at 240 g. After repeating the washing step cells were resuspended in stem cell expansion media (containing Pen-Strep and Amphotericin B) and seeded in flasks coated with 0.1% fibronectin. All steps were performed under sterile conditions.

3.2.1.4 Isolation of primary cancer cell spheroids

Malignant tissue was obtained from patients under the approval of the ethical board of the University of Heidelberg. Diagnoses were established by conventional clinical and histological criteria according to the World Health Organization (WHO). All surgical resections were indicated by principles and practice of oncological therapy. Specimens of surgically removed PDAC were minced mechanically and diluted in Matrigel. The probes were injected subcutaneously in the flanks of 6-week old female NMRI (nu/nu) mice. Developing tumours were resected and subtransplanted to new mice. After 3 subtransplantations a stabile growing xenograft line was established. For establishing primary cancer cell spheroids resected xenografts were rinsed with PBS and minced to ca. 1mm sized pieces under sterile conditions. The minced tumour was resuspended in Single Cell Isolation Medium supplemented with Collagenase Typ IV (200 U/ml) and DNase (50 U/ml). After 60min incubation at 37°C the suspension was filtered through a 100 µm cell strainer to obtain a single cell suspension. Cells were centrifuged and resuspended in fresh Single Cell Isolation Medium. Cells were used for experiments within 7 d after isolation.

3.2.2 Differentiation of MSC

For verifying differentiation potential MSC were trypsinized and seeded in 6-well plates coated with 0.1% fibronectin to 80% confluence. Differentiation was induced by cultivating the MSC in NH Osteodiff Medium (osteogenic induction) for 2 weeks or NH Adipodiff Medium (adipogenic induction) for 3 weeks. MSC cultivated in NH Expansion Medium were used as a control. For staining cells were washed with PBS and fixed for 5 min with methanol for at RT (adipogenic induction) or with pre-cooled methanol at -20°C (osteogenic induction). After fixation cells were washed twice with

deionized water and stained to detect differentiated cells. Osteoblasts were stained with Sigma Fast BCIP/NBT and adipocytes were stained with Oil Red O solution. For chondrogenic induction trypsinized MSC were seeded into a 15 ml Falcon tubes (5×10^5 cells per tube) and centrifuged. Differentiation was induced by cultivation in NH Chondrodifff Medium for 3 weeks. Medium was changed every 2-3 days. Pellets cultivated in NH Expansion Medium were used as a control. For detection the pellets were dehydrated and embedded in paraffin as described under 3.2.5. Slides were rehydrated and stained with Weigert's Iron Hematoxylin for 5 min. The Slides were then washed 4 times with distilled water and dipped for a few seconds in Acid EtOH. Afterwards probes were washed twice with distilled water and stained with 0.02% fast green solution in distilled water for 5 min. Then the slides were rinsed in 1% acetic acid solution before being stained with 1% safranin O solution for 30 min. Stained slides were cleared twice in 95% ethanol and dehydrated two times in absolute ethanol and xylene for 2 min. Dehydrated slides were mounted in Entellan.

3.2.3 Adenovirus infection of cells

For cell infection viruses were diluted in DMEM containing 2% heat-inactivated FCS and 10 mM HEPES to the respective infection titer. Virus titers were measured in TCID₅₀. The cells were infected 24 h after seeding as adherent cells. After removing the culture medium cells were incubated for 2 h with the virus solution. Afterwards medium was changed to the respective culture medium or cells were used immediately for experiments.

3.2.4 Colony formation assay

Cells were trypsinized 24 h after virus infection. After determination of cell number cells were seeded in 6-well culture plates at a density of 400 cells per well. Cells were incubated for two weeks without changing medium. Afterwards the cells were fixated with 3.7% PFA for 10 min and with 70% ethanol for 10 min. After washing with distilled water the cells were stained with 0.05% Coomassie Blue for 5 min. Then cells were washed with distilled water and dried over night. The number of colonies with more than 50 cells was counted under a dissecting microscope.

3.2.5 *In vitro* invasion assay

MSC were trypsinized and cell number was determined. After marking the cells with PKH26 dye (per manufacturers recommendations) 5×10^4 cells per well were seeded into a 24-well plate coated with 0.1% fibronectin. For creation of tumour cell spheroids adherent tumour cells were trypsinized and cell number was determined. Cells were resuspended in DMEM medium containing 2% heat-inactivated FCS and 0.25% methylcellulose. 1×10^4 cells were seeded into 1 well of a 96-well suspension culture plate and formed a single spheroid. The MSC were infected with the adenovirus constructs 24 h after seeding. Then the MSC were washed four times with PBS and layered with 250 μ l gel-layer (containing DMEM medium with 2% FCS and 0.25% methylcellulose, collagen solution and Matrigel in equal parts). Spheroids were placed on top of the gel layer and invasion occurred over night. Then the spheroids were removed and placed into a fresh 96-well suspension culture plate. Invasion of MSC was evaluated under a BIOREVO BZ-9000 microscope 42 h after MSC infection.

3.2.6 Cytospins

Cells were fixed with 4% PFA for 10min at RT to remove active virus particles. Afterwards cells were washed with TBS and with PBS. For centrifugation 100 μ l cell suspension was placed in a slide chamber. The cells were centrifuged for 4 min at 400 rpm. Slides were air dried and stored at -20°C .

3.2.7 Immunohistochemistry

The paraffin-embedded sections were deparaffinised and rehydrated prior to staining. They were incubated at 60°C for 15 min, before being transferred to xylene and incubated for 15 min. Afterwards the slides were incubated twice in Roti-Histol for 10 min to remove xylene. For rehydration the slides were incubated twice in 100%, 96% and 70% ethanol for 5min each. Finally they were washed with PBS.

Antigen retrieval was performed by boiling the slides in Bull's Eye decloaker solution in a decloaking chamber at 125°C for 10 min. Antigen retrieval was not performed on slides stained with the primary anti-adenovirus antibody. These slides were incubated with 0.1% trypsin for 10 min at RT after rehydration.

The slides were incubated for 30 min at RT in PBST with 20% goat serum and 4 drops/ml biotin solution to block unspecific antibody binding. Afterwards the slides were incubated for 1 h with the primary antibody in PBS with 4% goat serum and 4 drops/ml avidin solution. After washing two times with PBST and one time with PBS endogenous peroxidase activity was removed by incubation with methanol (containing 3% H₂O₂) for 10 min. After repeating the washing steps the slides were incubated with the secondary antibody (diluted 1:200) for 30 min. Subsequently the samples were washed and ABC solution (2.5ml PBS containing 1 drop biotin and 1 drop avidin) was added. After 30 min incubation the sections were washed three times with PBS and stained with AEC solution. The staining was stopped after 3-10 min, when red colouring was detected under a microscope. For stopping the reaction slides were put in distilled water. Then a counterstaining with haematoxylin for 3 min was performed. The samples were put under tap water to remove the dye and washed twice with distilled water before being mounted in ProTaq. Samples without the primary antibody were used as negative controls. The samples were evaluated under a Leica DMRB microscope. A SPOTTM FLEX 15.2 64Mp camera was used for taking pictures. The images were analysed using SPOT Basic/Advanced 4.6 software.

3.2.8 Immunocytochemistry

To prevent unspecific antibody binding slides were first incubated in PBST with 20% goat serum for 30 min. Afterwards slides were incubated with the first primary antibody for 1 h. After washing (two times with PBST, one time with PBS) samples were incubated with the appropriate fluorophore-linked secondary antibody for 30min. Subsequently the samples were washed and incubated with the second primary antibody for 1 h. After washing the slides were incubated with an appropriate secondary antibody and DAPI (1:50) for 30 min. The samples were washed again and mounted in Fluoromount G. Samples in which the respective primary antibody was omitted served as a negative control. All steps were performed in the dark. Antibodies were diluted in PBS with 4% goat serum. The stained samples were examined under a Leica DMRB microscope. Images were captured using a SPOTTM FLEX 15.2 64Mp camera and analysed with SPOT Basic/Advanced 4.6 software.

3.2.9 Grafting of tumour cells to fertilized chicken eggs

Tumour cells can be transplanted to the chorioallantoc membrane (CAM) of fertilized chicken eggs as a substitute for the use of xenografts grown on mice for in vivo experiments. The fertilized chicken eggs were incubated in an egg incubator at 37.8°C and 45-55% humidity. Eggs were disinfected with pre-warmed 70% ethanol before incubation. Eggs were opened 4 d after beginning of incubation. For this eggs were punched at the round end of the egg and ca. 3 ml of albumen was removed with a syringe. Then a hole with a diameter of ca. 2 cm was cut out in the middle part of the egg. After injecting 2 ml of the previously removed albumen into the egg the hole was covered by a strip of Leukosilk. The cells were grafted onto the CAM 9 d after beginning of incubation. Trypsinized cells were resuspended in medium and mixed 1:1 with Matrigel. Rings, which had been cut out of plastic cover slips, were placed on the CAM near a blood vessel and 50 µl of cell solution was seeded inside the rings. 5×10^5 cells were seeded per egg. Afterwards the holes were sealed again with Leukosilk. 18 days after beginning of incubation tumours were removed and the chicken embryos were euthanized with Narcoren injection. Tumour take and tumour volume were determined. The volume of tumours was calculated as

$V = \frac{4}{3} \pi r^3$, $r = \frac{1}{2} \sqrt{d_1 d_2}$. Tumours were fixed in 4% PFA solution over 2-3 days.

Subsequently the tissue was washed in tap water for 2 h to remove PFA. For dehydration the tissue was incubated for 2 h each in ascending strength of alcohol (70%, 96%, absolute). After incubation in absolute ethanol over night the tissue was incubated with chloroform twice for 2 h. Finally, the tissue was impregnated with melted paraffin wax at 60°C over night. Tissue sections were performed with a microtome.

3.2.6 Statistical analysis

The data of the colony-forming assay is presented as mean \pm SD of a representative experiment with 6 wells per group. The experiment was performed three times independently with similar results. Infection of primary CSC spheroids was performed three times with similar outcomes. Experiments with transplants in fertilized chicken eggs were performed twice with similar results. Representative experiments are shown. Groups were compared for tumour volumes of cells transplanted with a defined percentage of infected cells using the Kruskal-Wallis test and Mann-Whitney

test with a Bonferroni correction. Significance of the data of tumour volumes after treatment with adenovirus-infected MSC was evaluated by student's *t* test. $P < 0.05$ was considered significant.

4. Results

The present work was performed in cooperation with the Oncolytic Adenovirus Group (DKFZ), which is headed by Dr Dirk Nettelbeck. The Nettelbeck group demonstrated a better transduction of both MSC and pancreatic cancer cells by oncolytic adenoviruses serotype 5, when a chimeric capsid containing the knob domain of serotype 3 was used compared to other examined capsid variants. Therefore this capsid was used in further experiments. Moreover, they could show an earlier and increased virus release in infected MSC for the modified viruses Ad-TRAIL and Ad-19K- compared to the Ad-IL virus without improved anti-tumourgenic potential. The two modified viruses also exhibited improved oncolytic activity in established and primary pancreatic cancer cells. A prodrug-activating virus (Ad-FCU1) showed increased oncolytic activity as well if used together with the respective prodrug 5-FC. Increase in oncolytic activity varied between different cancer cell lines, indicating different sensitivity in the cancer cell lines. The results of our cooperation partner are being prepared for publication in an international peer-review journal. The oncolytic adenoviruses were provided to our group for further investigation. I tested in my part of the project the invasion ability of infected MSC, as well as infection and elimination efficiency, especially in regard to CSC. Furthermore I expanded the obtained *in vitro* results in an *in vivo* model.

4.1 Oncolytic adenovirus infection eliminates tumour-initiating cells *in vitro*

The elimination of the highly drug-resistant CSC population is essential for a successful therapy against pancreatic cancer. A colony formation assay was performed to examine if oncolytic adenovirus infection eradicates CSC and not only the differentiated cancer cells. As CSC are defined by their ability to grow autonomously and to form daughter populations, colony formation is correlated to the presence of CSC. An established cell line that harbours a high CSC potential, MIA-PaCa2, was used for this study. The cells were infected with different virus titres and seeded 24 h after infection at low density in cell culture plates. After two weeks no colonies had developed in infected populations, even when infection occurred with low virus titres (Fig. 11 A, C). If the infected cells were seeded in culture medium

containing methylcellulose the number of colonies decreased in a dose-dependent manner (Fig. 11 B, C). This reflects initial infection rates, as the methylcellulose matrix prevents the spread of released virus particles. Thus, the tumour-initiating CSC are infected and eliminated by the oncolytic adenovirus.

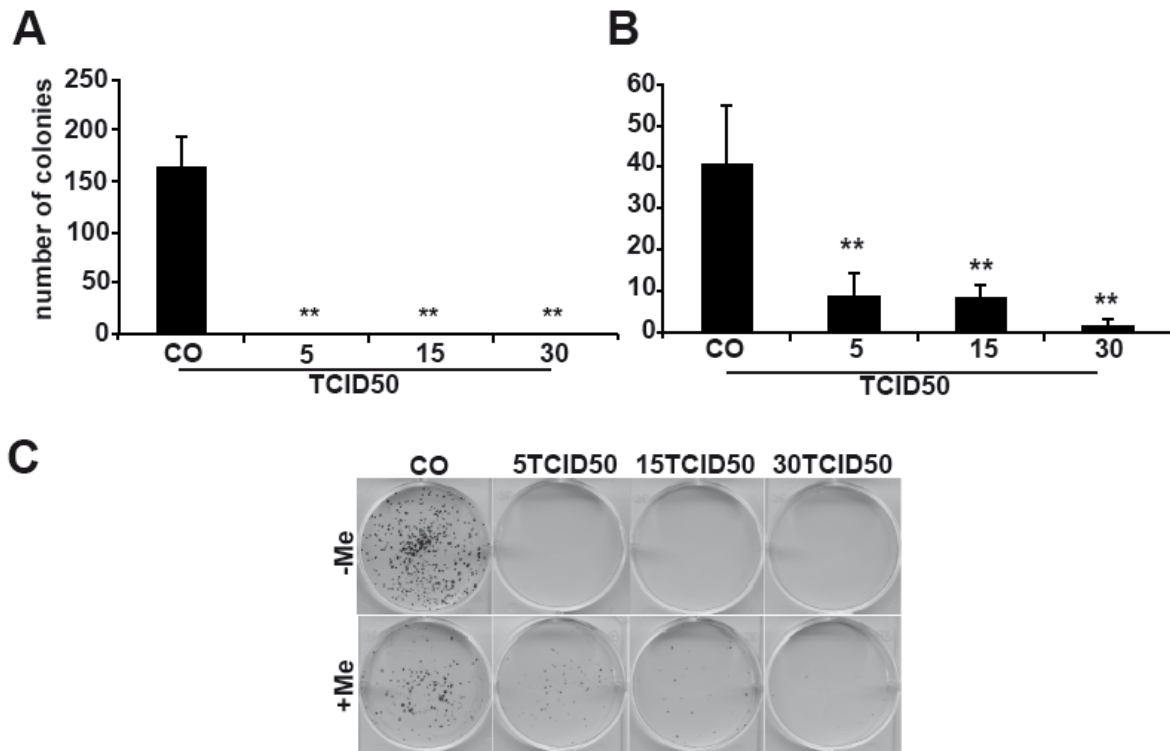


Figure 11: Infection with an oncolytic adenovirus eliminates the CSC population. (A) MIA-PaCa2 cells were infected with a virus titre of 5, 15 and 30 TCID₅₀ of the oncolytic Ad-SA GFP virus. Uninfected cells were used as a control. 200 cells per well were seeded in 6-well plates 24 h after infection. Colony formation was evaluated after two weeks. Means \pm SD are shown (**P<0.01). **(B)** Cells were seeded after infection like previously described in medium with 0.25% methylcellulose. Means \pm SD of each group are shown (**P<0.01). **(C)** Representative images of plates seeded with or without methylcellulose (+/- Me) and infected with different virus titres are shown.

No resistant surviving CSC were observed after infection. Additionally, the surviving colonies growing in the methylcellulose-containing medium were eliminated completely by a second infection with the oncolytic adenovirus (Fig. 12).

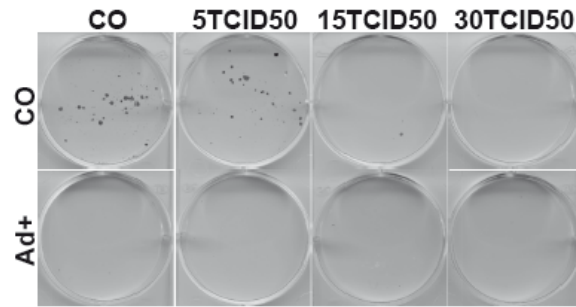


Figure 12: Infection with an oncolytic adenovirus eliminates surviving colonies. Surviving colonies infected with 5, 15 and 30 TCID₅₀ of the oncolytic Ad-SA GFP virus grown in medium with 0.25% methylcellulose were infected with a titre of 5000 TCID₅₀ per plate of Ad-SA GFP virus 14 d after initial infection. Plates were fixed 3 d after second infection and presence of surviving colonies was evaluated. Representative images of plates with (Ad+, lower row) or without (CO, upper row) a second infection are shown.

4.2 MSC isolation and confirmation of identity

The MSC used in this project were isolated from bone marrow samples obtained from healthy donors. The stem cells were isolated from hematopoietic cells by gradient centrifugation with Biocoll and subsequent cultivation as plastic-adherent cells. The isolated cells grow as a monolayer and exhibited a fibroblast-like morphology. To confirm the identity of the cultured bone marrow-derived cells as MSC differentiation into cell types from different lineages was induced. The cells differentiated readily into osteocytes, adipocytes and chondrocytes (Fig. 13 A). Additionally a characteristic marker profile for MSC was verified by FACS analysis (Fig. 13 B). The MSC expressed the surface markers CD44, CD90 and CD105, while the hematopoietic markers CD34, CD45 and CD166 were absent.

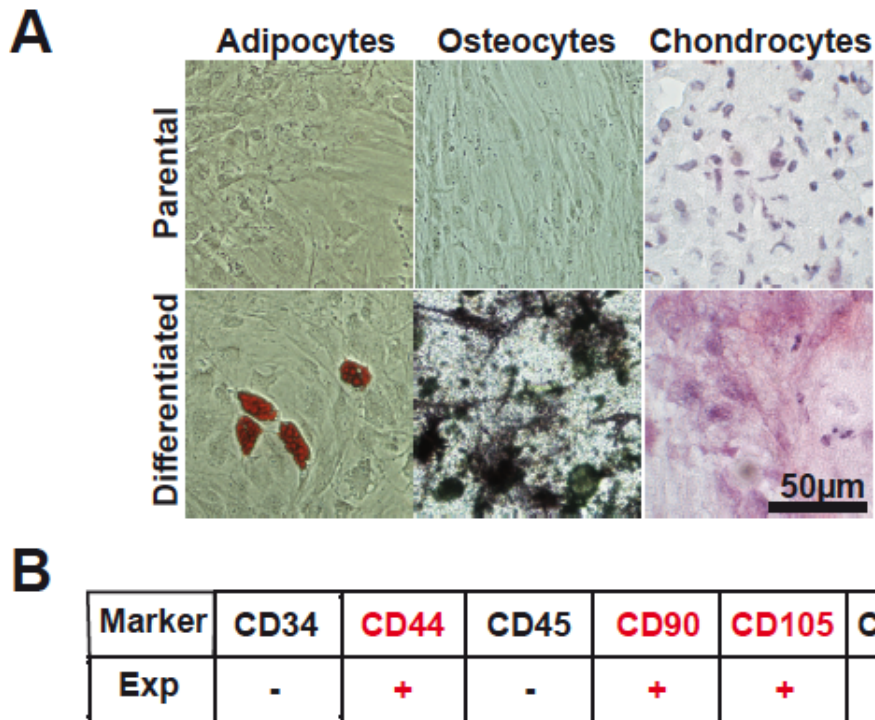
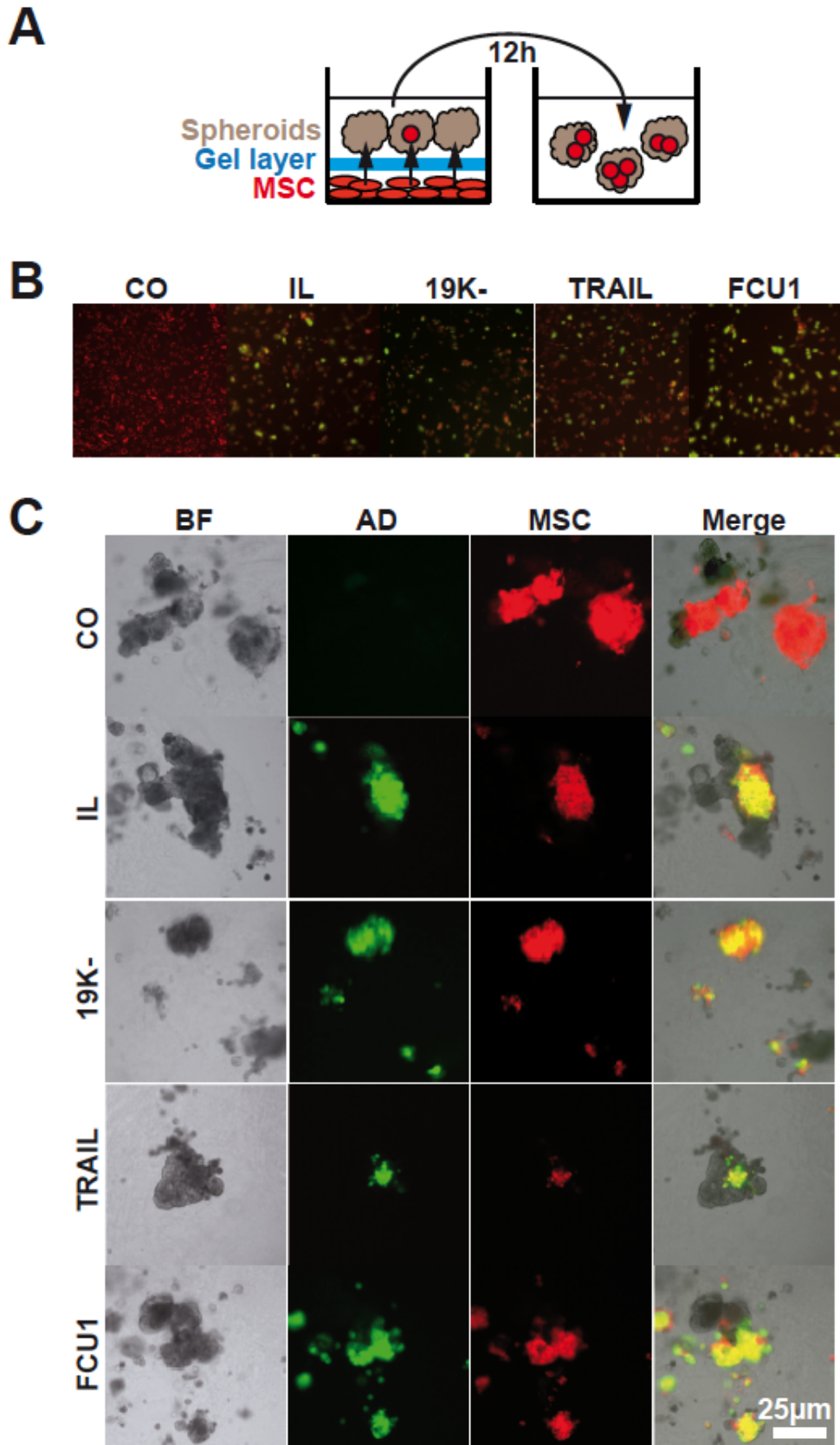


Figure 13: MSC properties of isolated bone marrow derived MSC (A) MSC were cultivated in NH expansion medium (parental cells) or in the respective differentiation medium. Differentiation was confirmed by staining with OilRed O (adipocytes), BCIP/NBT (osteocytes) and Fast Green/Safranin O (chondrocytes). (B) MSC were stained with FITC-conjugated antibodies for characteristic surface markers. Presence of the markers was analysed by FACS analysis.

4.3 Oncolytic adenovirus-infected MSC invade tumour spheroids *in vitro*

For the role as virus carriers for the therapy of PDAC, infection with the oncolytic adenovirus is not allowed to interfere with the natural homing ability of the MSC. An *in vitro* invasion assay was performed to ascertain that infected MSC still invade tumour tissue. The experiment was performed in medium containing 2% FCS to minimize interference of attractants present in the serum with signals released by the tumour cells. MIA-PaCa2 and PaCaDD spheroids were formed in methylcellulose containing medium. PaCaDD formed globular spheroids with sharp margins, while MIA-PaCa2 spheroids were more irregularly shaped. MSC growing as a monolayer were marked with a red fluorescence dye and infected with different oncolytic Ad strains. The tumour spheroids were separated from the MSC by a gel layer (Fig. 14 A) consisting of Matrigel, collagen and methylcellulose. The MSC had to migrate through the gel layer to reach the tumour spheroids. The spheroids and MSC were

co-incubated over night to enable invasion. Afterwards the spheroids were removed and placed in fresh wells. Microscopic evaluation occurred around 42 h after infection. Infected MSC were detected by green fluorescence due to virus-induced GFP expression. The presence of GFP-positive MSC was verified in spheroids from each group (Fig. 14 B), demonstrating that infection does not prevent the MSC from homing. Infiltration with infected MSC varied strongly between single spheroids. No significant differences were noticed between MSC infected with different Ad strains.



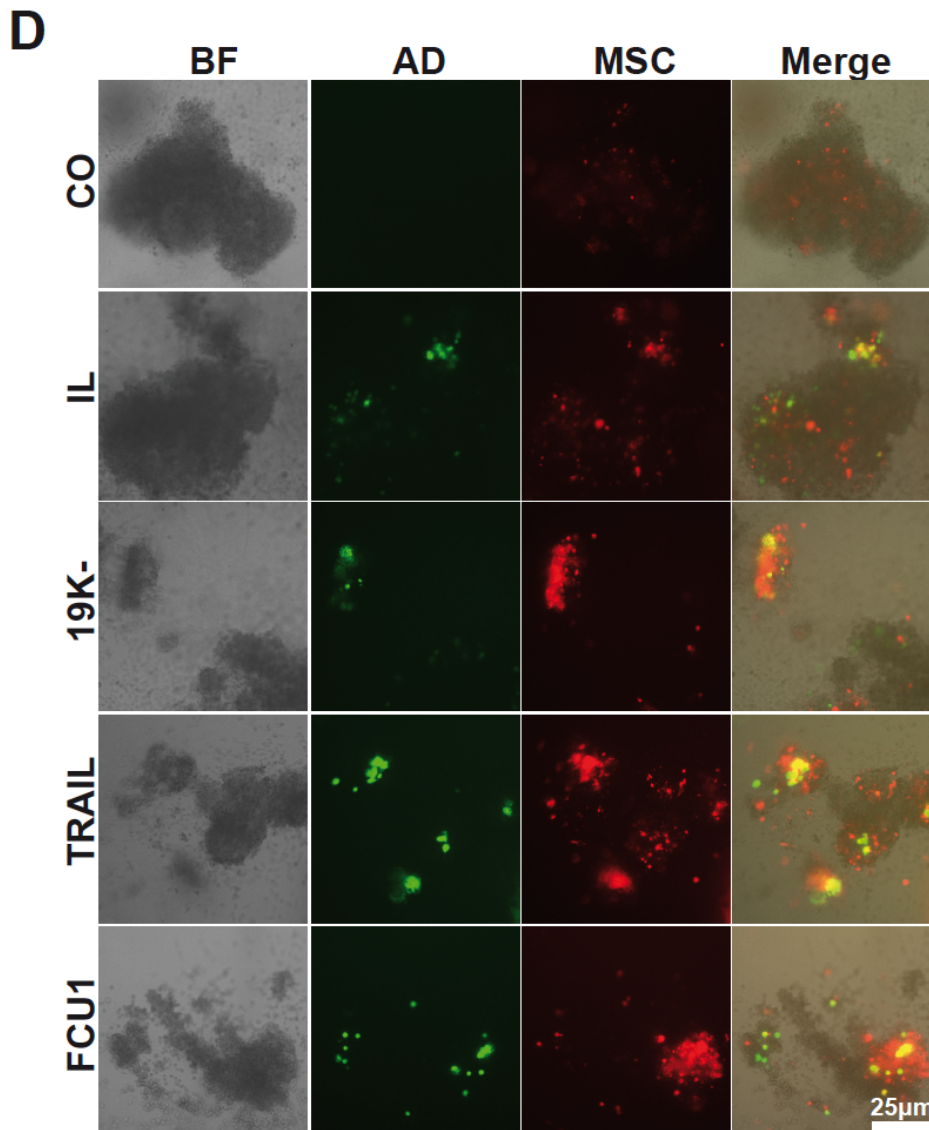


Figure 14: Invasion of tumour spheroids by oncolytic adenovirus-infected MSC. (A) MSC growing as a monolayer were infected with different oncolytic adenoviruses with a virus titre of 2000 TCID₅₀ and stained with a red fluorescent dye. After infection the MSC were overlaid with a Matrigel/collagen layer. Tumour spheroids were seeded on top of the gel layer. Co-incubation occurred over night to allow the MSC to invade the spheroids through the gel layer. Afterwards the spheroids were removed and cultivated until 42 h after infection before being evaluated under a microscope. (B) Infection control of MSC 42 h after infection. Infected cells are identified by GFP expression. GFP is coded by the viral genome. (C) Evaluation of invasion in PaCaDD spheroids 42 h after infection. Representative images from every group are shown. MSC appear red, virus-infected cells green. (D) Representative images of MIA-PaCa2 spheroids with invaded MSC 42 h after infection from every group are shown.

4.4 Oncolytic adenoviruses infect primary pancreatic CSC

The ability of the different virus constructs to infect and eliminate pancreatic cancer stem cells had been only examined in established cell lines so far. Consistent with their previously shown ability to eliminate tumour-initiating cells the control virus Ad-SA GFP infected CSC in the established pancreatic cell line Panc-1 (Fig. 15). CSC were identified by expression of the marker gene c-Met.

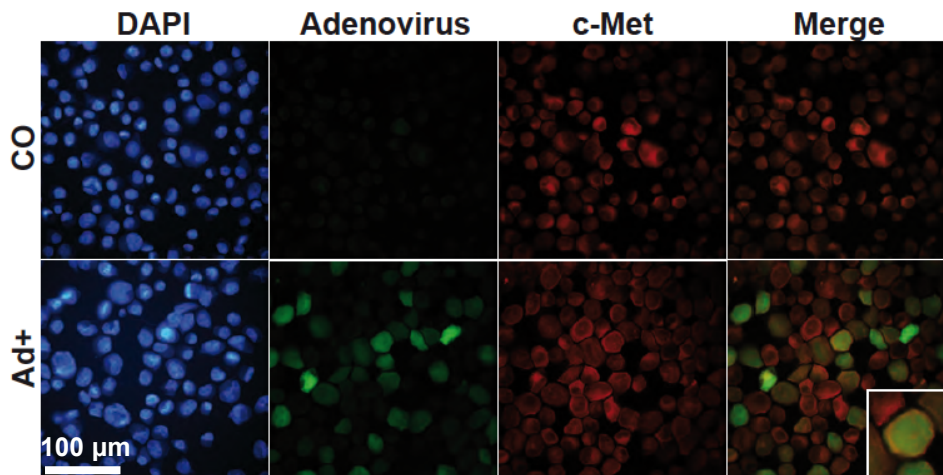


Figure 15: Oncolytic adenovirus infection of pancreatic CSC. Panc-1 cells were infected with a titre of 200 TCID₅₀ of the oncolytic adenovirus Ad-SA GFP. Uninfected cells served as a control. Cells were fixed and centrifuged on slides 42 h after infection. Adenoviral capsid and c-Met were detected with antibodies by immunocytochemistry. Representative images at 400x magnification are shown.

To investigate if the viruses are also able to infect CSC in a primary pancreatic cancer model, spheroid cultures established from primary mouse xenografts were used. The xenografts were established from freshly resected human PDAC tissue and were serially subtransplanted on immune deficient nude mice. With increasing passage numbers the xenograft tumours exhibited enhanced aggressiveness and an enrichment of the CSC markers c-Met and CD133, while maintaining the typical morphology of PDAC (Fig. 16 A). The spheroids were infected with oncolytic adenovirus after being in culture for two days. Forty-two h after infection the cells were centrifuged on slides and Ad infection was detected by staining with an antibody against adenoviral capsid proteins. c-Met was used to identify CSC. All of the examined adenovirus constructs infected primary CSC spheroids, as seen by c-Met/adenovirus double positive cells (Fig. 16 B).

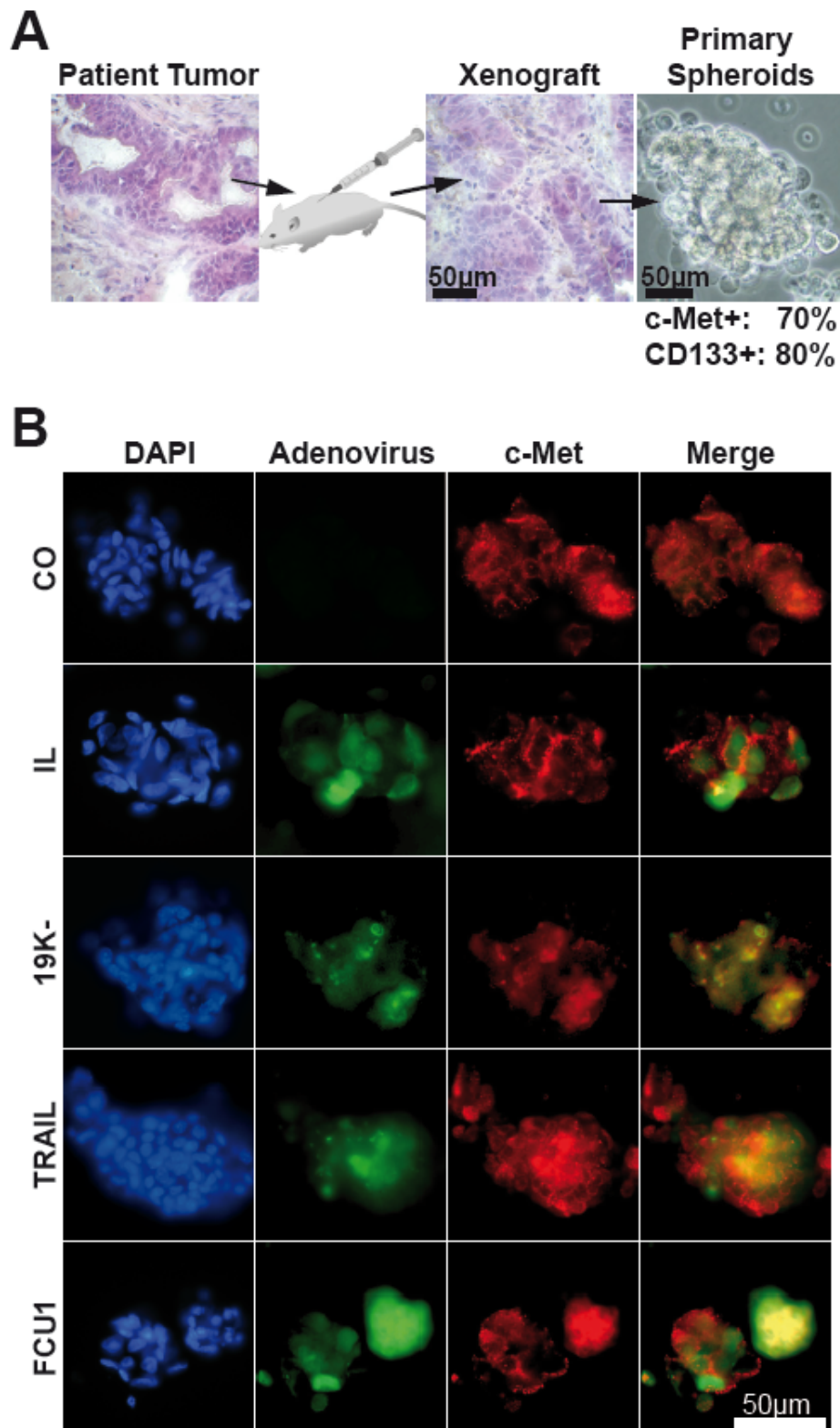


Figure 16: Infection of primary pancreatic CSC by oncolytic adenoviruses. (A) Resected patient PDAC were serially transplanted on mice. The xenografts retained morphology of the patient tumour. Tumour spheroids were established from freshly resected mouse xenografts and contained a high number of cells positive for the CSC markers c-Met and CD133. **(B)** Primary tumour spheroids were infected with a titre of 200 TCID₅₀ with four different oncolytic viruses (Ad-IL, Ad-19K-, Ad-TRAIL, Ad-FCU1). Uninfected spheroids

served as a control. Spheroids were fixed and centrifuged on slides 42 h after infection. Adenoviral capsid and c-Met were detected with antibodies by immunocytochemistry. Representative images at 400x magnification from each group are shown.

4.5 Oncolytic adenovirus infection reduces tumour growth *in vivo*

To investigate the effect of oncolytic adenoviral infection in an *in vivo* model MIA-PaCa2 cells were transplanted on the CAM membrane of fertilized chicken eggs (Fig. 17 A). The transplanted tumour cells are innervated and supplied by the blood vessels of the CAM and form tumour tissue that exhibits a morphology similar to the one found in mouse xenografts, thus allowing to investigate the effect of therapies in a highly patient-related model. The tumour cells were infected *in vitro* prior to engraftment with a high titre of the Ad-SA GFP virus. The transplanted cells contained a defined fraction of infected cells (0% (=CO), 1% or 5%). Oncolytic adenovirus infection reduced tumour size considerably (Fig. 17 A). Whereas tumour size showed large variability in the control group, only small tumours (with the biggest being around 200 mm³, compared with around 1000 mm³ for the control group) occurred in infected samples. Tumour take (percentage of grafted tumours having developed) was only reduced with 5% infected cells. The tumour take of the 1% infected cells was comparable to that of the control group. Additionally to smaller size infected tumours exhibited a radically altered morphology (Fig. 17 B). While cells were organized as a loose tissue in uninfected tumours infected tumours showed clusters of densely packed small cells. Both structures contained human cells, as confirmed by the presence of human cytokeratin 19. The presence of adenovirus was detected only in these clusters (Fig. 17 C) in tumour samples from the 5% group. Adenovirus particles could not be attested in the 1% group. To evaluate the effect of virus infection on CSC characteristics CD24 was used as a CSC marker. While control tumours contained many CD24 positive cells less cells expressing CD24 were found in infected tumours. A reduction of the amount of CSC seems to be evoked by virus infection (Fig. 17 C). In addition the presence of the proliferation marker Ki67 was also weaker in the infected tumours (Fig. 17 D). These results suggest that oncolytic adenovirus infection reduces proliferation and the amount of cells exhibiting CSC characteristics during the development of tumours under *in vivo* conditions.

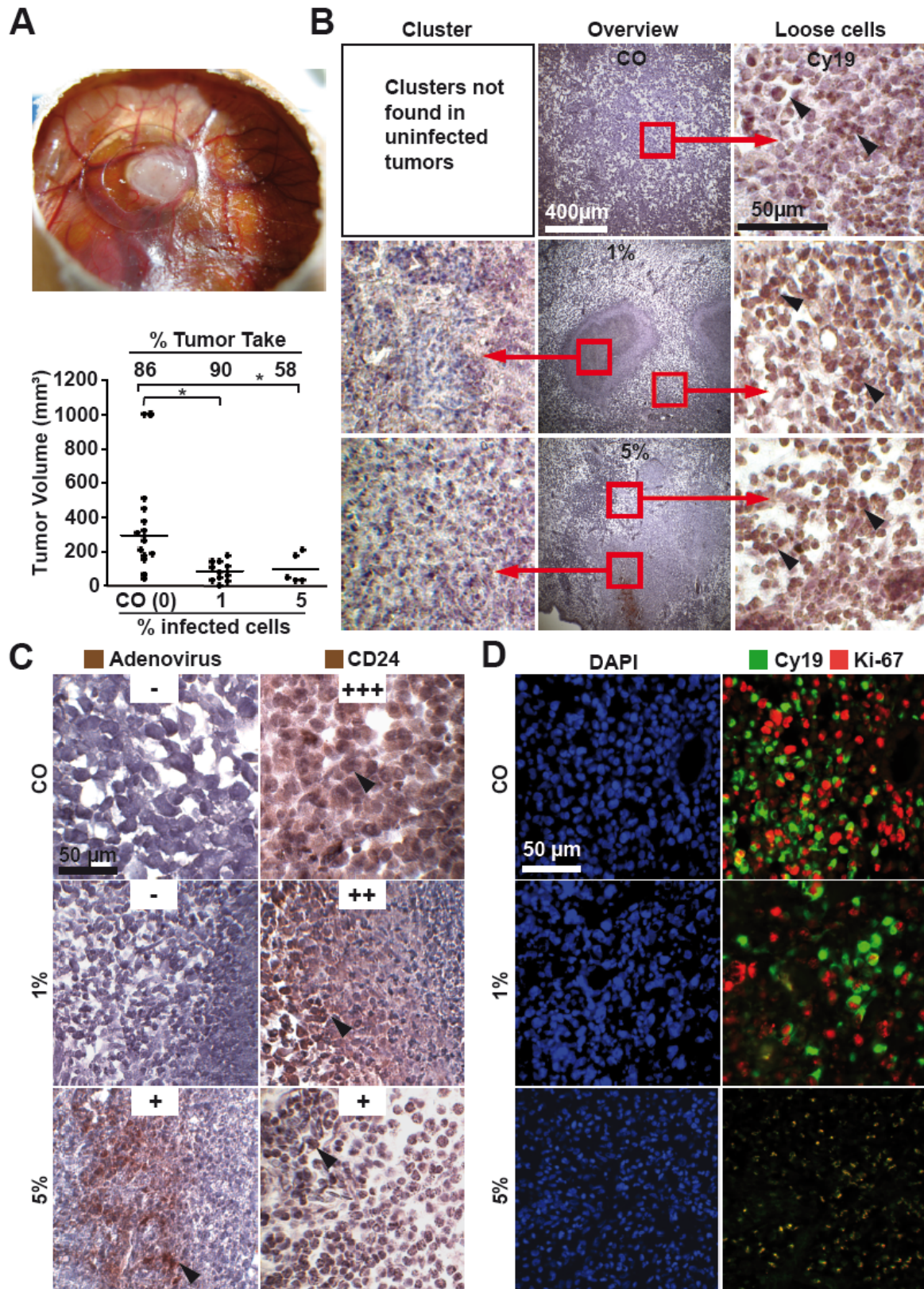


Figure 17: Effect of infection with an oncolytic adenovirus on tumour growth *in vivo* (A) MIA-PaCa2 cells containing 0% (=CO), 1% or 5% cells infected with Ad-SA GFP were transplanted on the CAM of fertilized chicken eggs at day 9 of development. Tumours were resected and tumour take (percentage of transplanted tumours which did develop) and tumour volume were determined. Mean volume is shown (* $P < 0.05$) (B) Representative images at 40x magnification of tissue sections from resected tumours are shown. Sections

stained with antibody against virus capsid are shown at magnification. Details of the different morphologies are shown at 400x magnification. Sections were stained with a human specific antibody against cytokeratin 19 (Cy19). Cy19 positive cells are indicated by arrowheads. (C) Location of adenovirus and expression of CD24 were evaluated by immunohistochemistry. Arrowheads indicate positive cells. The amount of positive cells is indicated in the white boxes. (D) Tumour sections were stained with antibodies against Cy19 and Ki67 for evaluation by immunocytochemistry. Nuclei were stained with DAPI. Representative images at 400x magnification are shown.

4.6 Ad-infected MSC exhibit tumour tropism *in vivo*

The homing of bone marrow-derived MSC to pancreatic tumour xenografts had been shown *in vivo* in previous studies. Transplants of established MIA-PaCa2 cells on the CAM of fertilized chicken eggs were used to investigate the invasion ability of MSC infected with the various oncolytic adenoviruses. The CAM is intensely vascularized. The capillaries found in the CAM form a part of the embryo's circulatory system. The MSC were injected into arteries from the CAM that were carrying blood towards the tumour. Prior to injection the MSC were infected *in vitro* by the miscellaneous Ad strains. MSC injection was performed two days before the tumours were resected (Fig. 18 A).

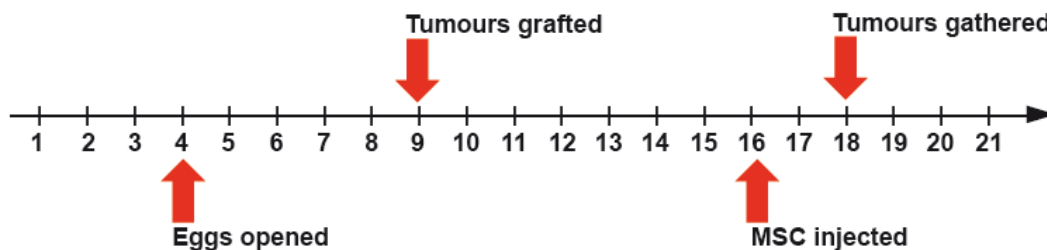


Figure 18: Timeline of *in vivo* invasion of tumour xenografts by infected MSC. Experiment is shown with specific steps indicated at respective days of development. Tumour cells were grafted at 9 days after beginning of incubation. MSC were injected two days before tumour resection, which was performed at incubation day 18, three days before the chicken would hatch.

A non-replicating adenovirus (Ad-CMV) was used in a first experiment to establish the technique (Fig. 19). The use of Ad-CMV ensured that invaded MSC were not lysated before detection. Additionally non-infected MSC were injected as a further control group. The presence of MSC did not influence tumour growth compared with the other groups. Also the injection procedure did not influence tumour development. Infected MSC were detected in tumour samples. Since the invasion of infected MSC

was successfully demonstrated in this experiment the replication competent oncolytic viruses were used in following experiments.

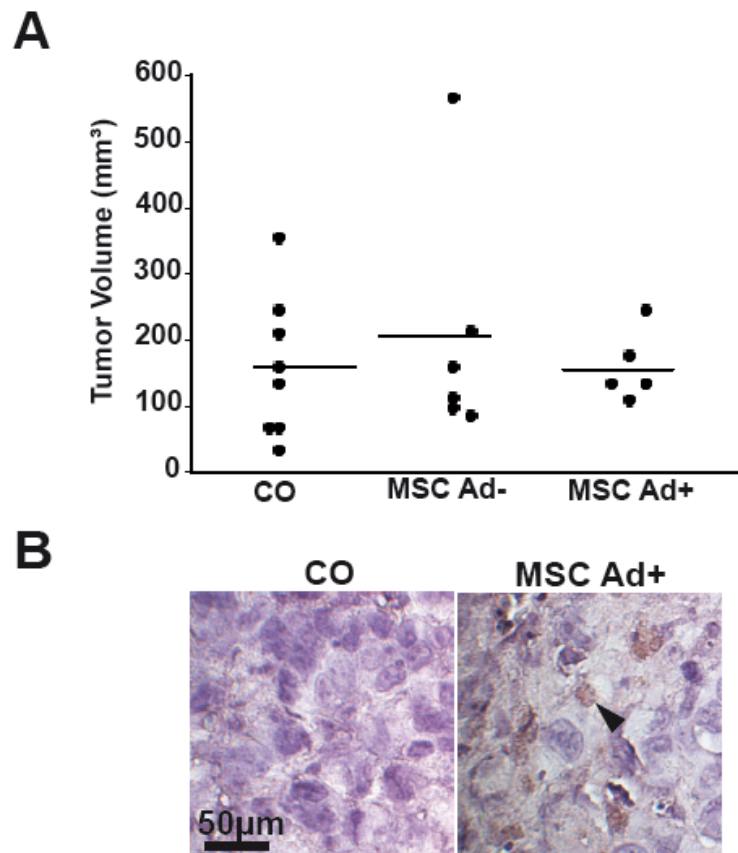


Figure 19: MSC infected with a non-replicating adenovirus invade tumours *in vivo*. (A) MSC were infected *in vitro* with a replication incompetent adenovirus (Ad-CMV) and injected into CAM arteries leading towards Panc-1 tumour xenografts 2 d before tumour resection. Tumour volumes were determined with mean tumour volumes shown. (B) Representative images of sections of resected tumours are shown at 400x magnification. Presence of infected MSC was detected by immunohistochemistry with antibodies against adenovirus capsid. Virus-containing MSC are indicated by arrowheads.

Cells infected with adenovirus were detected in tumour samples from each group after injection of infected MSC (Fig. 20) at a very low dissemination. The Ad-positive cells were mostly found near the margins of the tumours. No positive cells were observed in the control group. Virus injected without MSC (V-CO) was used as a control to investigate if the detected infected cells were indeed MSC. The identity of infected cells had to be proofed, as the injected MSC could have released virus particles into the blood stream. These viruses could possibly be carried to the tumour and infect malignant cells there. No infected cells were observed in samples from the V-CO group, suggesting that all the detected infected cells were indeed MSC. The

obtained results showed that injected, virus-infected MSC reached the tumour and where present in the tumour stroma.

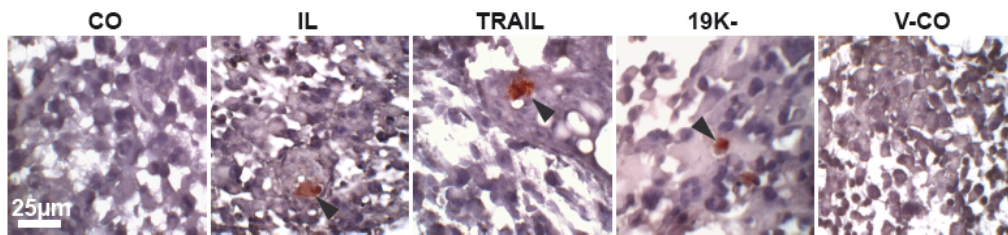


Figure 20: Oncolytic adenovirus-infected MSC invade tumours *in vivo*. Tumour cells were grafted at 9 days after beginning of incubation. MSC were injected two days before tumour resection, which was performed at incubation day 18, three days before the chicken would hatch. Representative images of paraffin-embedded tissue sections are shown at 400x magnification. Adenovirus infected cells were detected by immunohistochemistry for virus capsid. Positive cells were detected in MSC infected with each of the three viruses (Ad-IL, Ad-19K- and Ad-TRAIL). No positive cells were observed when virus was administered systemically without MSC (V-CO).

4.7 Application of Ad-infected MSC reduces tumour growth *in vivo*

Finally, the effect of a treatment by MSC-delivered oncolytic virus was evaluated *in vivo*. Oncolytic adenovirus-infected MSC were injected in CAM blood vessels two days after tumour transplantation. For injection, arteries leading to the tumour were chosen. The tumours were collected seven days after MSC injection. As the adenovirus lifecycle is two days long, this time should be sufficient for several replication and infection cycles. Ad-TRAIL was used as a further control being injected without MSC (V-CO) to compare its effect with the respective MSC-delivered virus. Whereas Ad-IL had no effect on tumour growth, the other two virus constructs, Ad-TRAIL and Ad-19K- reduced tumour growth *in vivo* (Fig. 21 A). The reduction in tumour size is only significant in the Ad-TRAIL treated group. Both Ad-TRAIL and Ad-19K- also reduced tumour take (percentage of tumours that developed after transplantation). The effect was more pronounced with Ad-TRAIL, which also strongly reduced tumour size. Tumour size reduction was also observed with V-CO treatment. In contrast to the MSC-delivered virus, V-CO treatment did not cause a reduction in tumour take and tumour size reduction was not significant. MSC-delivered infection with Ad-TR and Ad-19K- also decreased proliferation, as evident by a lower occurrence of the proliferation marker Ki67 (Fig. 21 B). Additionally, fewer cells expressed the CSC marker CD24. Ad-IL infected tumours exhibited less CD24

positive cells, but Ki67 presence was not lower than in uninfected tumours. No changes in regard to the control group were observed in samples from the V-CO group in quantity of these markers. Tumours from the Ad-TR and Ad-19K- groups displayed more cells positive for active caspase 3 than found in the other groups, indicating induction of apoptosis by infection with these viruses. These findings demonstrate that delivery of MSC infected with Ad-TR or Ad-19K- both reduce tumour growth, proliferation, and the amount of CSC and elevate apoptosis *in vivo*, with Ad-TRAIL having a more pronounced effect. MSC-delivered infection of Ad-IL in contrast showed no effect on the tumour. Compared with MSC-delivered Ad-TR, the systemically applied virus exhibited weaker antitumor effects, pointing to a more efficient virus delivery by the cell carriers.

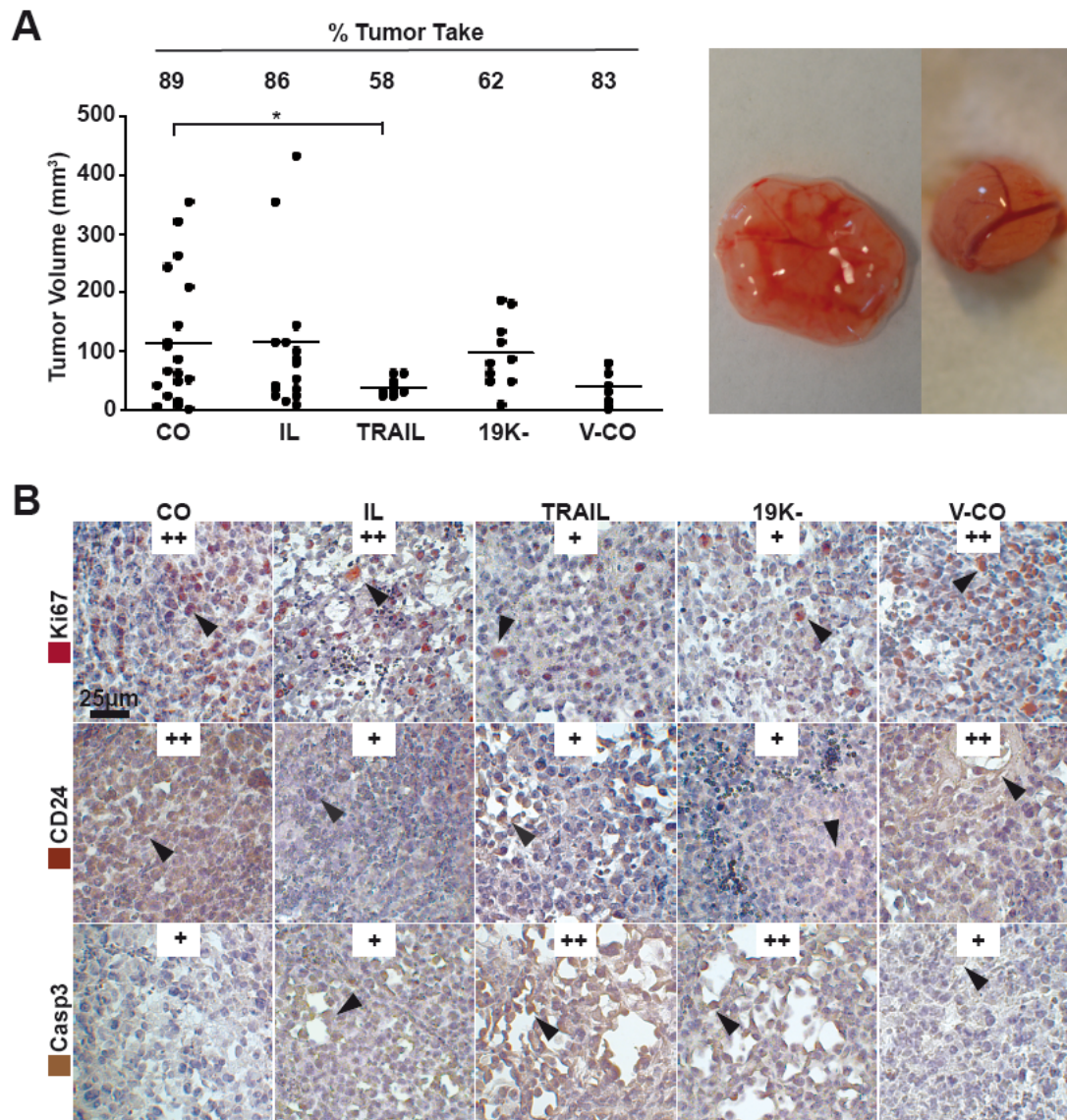


Figure 21: MSC-delivered oncolytic adenoviruses reduce tumour growth *in vivo*. (A) MSC infected with Ad-IL, Ad-TRAIL or Ad-19K- or Ad-TRAIL without MSC (V-CO) were injected in CAM blood vessels at day 11 of embryonic development, 2 days after tumour transplantation. MSC were infected with 2000 TCID₅₀. Tumours were resected at day 18. Tumour size was measured and tumour volume was calculated. Single tumours are represented by dots and mean volume is shown for each group (*P<0.05). (B) Immunohistochemical evaluation of sections from paraffin-embedded tumours. Representative images of each group are shown at 400x magnification. Positive cells are indicated by arrowheads.

5. Discussion

5.1 Elimination of pancreatic CSC by oncolytic adenoviruses

Despite recent progress in pancreatic cancer therapy the survival rates for the disease remain among the poorest for major malignancies [3]. The only curative treatment available is surgery, which is only possible in early stages of the disease. Other present therapies have only palliative functions [2]. The high amount of cells with CSC-like characteristics found in pancreatic tumours are thought to be mainly responsible for the poor success therapies have had so far [14]. These cells are composed of a population that is highly resistant to chemo- and radiation therapy [12]. Thus the elimination of the CSC population is a prerequisite for every effective therapy [85]. Oncolytic viruses like oncolytic adenovirus represent one promising agent being developed for this purpose [30, 31]. In the present study, the use of oncolytic adenoviruses for the elimination of pancreatic CSC was investigated. A main focus laid on virus delivery using bone marrow derived MSC. The study was performed in cooperation with the group of Dr Dirk Nettelbeck (DKFZ), who provided and optimized the oncolytic viruses that were used in my work. The group of Dr Nettelbeck demonstrated a better transduction of MSC and pancreatic cancer cells by viruses with a chimeric capsid, which was subsequently employed for all viruses used in this study. They also demonstrated a higher production and release of virus particles in MSC for certain armed viruses. These viruses exhibited in addition better oncolytic activity in both primary and established pancreatic cancer cells (Data is being prepared for publication). However, the assays performed by the group of Dr Nettelbeck showed mainly the effects on the bulk of the cancer cell population. Therefore, I examined in this work the elimination and infection of CSC. Besides elimination of CSC *in vitro* I demonstrated the infection of highly aggressive primary pancreatic CSC spheroids by all examined oncolytic viruses. Further, I successfully showed the invasion of tumour cells by oncolytic adenovirus-infected MSC both *in vitro* and *in vivo*. Finally, the effect of direct oncolytic adenovirus infection as well as the application of infected MSC was investigated in an *in vivo* model. In this model a reduction of tumour growth was shown.

In the present study, a control oncolytic adenovirus (Ad-SA GFP) was able to eliminate CSC *in vitro* completely, as defined by their tumour-initiating potential. The

fact that no surviving colonies could be detected even when a virus with no modifications increasing anti-tumour activity was used shows the potential of a therapy utilizing oncolytic adenovirus. Additionally, this assay ascertains that no CSC are resistant to adenovirus infection. This is further highlighted by the case of surviving uninfected colonies where methylcellulose prevented infection by released virus particles. Here a second infection with a very low virus concentration killed all surviving colonies. The results obtained by me contrast with the efficiency of gemcitabine, the most common drug used for pancreatic cancer therapy. Being the standard agent for chemotherapy of advanced pancreatic cancer it prolongs patient survival only for some months and is unable to efficiently eliminate the malignant cell population. Gemcitabine did not eliminate pancreatic CSC completely in reported similar assays, even when a sensitising agent was used [86]. This is especially the case as MIA-PaCa2, the cell line used in the present work, exhibits a high tolerance for gemcitabine. Such a resistance was also observed to build up in cells initially vulnerable to gemcitabine treatment [87]. It is a major problem in the clinical setting, where build-up of resistance is augmented by certain cell types found in the tumour stroma, like macrophages [88]. Thus, oncolytic adenovirus infection shows superior efficiency to gemcitabine in the elimination of pancreatic CSC. Moreover a virus therapy could be used in cases where the malignant cells have developed a resistance to gemcitabine. A resistance to gemcitabine often also results in less efficiency when the cells are treated with other cytotoxic agents [89]. The expression of multidrug resistance (MDR) proteins by the cancer cells is mostly responsible for this resistance to various, even unrelated drugs. This mechanism appears to have no effect on virus infection and virus-induced cell lysis [90, 91]. A co-treatment of oncolytic adenovirus and cytotoxic drugs like gemcitabine could be proved to have a synergistic effect *in vivo* even in a highly drug- and virus-resistant cell line [92]. Co-treatment in a mouse xenograft model of ovarian cancer with a combination of a modified adenovirus and gemcitabine resulted in an improved survival when compared with gemcitabine only treatment [93]. These previous studies and the present results suggest that oncolytic adenoviruses can efficiently eliminate pancreatic CSC and overcome resistance to conventional cytotoxic agents.

5.2 Infection of primary pancreatic CSC by oncolytic adenoviruses

While oncolytic adenovirus infection showed an excellent performance in established cell lines in this study, primary cancer cell lines often differ in their sensitivity to therapeutic agents from long established cancer cell lines like MIA-PaCa2 or Panc-1. A prolonged cultivation as essentially single-cell organisms can lead to selection of cells with specific mutations. The cell culture medium used can also alter cell characteristics from the original specimen [94]. Therefore the capability of the adenoviruses to infect CSC from primary cell lines was investigated in the present work. I examined this ability of four different oncolytic adenovirus strains, a control strain containing a luciferase gene (Ad-IL) and three virus constructs with enhanced oncolytic activity (Ad-TRAIL, Ad-19K- and Ad-FCU1). The cancer cell spheroids were established from resected mouse xenografts of primary human tumours. As only CSC can grow as anchorage-independent cells the spheroids represent an almost pure CSC population [95]. All of the oncolytic adenoviruses infected the primary CSC spheroids. The infection of the CSC cells in particular was demonstrated by the presence of cells positive for both adenovirus and c-Met. This demonstrates that also patient-derived primary CSC are infected by the viruses. However, the infection rate appeared to be lower than in the established cell lines. Thus, the virus concentration has likely to be adjusted when primary tumours will be targeted with the oncolytic adenoviruses.

5.3 Reduction of tumour growth *in vivo* by oncolytic adenovirus infection

Malignant cells cultivated in cell culture are often more sensitive to chemotherapy agents than cells in tumour tissue *in vivo* [96]. One cause is the better accessibility to cytotoxic agents of cells growing as a monolayer or as a spheroid, as most cells have direct contact to the drug-containing medium. This leads also to a better nutrition and oxygenation of the cells than in often poorly vascularized tumours. This stressed conditions and interaction with host cells increase therapy resistance in tumours. In addition, tumour tissue is composed of malignant cells and tumour stroma containing necrotic areas and fibrous tissue shielding many tumour cells from chemotherapy [9]. Tumour stroma could also be a serious obstacle for virus spread in the tumour. Tumour transplants in fertilized chicken eggs form a complex tissue containing

stromal cells and blood vessels formed by invaded chicken cells [97, 98]. Therefore, it is an excellent model to study therapy delivery and effect in a tumour tissue with a morphology resembling that found in patient samples [99]. This model was used in the present study to explore the efficiency of the oncolytic viruses *in vivo*. When malignant cells containing a small fraction of oncolytic adenovirus-infected cells were transplanted on the CAM of fertilized chicken eggs, the infection reduced tumour size considerably. The effect on tumour size correlated with a striking change in tumour morphology. While MIA-PaCa2 cells formed a tissue that was mainly composed of loosely organised cells, the infected tumours contained several clusters of densely packed cells. These clusters contained cells, which were smaller than the cells outside of the clusters. While chicken cells, which are present in CAM transplants, are smaller than the human malignant cells, the majority of the small cells in the clusters were human cells. This was confirmed by their expression of human proteins like cytokeratin 19. A rounding of cells after infection with adenovirus has been reported previously [100]. Therefore, the cytopathic effect induced by the viral infection appears to cause the phenotype observed in my experiment. This is also indicated by the fact that virus protein could only be detected in these clusters. Virus was not present in each cluster and could only be detected in tumours transplanted together with 5% infected cells. So it is likely that a certain threshold exists for the detection of virus capsid proteins by the antibody. As virus particles are not big enough to be seen directly with optical microscopes, a high concentration is needed for a positive staining. Consistent with these results virus infection decreased proliferation, as seen by decreased levels of Ki67. Additionally, there were fewer cells positive for the CSC marker CD24 in infected tumours. This effect was especially pronounced in the dense clusters, where hardly any positive cells were found. The absence of CD24 in the clusters points at an efficient elimination of CSC cells by the virus also taking place *in vivo*. My findings are in line with other studies where a reduction of the CSC population has been described for several adenoviral vectors [101, 102]. The present results show not only the anti-tumorigenic effect of the oncolytic virus, but it shows as well that tumour transplants on the CAM of fertilized chicken eggs are an excellent model for evaluation of virotherapy *in vivo*.

5.4 Invasion capability of oncolytic adenovirus-infected MSC

MSC can be easily isolated from adult humans and have been reported to invade tumour tissue [74]. These factors make them excellent candidates for transportation of cytotoxic agents to tumours [76]. The ability of MSC to invade tumour tissue is crucial for their usage as virus carriers. While virus delivery by MSC had been demonstrated previously [64, 65] modifications of the used viruses could have had an impact on MSC migration. To demonstrate that this ability is still intact after infection with the different virus constructs, an *in vitro* invasion assay was performed by me. The infected MSC had to invade tumour spheroids through a gel layer. MSC infected with the four different oncolytic viruses (AD-IL, Ad-TRAIL, Ad-19K- and Ad-FCU1) all invaded tumour spheroids after infection. No significant differences were observed in regard to uninfected control cells. Although a stimulation of MSC migration by TRAIL was reported [103], no superior migration ability was validated for the Ad-TRAIL infected population in the present study. As TRAIL is expressed mainly in the late stage of infection it is very probably not present during the main time point of invasion. MSC infected with Ad-19K- were able to invade the tumour spheroids similar to MSC from the other groups. Ad-19K- contains a deletion of the anti-apoptotic E1B19K gen causing an earlier killing of infected cells [54]. MSC infected with this virus exhibited a rounded, apoptotic morphology at later time points of infection, while cells infected by the other viruses still showed a normal phenotype at similar time points. But as MSC migration and invasion occurred right after infection the earlier cell death apparently did not interact with the invasion, as shown in this study. However, MSC invasion varied strongly between different spheroids. Therefore, an exact quantitative analysis of invaded cells could not be performed. This was especially true for PaCaDD spheroids, where also many single, floating MSC were observed. An explanation could be the more tight and compact morphology of these spheroids when compared to MIA-PaCa2 spheroids. This could have impeded MSC integration into the spheroids. But also in this situation some MSC invaded the PaCaDD spheroids, which demonstrates the homing ability of oncolytic adenovirus-infected MSC. These results show that MSC exhibit tumour tropism after infection with each of the oncolytic adenoviruses and are suitable vectors for their delivery. The present study confirms previous results where homing of virus-infected MSC to different tumour types, even ones with poor accessibility like glioma, has been demonstrated [80, 81].

5.5 *In vivo* tumour invasion by oncolytic adenovirus-infected MSC

The homing ability of infected MSC was studied on tumour transplants in fertilized chicken eggs in the present work. The grafted tumours are highly vascularized by blood vessels from the CAM and integrated into the blood circulatory system of the chicken embryo. The CAM is the place of gas exchange in the developing egg and is used for the examination of angiogenesis processes [104]. Therefore it is an ideal model for the investigation of MSC homing *in vivo*. In the present case, infected MSC were injected into arteries leading towards the tumour. As the MSC should be transported directly to the tumour, they should not have to pass through the whole circulatory system. Passing through the embryo would very likely reduce the amount of MSC, as they would be stuck in the liver and lung of the embryo. While bleeding occurred after injection, it soon stalled and did not harm the embryo. The injected MSC could be detected two days after injection in resected tumours. Only a few, dispersed infected cells were observed on each section. All MSC were present at the margins of the tumours and no MSC were found in central areas of the tumours. No infected cells were detected at this time point if virus had been administered without MSC. Therefore, delivery with MSC appears to be more efficient than systemic injection of virus particles. Moreover, as virus is replicating and released from infected cells, a relative small number of MSC could be sufficient for a measureable therapeutic effect. My results confirm previous studies done in the mouse model where tumour infiltration by oncolytic adenovirus-infected MSC has been demonstrated and where MSC-delivered virus was present in the tumour microenvironment, while systemic spread was reduced compared to virus injected without MSC [105]. Similar results have been achieved with measles virus-infected MSC in a mouse model for ovarian cancer [79]. The highly dispersed invasion pattern exhibited by the infected stem cells in the present study could be an advantage. In another study the direct injection of an oncolytic adenovirus into a tumour xenograft on the CAM resulted in only very localized infection [99]. Therefore, a wide distribution of initially infected cells should cause a wider dissipation of the virus and thus produce many infection hosts throughout the tumour. The ideal cell number for a therapy has to be evaluated in a more complex model like rodents and ultimately in trials with human patients to establish an efficient therapy protocol for the clinical setting.

5.6 Therapy with MSC-delivered oncolytic adenoviruses

A successful virus delivery through bone marrow-derived MSC was successfully verified in CAM tumour transplants in the present study. In addition to effective virus delivery, the efficacy of the oncolytic viruses in eliminating tumours is a major issue determining the overall potency of the therapy. Previous studies reported an anti-tumour effect of therapies using oncolytic adenovirus, both *in vitro* and in pancreatic cancer xenograft models [106, 107]. In the present study, pancreatic cancer cell transplants on the CAM were used to evaluate therapy efficiency and to compare the different used adenoviruses. Virus-infected MSC were injected 7 d before tumour resection. As the initially infected cells are lysated after 48 h, this time should be enough for the spread of the infection. A clear contrast between the efficiency of the different used viral constructs was detected. Infection with Ad-IL caused no differences in tumour growth when compared to uninfected tumours. Ad-IL does not possess enhanced anti-tumour activity. However, a similar virus (Ad-SA GFP) exhibited clear CSC eradication *in vitro* and also *in vivo*, when the infection was present upon transplantation. Reasons for the poor performance *in vivo* could be a low virus spread. This would be especially hindered in this case by the stroma, which is composed of chicken cells that cannot be infected by the human-specific virus. In addition, existing data points to an important role of the immune system in enhancing the anti-tumour activity of oncolytic viruses [36, 108]. The developing chicken embryo lacks a fully functional immune system. Therefore, the CAM membrane represents an immunologically incompetent model [109]. The immune-compromised state of the tumours could therefore lower therapy efficiency, especially in the case of non-armed oncolytic viruses. Another critical factor is time or more specifically the duration of the therapy. As viral replication takes at least two days viral spread needs a certain time. Especially if obstacles like non-infective stroma cells are taken into account. Treatment and evaluation in mouse tumour models takes place in a period of a few weeks allowing more time for virus replication and spread. In other studies, the effect of a therapy has been shown to be more pronounced at later time points exceeding one week [106, 110]. An administration of several low doses also proved effective against pancreatic cancer tumours [111]. In the present study only a single administration was used. In contrast, several treatment rounds would be used presumably in a cancer therapy with oncolytic viruses in the clinical setting. In

contrast to Ad-IL, the other two viruses, which possess enhanced oncolytic activity, displayed a pronounced effect on tumour growth in our model. Both viruses reduced tumour size and tumour take. Ad-TRAIL caused a stronger anti-tumorigenic effect than Ad-19K-. While both viruses are armed, Ad-19K- is only able to kill directly infected cells. Ad-TRAIL on the other hand possesses a prominent bystander effect, as released TRAIL proteins can induce apoptosis in non-infected cells. The enhanced efficiency of oncolytic adenoviruses expressing TRAIL has been demonstrated in several studies [39, 112, 113]. While Ad-19K- proved less efficient on its own, a synergistic effect of an E1B19K mutant oncolytic adenovirus together with gemcitabine has been described [114]. Therefore Ad-19K- could be suited for use in a combination therapy together with DNA-damaging drugs. The anti-tumorigenic activity of both armed viruses was additionally confirmed in the present study by a reduction of proliferation and CSC markers and elevation of an apoptosis marker. Directly administered Ad-TRAIL demonstrated also some therapeutic potential. But the effect was not as pronounced as with the MSC-delivered virus. This is shown clearly by the lack of influence on the examined markers or on tumour take by the directly administered virus. The current data confirms a previous report from a clinical trial, where only the MSC-delivered oncolytic viruses increased patient survival [79]. In another study a MSC subpopulation even decreased virus immunity in melanoma xenografts [115]. Therefore, MSC can likely not only act as virus carriers, but also even enhance their therapeutic potential in certain circumstances. Additionally MSC prevent virus neutralisation by antibodies. While MSC-delivered oncolytic viruses prolonged survival in an immunized mouse model of ovarian cancer naked virus did not [65]. This result points to an even more important role of virus carriers in immune-competent models. A possible immune-competent model for future studies is the Syrian hamster. Syrian hamster tissue supports the replication of adenovirus serotype 5 well [116]. Therefore the Syrian hamster is a capable model for the study of virotherapy. A study in Syrian hamster could demonstrate the induction of tumour-specific immunity by treatment with adeno- and/or vaccinia virus [111]. The use of this model would also enable the study of toxicity and side effects of adenoviral treatment. Side effects cannot be assessed in the CAM transplants or in other, more commonly used rodents, like mice or rats, as none of these models supports adenovirus replication. However, similar oncolytic adenoviruses have been used in clinical trials without exhibiting severe toxicity in patients [40, 41]. These results point

to an excellent safety profile of oncolytic adenoviruses as therapeutic agents. From the present data, the two viruses with enhanced oncolytic activity, especially Ad-TRAIL, appear to be the best candidates for further evaluation and therapy development. It also shows that the approach with MSC-delivered oncolytic adenovirus as a therapeutic agent is superior over directly applied virus and exhibits strong anti-tumorigenic effects.

5.7 Conclusion

The present study demonstrates the efficacy of human bone marrow-derived MSC as carriers for oncolytic adenovirus in pancreatic cancer treatment. Oncolytic adenoviruses eliminated pancreatic CSC *in vitro* completely, as was shown by the abolition of their colony forming ability. Further, all examined adenoviruses could infect highly aggressive CSC from primary tumour spheroids. Therefore, they are likely to exhibit a profound activity against pancreatic CSC in a clinical setting. Infection with adenoviruses did not interfere with MSC homing *in vitro*, demonstrating the suitability of MSC as virus carriers. Moreover, adenovirus-infected MSC were able to invade pancreatic tumour cells and to deliver the virus after injection into blood vessels in an *in vivo* setting. Two evaluated MSC-delivered adenoviruses, Ad-19K- and Ad-TRAIL, reduced tumour growth *in vivo*. Additionally they reduced the amount of CSC and elevated apoptosis. However, Ad-TRAIL exhibited a superior effect. Therefore, MSC-delivered oncolytic adenoviruses are a novel promising treatment for the currently incurable pancreatic cancer. A therapy is likely to be especially promising if armed viruses with superior anti-tumorigenic potential like TRAIL expressing viruses are used. This therapy has the potential to improve the poor performance of today's pancreatic cancer treatment either on their own or in combination with classic cytotoxic agents, like gemcitabine. However, further evaluation of therapy parameters like toxicity and virus distribution in mouse or similar models will be needed for establishing an effective and safe therapy protocol before clinical trials can be conducted.

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7. Publications

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2. Appari M, Kamesh RB, **Kaczorowski A**, Groß W, Herr I (2014). Sulforaphane, quercetin and catechins complement each other in elimination of advanced pancreatic cancer by miR-let-7 induction and K-ras inhibition. *Int J Oncol* Accepted for publication.
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