ONCOLYTIC ADENOVIRUS CODING FOR GM-CSF IN TREATMENT OF CANCER

Simona Bramante

Cancer Gene Therapy Group

Department of Pathology

Doctoral Programme in Clinical Research

Faculty of Medicine

University of Helsinki

Finland

ACADEMIC DISSERTATION

To be publicly discussed with the permission of the Faculty of Medicine of the University of Helsinki, in Haartman Institute, Lecture Hall 2, on 2nd of October 2015, at noon.

Helsinki 2015

Supervised by

Akseli Hemminki, MD, PhD,
Erkko Professor of Oncology

Cancer Gene Therapy Group,
Department of Pathology,
Faculty of Medicine,
University of Helsinki, Finland

Anniina Koski, MD, PhD

Cancer Gene Therapy Group,
Department of Pathology,
Faculty of Medicine,
University of Helsinki, Finland

Thesis Committee

Satu Mustjoki, MD, PhD, Docent

Hematology Research Unit Helsinki,
Department of Medicine,
Helsinki University Central Hospital,
University of Helsinki, Finland

Hanna Jarva, MD, PhD, Docent

Department of Bacteriology and
Immunology
University of Helsinki, Finland

Reviewers appointed by the Faculty

Kimmo Taari, MD, PhD,
Professor of Urology

Department of Urology,
Helsinki University Central Hospital,
University of Helsinki, Finland

Hanna Jarva, MD, PhD, Docent

Department of Bacteriology and
Immunology,
University of Helsinki, Finland

Official Opponent

Eivind Per Thor Straten, PhD,
Clinical Professor

Center for Cancer Immunotherapy,
Department of Haematology,
Copenhagen University Hospital Herlev, Denmark

Dissertationes Scholae Doctoralis Ad Sanitatem Investigandam Universitatis Helsinkiensis

No. 73/2015
ISBN 978-951-51-1504-1 (paperback)
ISBN 978-951-51-1505-8 (PDF)

http://ethesis.helsinki.fi
ISSN 2342-3161 (print)
ISSN 2342-317X (online)

“Above all, don't fear difficult moments. The best comes from them.”
Rita Levi-Montalcini

To mamma&papá

TABLE OF CONTENTS

PART A

List of original publications

The thesis is based on the following publications:

I. **Bramante S**, Koski A, Kipar A, Diaconu I, Liikanen I, Hemminki O, Vassilev L, Parviainen S, Cerullo V, Pesonen SK, Oksanen M, Heiskanen R, Rouvinen-Lagerström N, Merisalo-Soikkeli M, Hakonen T, Joensuu T, Kanerva A, Pesonen S, Hemminki A. Serotype chimeric oncolytic adenovirus coding for GM-CSF for treatment of sarcoma in rodents and humans. *Int J Cancer*. 2014 Aug 1;135(3):720-30.

II. Siurala M, **Bramante S**, Vassilev L, Hirvinen M, Parviainen S, Tähtinen S, Guse K, Cerullo V, Kanerva A, Kipar A, Vähä-Koskela M, Hemminki A. Oncolytic adenovirus and doxorubicin-based chemotherapy results in synergistic antitumor activity against soft-tissue sarcoma. *Int J Cancer*. 2015 Feb 15;136(4):945-54.

III. **Bramante S**, Kaufmann JK, Veckman V, Liikanen I, Nettelbeck DM, Hemminki O, Vassilev L, Cerullo V, Oksanen M, Heiskanen R, Joensuu T, Kanerva A, Pesonen S, Matikainen S, Vähä-Koskela M, Koski A, Hemminki A. Treatment of melanoma with a serotype 5/3 chimeric oncolytic adenovirus coding for GM-CSF: Results in vitro, in rodents and in humans. *Int J Cancer*. 2015 Oct 1;137(7):1775-83.

IV. **Bramante S**, Koski A, Liikanen I, Vassilev L, Oksanen M, Siurala M, Heiskanen R, Hakonen T, Joensuu T, Kanerva A, Pesonen S, Hemminki A. Oncolytic virotherapy for treatment of breast cancer, including triple-negative breast cancer. *OncoImmunology*. In press, accepted on July 22nd, 2015.

The publications are referred to in the text by their Roman numerals.

Abbreviations

4-HP-CP	4-hydroperoxycyclophosphamide
Ad	Adenovirus
ALT	Alanine amino transferase
AST	Aspartate amino transferase
AR	Adverse reaction
ATAP	Advanced Therapy Access Program
BRAF	v-Raf murine sarcoma viral oncogene homolog B
ATP	Adenosine triphosphate
CAR	Coxsackie-adenovirus receptor
CAR T-cell	Chimeric antigen receptor (CAR) T-cell
CEA	Carcinoembryonic antigen
CMR	Complete metabolic response
CMV	Cytomegalovirus
Cox	Cyclo-oxygenase
CP	Cyclophosphamide
CPE	Cytopathic effect
CR	Complete response
CRAd	Conditionally replicating adenovirus
CT	Computed tomography
CTCAE	Common Terminology Criteria for Adverse Events
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T-lymphocyte-associated antigen 4
DC	Dendritic cell
dsDNA	Double stranded DNA
DSG-2	Desmoglein 2 protein
ER	Estrogen receptor
FBS	Fetal bovine serum
FIMEA	Finnish Medicines Agency
FTV	Fractional tumor volume
GM	Growth medium
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HER-2	Human epidermal growth factor receptor 2
HMGB1	High-mobility group box 1 protein
HNC	Head and neck cancer
hTERT	Human telomerase reverse transcriptase
ICD	Immunogenic cell death
IFN	Interferon
IL	Interleukin

i.p.	Intraperitoneal
Luc	Luciferase
MHC	Major histocompatibility complex
MMR	Minor metabolic response
MR	Minor response
MRI	Magnetic resonance imaging
NK	Natural killer
PD	Progressive disease
PD-1	Programmed cell death-1 protein
PET	Positron emission tomography
pfu	Plaque-forming units
PMD	Progressive metabolic disease
PMR	Partial metabolic response
PR	Progesteron receptor
PR	Partial response
PSA	Prostate-specific antigen
Rb	Retinoblastoma
RECIST	Response Evaluation Criteria in Solid Tumors
RGD	Arginine-Glycine-Aspartic acid motif
SAE	Serious adverse event
SD	Stable disease
SMD	Stable metabolic disease
STS	Soft-tissue sarcoma
TAA	Tumor associated antigen
TCC	Transitional cell carcinoma
$TCID_{50}$	Tissue culture infectious dose 50%
TCR	T-cell receptor
TIL	Tumor-infiltrating lymphocyte
TK	Thymidine kinase
TLR	Toll-like receptor
TNBC	Triple-negative breast cancer
TNF	Tumor necrosis factor
TP53	Tumor protein p53
T-reg	Regulatory T-cell
UV	Ultraviolet radiation
VEGF	Vascular endothelial growth factor
VP	Viral particles
wt	Wild-type
WHO	World Health Organization

Abstract

Despite the numerous advances in cancer therapy over the past 50 years, cancer still remains the major cause of mortality worldwide, and thus new and more efficient treatments are needed. Oncolytic viruses have shown promising results in preclinical and clinical studies for the treatment of solid tumors, but their efficacy often remains low.

A multitude of viral strains, such as adenovirus, have been engineered to become tumor-selective and to stimulate the immune system against the tumors. Alteration of viral surface proteins that recognize specific cellular receptors, modification of viral genes required for viral replication and insertion of immunostimulatory molecules in the viral genome are promising ways to allow the virus to specifically enter and kill cancer cells by oncolysis, and to stimulate antitumoral immune responses. One example of oncolytic virus featuring these three modifications is Ad5/3-D24-GMCSF, a tumor-selective 5/3 chimeric oncolytic adenovirus armed with the immunostimulatory granulocyte-macrophage colony-stimulating factor (GM-CSF). In preclinical models, Ad5/3-D24-GMCSF has displayed good antitumor efficacy, production of functional GM-CSF and tumor-selective replication. Good tolerability, possible treatment benefits and activation of immune responses have also been observed in cancer patients.

In this thesis, we studied the utility of Ad5/3-D24-GMCSF in the treatment of sarcoma, melanoma and breast cancer. The data is promising with regard to future clinical trials and for personalized cancer treatments.

The virus showed strong oncolytic potential *in vitro* and antitumor efficacy in immunodeficient animal models. Furthermore, replication-linked GM-CSF production stimulated the differentiation of human monocytes into macrophages,

important for induction of antitumor immune responses. In immunocompetent Syrian hamsters with soft-tissue sarcoma (STS) tumors, Ad5/3-D24-GMCSF reached non-injected tumors through vascular circulation, suggesting its utility for the treatment of metastatic disease.

Combination of Ad5/3-D24-GMCSF with chemotherapeutics that possess immunogenic properties (doxorubicin and ifosfamide) and that selectively reduce circulating regulatory T-cells (cyclophosphamide) was studied to enhance antitumor efficacy. Our results showed that chemotherapeutic agents may have a useful combination effect with the virus, due to enhancement of adenoviral replication and induction of immunogenic cell death, setting the stage for clinical testing of combination regimens.

Finally we reported safety and possible signs of efficacy in 40 patients with advanced sarcoma, melanoma and breast cancer, who were treated in the context of an Advanced Therapy Access Program (ATAP). Treatments were overall well-tolerated, and objective signs of treatment benefits were also observed. Therefore, our results confirm previous good data regarding Ad5/3-D24-GMCSF as a promising agent for treatment of cancer. Furthermore, our data may prove useful for clinical development of oncolytic adenoviruses combined with low-dose chemotherapy for the treatment of advanced sarcoma, melanoma and breast cancer, and may help to design optimal clinical trials, selecting the right patient populations. A phase I clinical trial studying Ad5/3-D24-GMCSF has been recently completed, and phase I/II trials studying combination regimens are in the planning stages.

PART B

1. REVIEW OF THE LITERATURE

1.1 Introduction

Cancer is among the most common causes of mortality worldwide, with approximately 8.2 million cancer-related deaths out of 14.1 million new cases in 2012 (World Cancer Report 2014, WHO, IARC). The five most common cancers in men are lung, prostate, colorectal, stomach, and liver cancer, while among women, the most common diagnosed cancers are breast, colorectal, lung, cervix, and stomach cancer. Cancer is more prevalent in less developed regions, including Africa, Asia, South and Central America (which account for 70% of the world's cancer deaths), which reflects the lack of adequate health care infrastructure and effective treatment options. Worldwide, it is expected that the annual number of cancer cases will rise from 14.1 million in 2012 to 22 million within the next two decades.

Cancer is a heterogeneous disease, related to many different factors, including genetics, age and potentially avoidable lifestyle risk factors, among which smoking, lack of physical activity, alcohol use, diet (low fruit and vegetable intake), overweight and obesity, and viral infections (Worldwide cancer Key Stats 2012). Many cancers can be cured if detected and treated early and adequately. The most common types of cancer treatment are surgery, chemotherapy, and radiation therapy. Because cancer is a generic term to indicate a large group of genetic disorders which can develop in any part of the body, the existence of only one cure for all cancer types is not expected. Instead, combinations of different therapies can be used to attack the tumors from different angles.

Cancer gene therapy is an emerging field which offers different treatment approaches aiming at modifying cells using genes. Oncolytic viruses showed great promise, and several vectors have been tested preclinically and clinically, with positive clinical phase III results obtained in 2013 (Kaufman et al., 2014), and an oncolytic adenovirus was approved in China already in 2005 for the treatment of head and neck cancer (Garber, 2006, Guo and Xin, 2006). In addition to their ability to replicate and lyse cancer cells, oncolytic viruses can also activate the immune system, inducing innate and antigen-specific adaptive immunity against the tumor (Tuve et al., 2009, Alemany, 2008). Viruses can also be armed with immunostimulatory transgenes, to further improve antitumor properties (Cerullo et al., 2010). However, despite the promising results with a wide range of tumors, their overall efficacy needs to be improved, and one method could be to combine them with current cancer treatment modalities.

1.2 Sarcoma

Sarcomas are malignant tumors of connective tissues. They comprise a heterogeneous group of neoplasms of mesenchymal origin and can be generally grouped into two categories, soft-tissue sarcoma (STS) and primary bone sarcoma (**Table 1**), each with different staging and specific treatment modalities (Skubitz and D'Adamo, 2007). Sarcomas are relatively uncommon in adults (1% of all adult solid cancers), but they are among the most frequent tumors in young adults and children, constituting over 20% of all pediatric solid malignant cancers (Burningham et al., 2012). The three most important prognostic variables are histologic grade, size, and location of the primary tumor (Ducimetiere et al., 2010). Primary tumors may remain essentially asymptomatic for extended periods of time. Therefore, a large proportion of patients develop metastatic disease, which is often incurable with available treatments, thus survival rate in sarcoma remains low (McClay, 1989). Although most sarcomas do not have known causes, some risk factors have been identified: exposure to ionizing radiation, for example radiation therapy for a previous cancer (Amendola et al., 1989); genetic syndromes, for example neurofibromatosis type I, Gardner syndrome, Werner syndrome, Li-Fraumeni syndrome, and familial form of retinoblastoma (Hawkins et al., 1987); exposure to chemicals (vinyl chloride, dioxin), and human herpesvirus 8 infection, which plays a role in the development of Kaposi sarcoma (Whitby et al., 1995).

Soft-tissue sarcoma (STS)

Soft tissue sarcomas represent less than 1% of malignancies, and may arise in soft tissues of the body. The most common types of STS are shown in **Table 1**. Localized resected tumors can often be controlled by surgery and radiotherapy, while for high-grade and metastatic disease, chemotherapy is the primary treatment modality (Reed and Altiok, 2011). Chemotherapy may be used before or after

surgery, to shrink the tumor or prevent its recurrence (Skubitz and D'Adamo, 2007). For the treatment of STS, the two most commonly used chemotherapeutic agents are doxorubicin and ifosfamide (discussed in section 1.5.2), but also gemcitabine, dacarbazine, temozolomide and combination of these have been used (Gottlieb et al., 1972, Awada et al., 2004). The benefits of a single agent vs combination therapy remain subject of study. More recently, immunotherapeutic strategies have been developed, which could affect both local and systemic disease, acting over a prolonged period of time (Finkelstein et al., 2012). Cytokines, interferon, tumor vaccines, immunologic checkpoint blockade antibodies, adoptive cell transfer have shown promising results by enhancing immune responses (D'Angelo et al., 2014). Recently, oncolytic viruses have been used for the treatment of pediatric sarcomas with encouraging results, and five clinical trials are currently ongoing (Lettieri et al., 2012).

Table 1. Sarcoma subgroups (Skubitz and D'Adamo, 2007)

Soft-tissue sarcomas	**Primary bone sarcomas**
Malignant fibrous histiocytoma	Osteosarcoma
Liposarcoma	Ewing sarcoma
Leiomyosarcoma	Giant cell tumor
Synovial sarcoma	Chondrosarcoma
Dermatofibrosarcoma protuberans	
Angiosarcoma	
Kaposi sarcoma	
Gastrointestinal stromal tumor	
Aggressive fibromatosis	
Rhabdomyosarcoma	
Primary alveolar soft-part sarcoma	

1.3 Melanoma

Melanoma is a type of skin cancer which develops from melanocytes. The incidence of melanoma is increasing rapidly, mainly due to increased ultraviolet radiation (UV) exposure, with around 232 000 new cases diagnosed in 2012 worldwide (2% of all cancers) (Ferlay et al., 2010). Due to its aggressive nature, malignant melanoma is the most common cause of mortality from skin cancer worldwide (Ferlay et al., 2010). The key risk factors for skin cancer are mainly linked to lifestyle: exposure to solar radiation and carcinogenics, including tobacco and alcohol, diet, lack of physical activity, overweight and obesity (Cogliano et al., 2011, Elwood and Jopson, 1997). Based on the stage of the cancer and other factors, melanoma treatments include surgery (for early-stage melanomas), chemotherapy, radiation therapy, targeted therapy, immunotherapy or a combination of immunotherapy and chemotherapy (biochemotherapy) (Bhatia et al., 2009). The most used chemotherapeutic agents for the treatment of melanoma are dacarbazine and temozolomide, despite modest efficacy and lack of data for survival benefit. With advances in the use of immunotherapy, chemotherapy gained a secondary role and it is generally not used as the initial treatment for patients with a late-stage disease. Immunotherapy with ipilimumab (anti-cytotoxic T-lymphocyte-associated antigen 4 antibody) and targeted therapy with BRAF (v-Raf murine sarcoma viral oncogene homolog B)-inhibitor vemurafenib, have been shown to improve survival of patients, becoming the preferred approaches for most patients with metastatic melanoma (Wolchok et al., 2010, Swaika et al., 2014). With regard to oncolytic viruses, T-VEC (discussed in section 1.8.2) has become the first oncolytic virus to successfully complete a phase III trial in advanced melanoma (Andtbacka et al., 2015), and the first oncolytic virus to be approved by the US Food and Drug Administration (FDA) (Dolgin, 2015).

1.4 Breast cancer

Breast cancer is the most common cancer in women worldwide, with over 508 000 breast cancer deaths in women in 2011 (Global Health Estimates, WHO 2013). Breast cancer incidence is particularly increasing in developing countries due to the lack of early detection programs. The most common risk factors associated with breast cancer are: familial history, in particular mutations in some genes, including *BRCA1*, *BRCA2* and *TP53*; prolonged exposure to endogenous and exogenous hormones, such as estrogens associated with reproductive factors (early menarche, late menopause, late age at first childbirth) and use of oral contraceptive and hormone replacement therapy; alcohol use, overweight and obesity, and inadequate physical activity (Lacey et al., 2009, Danaei et al., 2005). Breast cancer is characterized by several distinct biological subtypes associated with specific biological behavior and different clinical outcomes. Based on the expression of some particular receptors, tumors can be classified in: estrogen (ER)-receptor and progesterone (PR)-receptor positive, human epidermal growth factor receptor 2 (HER2)-positive, triple positive (positive for ER, PR and HER2), and triple negative (not positive for ER, PR and HER2) (Sandhu et al., 2010). Thus, the first critical step in diagnosis is to determine the stage, the receptor status and the spread of the tumor, in order to plan the most effective treatment program. Surgery, radiotherapy and chemotherapy are the most common treatment modalities, but they can be effective as single therapies only for a small number of cancers, usually localized and small in size (World Health Organization, 2015). Other common therapies such as hormone therapy (i.e. aromatase inhibitors or tamoxifen) and biological treatments (i.e. trastuzumab), are effective only for hormone receptor-positive and HER2+ breast cancers, often in combination with surgery and radiotherapy (Buzdar, 2009). With regard to breast cancer treatments using oncolytic viruses, a herpes simplex oncolytic virus has recently been shown to be

effective against breast cancer stem cells and for the treatment of metastatic breast cancer *in vitro* and *in vivo* (Wang et al., 2012a, Li et al., 2012).

Triple-negative breast cancer (TNBC)

Triple-negative breast cancer (TNBC) is a subtype of breast cancer defined as ER-, PR- and HER2- (Sandhu et al., 2010). TNBC (10–15% of breast cancers) is insensitive to the treatment modalities available for hormone receptor positive and HER2+ diseases, and thus it is the most aggressive breast cancer subtype, associated with a poor prognosis (Ovcaricek et al., 2011). TNBC is typically currently treated with a combination of surgery, radiotherapy and chemotherapy (Kern et al., 2013, Liedtke et al., 2008, Andre and Zielinski, 2012), but treatment choices are still limited, highlighting the need for new treatment regimens in this patient group.

1.5 Conventional and novel cancer therapies

1.5.1 Surgery

Surgery has been the predominant form of treatment for most types of cancer since 1846, when ether was first used as a surgical anesthetic (Gallucci, 2008). For patients with early-stage cancer, surgery is almost always the first step, and it may be the only treatment needed. However, following surgery, patients frequently relapse. Paradoxically, scientific evidence has revealed that surgery itself can contribute to the development of both local recurrences and distant metastases (van der Bij et al., 2009), emphasizing the need for other treatment options.

1.5.2 Radiotherapy

Radiotherapy has been used for treatment of cancer widely since 1895, when X-rays were discovered, with approximately 40% of cancer patients having it as part of their treatment (Robinson, 2008). Radiotherapy works by damaging the DNA directly or indirectly (by creating free radicals within the cells), and thus depriving cancer cells of their multiplication potential (Baskar et al., 2012). Radiations can be administered to the tumor from outside the body (external-beam radiation therapy), from inside (internal radiation therapy, or brachytherapy), or *via* the systemic circulation, according to the tumor type, size and location, and to the patient´s general condition (Robinson, 2008). Radiotherapy can also been used as palliative treatment (to relieve symptoms and reduce the suffering caused by cancer) or in combination with other treatment modalities such as surgery (as neoadjuvant or adjuvant therapy), chemotherapy, or more recently, with immunotherapy (Robinson, 2008, Warde, 2008, Vatner et al., 2014). Radiotherapy can also affect normal cells causing side effects, which can occur during the treatment (acute side effects) or months/years after the treatment (chronic side effects) (Lawrence et al., 2008). The most common acute side effects include skin irritation, damage to the salivary glands, hair loss and urinary problems, according to the area which receives the treatment (Warde, 2008). However, normal cells have more efficient repair mechanisms than cancer cells, and most acute effects disappear after treatment ends (Baskar et al., 2012). Other common side effects are nausea, fatigue and vomiting (Warde, 2008). Chronic side effects can include memory loss, infertility, fibrosis, and bowel damage (Warde, 2008). Nowadays, researchers are studying ways to improve the efficacy of radiotherapy while minimizing side effects. Radioprotectors can be used to protect normal cells from damage caused by radiation; radiosensitizers can be used to make cancer cells more sensitive to the effects of radiotherapy (Raviraj et al., 2014). Cisplatin and 5-fluorouracil are two examples of antitumor drugs which make cancer cells more sensitive to radiation

(Nagy et al., 2002). Furthermore, preclinical evidence indicated that serotype 5 adenovirus replication *per se* can also sensitize cancer cells to radiotherapy (Kim et al., 2009, Rajecki et al., 2009), and a year later Liikanen et al. showed that some endogenous adenoviral proteins play an important role in radiosensitization *in vitro* and *in vivo* (Liikanen et al., 2010).

1.5.3 Chemotherapy

The era of modern chemotherapy started in early 1940s, when Goodman and Gilman first administered nitrogen mustard to patients with lymphoma (Goodman et al., 1984). Chemotherapeutic drugs attack tumors at the cellular level by interrupting processes necessary for cellular replication (Malhotra and Perry, 2003). The most common chemotherapeutic drugs are "cell cycle phase-nonspecific", and thus they have significant activity in multiple phases of the cell cycle, with a broad spectrum of activity (Malhotra and Perry, 2003). Because of cytotoxic action on rapidly dividing cells, chemotherapeutic drugs are toxic to normal cells that are actively multiplying, often depending on the dose used (Malhotra and Perry, 2003). Low-dosage of certain chemotherapeutic drugs has been found associated with immunostimulatory properties, instead of increased cytotoxicity and immunosuppression (Ghiringhelli et al., 2004), providing a good rationale for combining oncolytic viruses with chemotherapy administered in metronomic low-dose.

Cyclophosphamide

Cyclophosphamide (CP) belongs to the class of alkylating agents, which interfere with DNA replication by forming DNA crosslinks (Lind, 2008). As a prodrug, CP is converted by liver cytochrome P450 enzymes to form the main active metabolite 4-hydroperoxy-cyclophosphamide (4-HP-CP) that has chemotherapeutic activity

(Huttunen et al., 2011). CP is commonly used to treat a variety of tumors, as single agent or in combination with other chemotherapeutic drugs (Shanafelt et al., 2007, Young et al., 2006, Lien et al., 2013). It has been shown that at higher doses, CP is associated with increased cytotoxicity and immunosuppression, while at low continuous dosage it shows immunostimulatory and antiangiogenic properties, as well as alteration of tumor microenvironment, and eradication of the cancer stem cell population (Loven et al., 2013). In particular, administration of low-dose CP (<100 mg/kg) to mice resulted in a significant reduction in regulatory T-cell (T-reg) frequency and function (Lutsiak et al., 2005). Therefore, the combination of oncolytic immuno-virotherapy with low-dose CP is an appealing approach.

Doxorubicin

Doxorubicin belongs to the class of anthracyclines, drugs which interact with DNA by intercalation, inhibiting macromolecular biosynthesis (Lind, 2008). Because anthracyclines have been recently proposed as inducers of immunogenic cell death (Casares et al., 2005, Obeid et al., 2007, Galluzzi et al., 2012), their combination with intrinsically immunogenic agents such as an oncolytic virus armed with granulocyte-macrophage colony-stimulating factor (GM-CSF), is an appealing approach. Doxorubicin has been used as first-line chemotherapy for the treatment of STS, typically in metastatic or unresectable disease, alone or in combination with ifosfamide (Reed and Altiok, 2011). Results from a large randomized phase III clinical trial in advanced or metastatic STS (EORTC 62012) show that the combination of doxorubicin with ifosfamide improves response rates with no clear benefit in overall survival (Judson et al., 2014).

Ifosfamide

Ifosfamide is also an alkylating agent, structurally similar to CP, which is converted by liver cytochrome P450 enzymes to form 4-hydroperoxyifosfamide (Lind, 2008). It is usually used to treat a wide range of tumors, including STS, as a single agent or in combination with doxorubicin (Reed and Altiok, 2011). Based on a large randomized phase III study (EORTC 62012), the combination of ifosfamide with doxorubicin for the treatment of STS is useful to shrink the tumor before other interventions or to relieve symptoms, but it does not improve overall survival (Judson et al., 2014).

1.5.4 Cancer gene therapy

The use of viruses as cancer treatment has been a promising possible therapeutic approach since the mid-19th century, when cases of tumor regression were observed in patients during other naturally acquired virus infections (Kelly and Russell, 2007). However, viruses started to be investigated as therapeutic agents in clinical trials only after development in basic virology in the 1950s. Results from these early clinical trials were not promising, leading to a period of decreased interest in virotherapy. In the 1990s, virotherapy gained again interest when viruses started to be engineered using six main strategies to defeat cancer: 1) viruses can be used as vectors to replace abnormal or missing genes with healthy ones (for example *TP53*) (Pearson et al., 2004); 2) oncolytic viruses are specifically directed to replicate in, and kill cancer cells *via* oncolysis; 3) genes coding for immunostimulatory molecules can be inserted into the viral genome to improve host´s antitumor immune responses; 4) viruses can be modified by inserting genes which would render cancer cells more susceptible to chemotherapy, radiotherapy or other treatments, or 5) by inserting genes that inactivate tumor angiogenesis; 6) employment of “suicide gene therapy” strategies, where a prodrug is converted in

the toxic metabolite by a virally-encoded prodrug conversion enzyme, which will be therefore produced only by virus-infected cancer cells.

The first virotherapeutic agents which received marketing approval for cancer treatment were a modified replication-deficient adenovirus coding for p53 protein (Ad-p53) and an oncolytic adenovirus (H101), both developed in China (Pearson et al., 2004, Garber, 2006). Recent success in clinical trials featuring oncolytic viruses makes this approach one of the most promising, and subject of this thesis.

1.5.5 Cancer immunotherapy

Immunotherapeutic approaches aim at the activation of patients´ immune system against cancer. Early immunotherapeutic strategies included infusion of recombinant cytokines such as interleukin-2 (IL-2), tumor necrosis alpha (TNF-α), interferon alpha (IFN-α), and granulocyte-macrophage colony-stimulating factor (GM-CSF), with promising preclinical and clinical results (Mocellin et al., 2005, Quesada et al., 1986, Arellano and Lonial, 2008, Atkins et al., 1999). More recently, the use of monoclonal antibodies, which target a specific antigen, has become a popular cancer treatment. A randomized phase III clinical trial with the anti-cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) antibody ipilimumab resulted in improved survival in patients with metastatic melanoma compared to conventional therapies (Hodi et al., 2010), and ipilimumab was approved in 2012 for the treatment of metastatic melanoma. Another example of monoclonal antibody which targets a specific immune checkpoint protein is nivolumab, which is directed against the programmed cell death-1 (PD-1) receptor on T-cells (Wolchok et al., 2013). In an early clinical trial, nivolumab showed good tolerability and durable response rate in patients with metastatic melanoma, renal and lung cancer (Topalian et al., 2012). Cell-based approaches have also gained attention in the recent years, when the first cancer vaccine sipuleucel-T was approved for the treatment of advanced prostate cancer (Kantoff et al., 2010).

Sipuleucel-T is a mixture of peripheral blood mononuclear cells supplemented with GM-CSF and a prostate-tumor associated antigen. Several other anticancer vaccines have been tested, with modest success in clinical trials (Aranda et al., 2013). Another form of cancer immunotherapy is adoptive T-cell therapy, which involves the use of autologous tumor-infiltrating lymphocytes (TILs) that have been genetically-modified and expanded *ex vivo* and then re-infused to the patient. The first clinical trial was performed in 1989 (Rosenberg et al., 1990), and since then, many strategies have been adopted to improve the therapy and circumvent the obstacles. Among these, the use of T-cell receptor-(TCR) modified T-cells and chimeric antigen receptor (CAR) T-cells are the most appealing approaches (Hinrichs and Rosenberg, 2014).

1.6 Adenoviruses

Adenoviruses are DNA viruses that were isolated for the first time in the 1950s in adenoid tissue-derived cells cultures (Rowe et al., 1953). They are members of the family *Adenoviridae*, which is divided into five genera. Human adenoviruses belong to the Mastadenovirus genus, which is divided into 7 species (A-G), based on their ability to agglutinate erythrocytes. So far, 59 serotypes of human adenoviruses have been identified by genotyping techniques (Khare et al., 2011, Liu et al., 2012).

The primary targets for adenovirus pathology are epithelial cells of the eye, respiratory and gastrointestinal tracts, leading to keratoconjunctivitis, respiratory infections and gastroenteritis (Kunz and Ottolini, 2010). They can also infect other tissue types, including liver and bladder, causing hepatitis (Ozbay Hosnut et al., 2008) and hemorrhagic cystitis (Manalo et al., 1971). Adenoviral infections are usually mild, but they can be severe and life-threatening in people with a weakened

immune system and in infants. Different serotypes have been associated with different diseases (Kunz and Ottolini, 2010). Human adenoviruses are species-specific regarding their replication cycle, and thus they are usually non-pathogenic to animals (Wold and Horwitz, 2007). Exceptions to the species-specificity include studies suggesting that serotype 5 adenoviruses can replicate in cotton rats, Syrian hamsters and New Zealand rabbits (Pacini et al., 1984, Thomas et al., 2006, Gordon et al., 1992).

Serotype 5 adenoviruses, which belong to the species C, have been mainly associated with self-limiting upper respiratory tract infections. They are the most used cancer gene therapy vectors, thanks to their well-known structure and functions, which makes cloning and modifications of the genome relatively easy. Furthermore, Ad5 is not oncogenic, which is an advantage for safety reasons. Serotype 3 adenoviruses, which belong to the species B, have been associated with upper and lower respiratory tract infections, as well as other diseases including conjunctivitis and pharyngo-conjunctival fever (Kunz and Ottolini, 2010). During the last 10 years, the use of serotype 3 and 5/3 chimeric adenoviruses (Kanerva et al., 2002, Kangasniemi et al., 2006, Zheng et al., 2007) for cancer gene therapy has gained a lot of attention. For example, a serotype 3 oncolytic adenovirus showed inhibition of tumor growth *in vivo*, and was well-tolerated in cancer patients with possible signs of efficacy, with the advantage that this serotype is not neutralized by neutralizing antibodies directed against adenoviruses of serotype 5 (Hemminki et al., 2011, Hemminki et al., 2012).

1.6.1 Structure of adenovirus

Adenoviruses are medium-sized (90–100 nm), non-enveloped (without an outer lipid bilayer) viruses with an icosahedral protein capsid containing a double-stranded DNA genome (Russell, 2000). The protein capsid is composed of hexon

proteins with penton proteins at the twelve vertices, and fiber proteins associated with each penton base of the capsid (**Figure 1**) (Smith et al., 2010). Fibers are responsible for adenovirus attachment to the host cell via a receptor on the surface of the host cell (Campos and Barry, 2007). In addition to these major capsid proteins, adenoviral capsid also contains minor structural proteins IIIa, VI, VIII and IX (Smith et al., 2010).

The adenovirus genome is linear, non-segmented double-stranded (ds) DNA that is circa 36 kilo-base pairs long, and it has a terminal protein associated with each of the 5' ends of the linear dsDNA, used as primers to initiate viral DNA replication (Rekosh et al., 1977). Adenoviral genome is associated with core proteins, important for creating links between genome and capsid, and for packaging of the virions (Campos and Barry, 2007). The genome consists of immediate early (E1A), early (E1B, E2, E3, E4), intermediate (IVa2, IX) and late genes (L1-L5) (Russell, 2000)

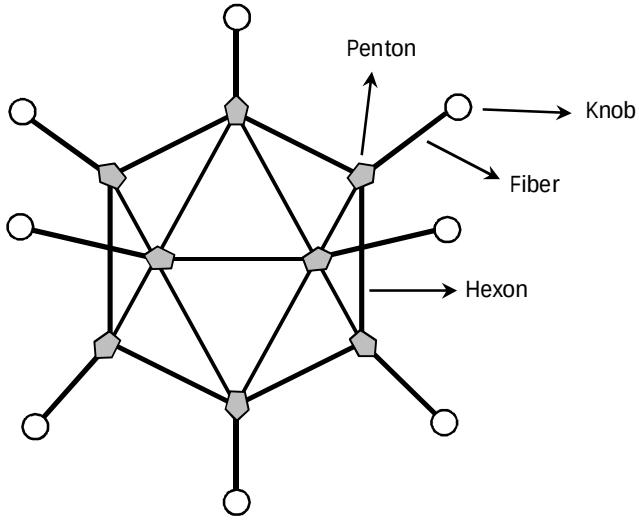

Figure 1. Adenovirus particle structure and major capsid proteins.

1.6.2 Adenovirus life cycle

Adenoviruses are obligate intracellular parasites, fully dependent on the host-cell´s replication machinery. The first step of the adenovirus life cycle is the entry into the host cell, which is initiated by the **binding** of virus knob proteins to the cell primary receptors (Campos and Barry, 2007). This is followed by secondary interactions between a penton base motif and cellular integrin molecules (Mathias et al., 1998), which mediate **internalization** of the virus particle via clathrin-coated vesicles, and following **endocytosis** (Wang et al., 1998). Once the virus is in the endosome, a process of acidification leads to digestion of pentons and capsid components, with subsequent release of the virus into the cytoplasm (Campos and Barry, 2007). The virus, surrounded by only hexon proteins, is then transported with the help of cellular microtubules to the nuclear pore complex, where the remaining capsid proteins are dissociated, and the **viral genome is released** into the nucleus (Greber et al., 1997). In the nucleus, **viral gene expression** is initiated, and new viral particles can be generated (Russell, 2009). The immediate early gene E1A is the first viral gene that is expressed and it activates the transcription of other early genes (Volpers and Kochanek, 2004). Through binding to retinoblastoma protein (Rb) and subsequent release of E2F factor, E1A activates adenoviral E2 gene expression and transcription of the other early genes (E1B, E2, E3, E4) (Russell, 2000). E1-E4 early genes are expressed before viral DNA replication (early phase) and they are responsible for expressing mainly regulatory proteins. They are involved in the activation of other viral genes and in the inhibition of anti-adenoviral immune responses and apoptosis to prolong host-cell survival (Berk, 2005). After transcription of the early genes, **replication of viral DNA** can occur. The primer for the replication is a terminal protein covalently bound to each of the 5' ends of the genome (Rekosh et al., 1977). Intermediate genes IVa2 and IX are expressed next (late phase) and they activate the major late promoter, with subsequent transcription of late genes L1-L5 for **structural**

proteins production (Russell, 2009). Once viral DNA has been replicated and sufficient quantities of structural proteins have been produced, the **new virions are assembled and released** from the cell, as a result of virally-induced **cell lysis**.

The primary high-affinity receptor identified for species C adenoviruses (which includes Ad5) is Coxsackie-adenovirus receptor (CAR) (Bergelson et al., 1997). CAR-receptor was subsequently identified to be the primary receptor for also other adenoviral species, including A, D, E and F (Roelvink et al., 1998). CAR belongs to the immunoglobulin superfamily, and it is involved in the formation of tight junctions between epithelial cells (Cohen et al., 2001). Thus, it is still unclear how the virus can reach the receptor, given its basolateral location. It has been proposed that binding between adenovirus fiber proteins and CAR facilitates the spread of the virus in the epithelium by disrupting the tight junctions (Walters et al., 2002). Secondary interactions involve an Arg-Gly-Asp (RGD) motif in the adenoviral penton base, which binds to αβ integrins on the cell surface (Mathias et al., 1998). In addition to CAR and integrins, also fiber shaft interactions with heparan sulfate proteoglycans on the cell surface are important for virus entry (Dechecchi et al., 2001).

Group B human adenoviruses (which include Ad3) do not use CAR as the primary receptor. The primary receptor for some species B serotypes was identified as CD46 (Gaggar et al., 2003), but for other species B serotypes, like serotype 3, another high-affinity receptor was recently identified as desmoglein-2 (DSG-2) (Wang et al., 2011). In addition, other receptors and molecules might play a key role in virus entry. For serotype 3 adenoviruses, CD80 and CD86 have been identified as additional receptors (Short et al., 2004), while for species C adenoviruses there are some reports suggesting that major histocompatibility complex (MHC) molecules (Hong et al., 1997), scavenger receptor A (Haisma et

al., 2009), and vascular cell adhesion molecule-1 (Chu et al., 2001) could function in this capacity as well.

1.6.3 Immune responses to adenoviral infection

When a virus infects host organisms, it is immediately recognized as "non-self invading pathogen", leading to the development of an antiviral immune response. The innate immune system is the first-line defense against pathogens. As soon as the adenoviral fiber proteins bind to the receptor on cell surface, the viral capsid is recognized as foreign, triggering a rapid induction of inflammatory cytokines (Tamanini et al., 2006). Thereafter, adenoviral DNA is sensed by receptors on dendritic cells (DCs), such as Toll-like receptor 9 (TLR-9), leading to activation of immune cells (macrophages and natural killer cells) (Huang and Yang, 2009, Cerullo et al., 2007) and secretion of large amounts of pro-inflammatory cytokines (IL-6, IL-8, IL-12, TNFα) and type I interferons (Wold and Horwitz, 2007, Huang and Yang, 2009). Cytokine and interferon responses recruit natural killer (NK)-cells, granulocytes, macrophages and DCs locally (Hendrickx et al., 2014). Later, an adaptive antiviral immune response is activated by recruitment of specific B and T lymphocytes, which is initiated by DCs (Jooss and Chirmule, 2003). DCs, after recognizing adenoviral DNA by TLR-9, process the internalized adenoviral fragments and present the viral antigens to CD4+ helper and CD8+ cytotoxic T cells (CTL), via MHC, providing also costimulatory signals (Muruve, 2004). This occurs in local lymph nodes. Activated CTL directly kill virus-infected cells which express viral antigens on MHC-I class molecules on their surface. Activated CD4+ helper T cells are involved in B lymphocyte activation, leading to antibody production against adenoviral proteins, in particular hexon and fiber proteins (Bradley et al., 2012). Furthermore, adaptive immunity creates an immunological memory which will protect the host from potential virus reinfections. If a second

infection reoccurs later, the immune system will more rapidly recognize viral antigens and activate antiviral immune responses (Nayak and Herzog, 2010).

1.7 Modified adenovirus vectors for treatment of cancer

Adenoviral vectors have been used extensively in gene therapy to treat diseases by transferring functional or therapeutic genes into cells. Adenoviral genome can be easily modified to target adenovirus to certain cell types, including cancer cells (Beatty and Curiel, 2012). Viral selectivity to cancer cells can be achieved by modifying the viral capsid (transductional targeting) or by controlling the expression of genes essential for virus replication, by gene-deletion or insertion of specific promoters (transcriptional targeting) (Barnett et al., 2002). The use of adenoviral vectors for gene therapy has many advantages: adenoviruses can infect both replication and non-replicating cells, adenoviral DNA does not integrate into the host genome, large transgene can be inserted into the adenoviral genome, reaching high levels of transgene expression (Russell, 2000).

1.7.1 Transductional targeting

Transductional targeting refers to alterations of the interactions by which the virus enters into the host cells. This can be achieved by modifications on the viral capsid proteins. Along with the findings that CAR, the primary receptor of serotype 5 adenoviruses, is expressed at low and variable levels on cancer cells and that it is often downregulated in tumors (Kanerva and Hemminki, 2004), much effort has been put in redirect the virus to other receptors, expressed at higher levels on tumor cells. One method, in order to achieve increased transduction of tumor cells, is the generation of chimeric adenovirus constructs, through replacement of the entire adenovirus knob with a knob from a different serotype. With the discovery that the serotype 3 primary receptor, later identified as DSG-2 (Wang et al., 2011) is highly

expressed on cancer cells (Tuve et al., 2006, Kanerva et al., 2002), Ad5/3 chimeras were generated through placement of Ad3 fiber knob into the Ad5 backbone (Krasnykh et al., 2001), with promising preclinical and clinical efficacy and safety data (Kanerva et al., 2003, Kangasniemi et al., 2006, Volk et al., 2003, Ranki et al., 2007, Guse et al., 2007, Zheng et al., 2007, Koski et al., 2010).

Another common modification to improve cancer cell transduction is the insertion on the virus capsid of ligands that bind adhesion molecules overexpressed on cancer cell surface (Nicklin et al., 2005, Hall et al., 2010). For example, polylysine residues and RGD-motif can be added to the virus fiber C-terminus or HI-loop, redirecting the virus attachment to heparan sulfate proteoglycans and cell surface integrins (Wickham et al., 1996, Dmitriev et al., 1998). Penton base and hexon modifications have also been investigated (Vigne et al., 1999, Wickham et al., 1995), as well as insertion of polylysine to the minor capsid protein IX (Dmitriev et al., 2002).

1.7.2 Transcriptional targeting

Transcriptional targeting refers to modifications of viral genes essential to viral replication, in order to control their transcription. Genetic deletions and insertion of tumor-specific promoters are the main approaches for transcriptional targeting (Berk, 2005, Robson and Hirst, 2003). Depending on the type of deletion, viruses can be divided in replication-deficient and conditionally replicating vectors (Lai et al., 2002, Dobbelstein, 2004).

Replication-deficient adenoviral vectors

Replication-deficient adenoviruses are generated by large deletions of their genes, so that they are unable to replicate in cells (Lai et al., 2002). These vectors are mainly used to deliver transgenes, for cancer therapy as well as other genetic

disorders. The first generation vectors were deleted of the E1 gene region and often also E3 regions, with a transgene inserted in place of the deleted E1 gene (Hall et al., 2010). Usually, these viruses can accommodate transgene sequences not larger than 8.2 kilo-base pairs (Alba et al., 2005), to avoid genomic instability (Bett et al., 1993). These vectors are unable to replicate in cells due to the lack of E1 gene, but they can infect cells normally and deliver therapeutic transgenes, such as tumor-suppressor proteins (Nielsen et al., 1998), anti-angiogenic molecules (Im et al., 2001), prodrug-converting enzymes (Tyynelä et al., 2002), monoclonal antibodies (Jiang et al., 2006), and cytokines (Sung et al., 2002). However the transgene expression is transient due to lack of virus replication and activation of host immune responses, which eradicate the vector (Muruve, 2004, Alba et al., 2005).

Second generation vectors were designed to overcome the limitations of the first-generation adenoviruses. These new-generation vectors have additional viral gene deletions in the E2 and E4 regions, to increase the cloning capacity of the vector and to decrease antiviral immune responses (Danthinne and Imperiale, 2000, Hall et al., 2010). However, these vectors still had the same problems of rapid immune eradication and transient gene expression, leading to the development of a third-generation of vectors, called “gutless” or “high capacity” vectors (Alba et al., 2005). These vectors lack of all coding viral genes except the 5´and 3´ inverted terminal repeats and the packaging signal. They can accommodate large transgenes up to 36 kilo-base pairs, but for virus production they require co-infection with a helper adenovirus that provides the missing essential genes. For this reason, they are often called helper-dependent adenoviruses (Alba et al., 2005). The advantages of these vectors include a minimal immune response against the virus and a longer transgene expression (Seiler et al., 2007). Thus, these vectors have been tested for gene replacement therapies where long-time transgene expression is needed (Ginn et al., 2013).

Conditionally replicating adenoviral vectors

Conditionally replicating adenoviral vectors (CRAds), or oncolytic adenoviruses, are viruses that selectively replicate in cancer cells (Jounaidi et al., 2007). These vectors are generated by modifications of the viral genome to prevent their replication in normal cells. These modifications include small deletions in essential genes which render the virus replication-deficient in normal cells but not in cancer cells that possess genetic deficiencies that complement the viral defects (Jounaidi et al., 2007). The first and the most known oncolytic adenovirus is ONYX-015. This virus has deletions in the E1B-55k gene, which is involved in inhibition of p53 protein (Martin and Berk, 1998). Thus, ONYX-015 was expected to replicate only in cells with deficiencies in the gene coding for p53 protein and not in cells with the intact gene (Bischoff et al., 1996). However, given the many functions of E1B-55k protein, deletions in E1B-55k gene also reduced cell killing potency (Dix et al., 2001), and it was later reported that the mechanism of selectivity is related to defects in viral mRNA export, rather than the lack of virus replication (O'Shea et al., 2004). Another strategy to direct virus replication to cancer cells is a partial genomic deletion. An example is a 24-base pair deletion in the constant region 2 of the E1A gene (Fueyo et al., 2000). The rationale behind this strategy is that E1A protein normally binds to the Rb protein, releasing the E2F transcription factor. When released, E2F activates genes that promote cell-cycle S phase, allowing virus replication (Whyte et al., 1988). The protein produced from the modified E1A gene, lacking the 24-base pair sequence, is unable to bind to Rb, and thus E2F remains bound to Rb, blocking viral DNA replication. In cancer cells, Rb/p16 pathway is often defective (Sherr and McCormick, 2002), and thus free E2F is constantly available in these cells, allowing adenoviral replication even without E1A. Oncolytic adenoviruses carrying a 24-base pair deletion in E1A gene have been successfully tested preclinically and clinically (Kanerva et al., 2003, Hakkarainen et al., 2009).

Oncolytic viruses can be also generated by the insertion of tumor-specific promoters that control viral replication. Expression of E1A gene can be controlled by the insertion of a promoter that is active in particular tumor cells. For example, E1A gene controlled by prostate-specific antigen (PSA) promoter resulted in selective virus replication in prostate tumors expressing PSA (Rodriguez et al., 1997). Other examples of tumor-specific promoters that have been used are carcinoembryonic antigen (CEA) for colorectal cancer (Li et al., 2003), α-fetoprotein for liver cancer (Kim et al., 2002) and tyrosinase for melanoma (Zhang et al., 2002). In addition to these tumor-specific promoters, which have limited applicability due to their tumor-type specificity, promoters active in a variety of cancer types, such as cyclo-oxygenase 2 (Cox-2) promoter (Kanerva et al., 2004, Bauerschmitz et al., 2006, Pesonen et al., 2010), vascular endothelial growth factor (VEGF) promoter (Kanerva et al., 2008), E2F promoter (Rojas et al., 2009, Hemminki et al., 2015) and human telomerase reverse transcriptase (hTERT) (Ito et al., 2006, Hemminki et al., 2012, Diaconu et al., 2012, Pesonen et al., 2012b) have also been employed.

1.8 Arming adenoviruses with immunostimulatory transgenes

The primary efficacy of oncolytic viruses was previously thought to be related to their selective replication and lytic effect on cancer cells. Later, preclinical and clinical evidence suggested that adenovirus replication activates immune responses against virus-infected cancer cells, mediating antitumor efficacy (Tuve et al., 2009). However, because virus-induced immune reactions are not sufficient to eradicate tumors (Tuve et al., 2009) researchers started to generate viruses armed with immunostimulatory molecules, to increase antitumor potency and to boost the immune system against tumor cells (Cerullo et al., 2010). Cytokines have a key role in immune reactions, and recombinant cytokines such as IL-2, GM-CSF,

TNFα and IFNα have been tested clinically for the treatment of certain tumor types (Lee and Margolin, 2011, Lejeune et al., 1998). However, the main problems with infusion of cytokines were related to adverse reactions and systemic toxicity, while the concentration at the tumor site often remained low (Li et al., 2005). Thus, an armed adenovirus would direct local cytokine-production by infected cancer cells, ensuring local concentration at the tumor site while minimizing systemic adverse reactions. A multitude of oncolytic and replication-deficient viruses have been armed with various immunostimulatory molecules, including TNFα, IL-2, GM-CSF, IL-12, IL-15, CD40-ligand, IFNα, IFNβ and IFNγ (Li et al., 2005).

1.8.1 GM-CSF

GM-CSF is one of the most potent cytokines that stimulates cells of the innate immune system including NK cells, DCs, and macrophages, potentially resulting in antitumor immunity (Dranoff, 2003). Increased levels of local GM-CSF also induce recruitment of monocytes and their maturation into macrophages and DCs (Chang et al., 2004). However, similarly to other cytokines, systemic use of GM-CSF is compromised by toxic side effects and induction of potentially harmful myeloid-derived suppressor cells, while its efficacy may remain limited due to the low local concentration in tumors (Serafini et al., 2004, Arellano and Lonial, 2008). Therefore, local production of GM-CSF by cancer cells, could ensure both sufficient concentration at the tumor site and minimal systemic exposure (Koski et al., 2010). Oncolytic adenoviruses coding for GM-CSF have previously shown promising antitumor efficacy and signs of immune response activation against the tumors in preclinical models (Ramesh et al., 2006, Lei et al., 2009, Koski et al., 2010). Furthermore, good tolerability and promising signs of antitumor efficacy have been observed in cancer patients, both in early clinical data (Koski et al., 2010, Cerullo et al., 2010) and in phase I clinical trials (Chang et al., 2009, Oncos Therapeutics Ltd.).

1.8.2 Oncolytic viruses armed with GM-CSF

A multitude of vectors (viral and non-viral, replication-deficient and oncolytic) have been armed with GM-CSF. With regard to conditionally replicating viruses, promising GM-CSF-coding oncolytic viruses include adenovirus, vaccinia and herpes simplex viruses.

CG0070 is a serotype 5 oncolytic adenovirus armed with GM-CSF, with the E2F promoter to control E1A expression (Burke, 2010). In 2006, *in vitro* and *in vivo* studies showed selective replication, cytotoxicity, production of GM-CSF, and antitumor efficacy of CG0070 in several human bladder transitional cell carcinoma (TCC) models, suggesting a potential utility of this oncolytic agent for the treatment of bladder cancer (Ramesh et al., 2006). Indeed, preliminary results of a phase I trial in 35 patients treated with single or multiple intravesical infusions of CG0070 at multiple-dose levels, showed tolerable safety profile and antibladder cancer activity, with GM-CSF expression and virus replication (Burke et al., 2012). An Integrated Phase II/III, open label, randomized and controlled study of the safety and efficacy of CG0070 in patients with non-muscle invasive bladder cancer is currently ongoing (ClinicalTrials.gov).

KH901 is a serotype 5 adenovirus coding for GM-CSF and with a modified hTERT promoter to control the E1A gene (Chang et al., 2009). In 2009, a phase I study of this oncolytic adenovirus in 23 patients with recurrent head and neck cancer (HNC) showed feasibility of intratumoral administration of the virus, biological activity (with virus replication and high GM-CSF levels in serum and two disintegrated tumors), and good tolerability. Despite the good data, no objective treatment responses were recorded, thus evaluation of efficacy would require a phase II trial (Chang et al., 2009).

JX-594 is an oncolytic vaccinia virus, with GM-CSF in the thymidine kinase (TK) gene. The first phase I clinical trial in 1999 showed promising results in 7 melanoma patients treated with intratumoral injections of the virus, with signs of safety, antitumor and immunological efficacy (Mastrangelo et al., 1999). Another phase I clinical trial was performed in 2008 in 14 patients with primary or metastatic hepatic cancer with intratumoral injections of the virus: the results were still promising, with disease control achieved in 9 out of 10 evaluable patients (Park et al., 2008). In 2011, ten patients with metastatic melanoma were treated with a low viral dose, equivalent to only 10% of the maximum tolerated dose in the previous trial ($1x10^9$ plaque forming units). Circulating virus was detected, suggesting virus replication and shedding into the blood. Furthermore infiltration of lymphocytes into the tumors and necrosis of tumor tissues were observed (Hwang et al., 2011). In 2013, a randomized phase II dose-finding trial in 30 patients with liver cancer showed good tolerability, oncolytic and immunotherapy mechanisms of action, tumor responses in injected and non-injected lesions, and dose-related survival (Heo et al., 2013).

Talimogene laherparepvec (T-VEC) is a conditionally replicating herpes virus coding for GM-CSF, which was first studied in a phase I clinical trial in 2006 (Hu et al., 2006). Preliminary results showed good tolerability, signs of virus replication and antitumor efficacy, tumor necrosis and GM-CSF expression in 30 patients with melanoma, HNC, breast and gastrointestinal cancer treated with intratumoral injections (Hu et al., 2006). In 2009 and 2010, T-VEC was further studied as a single agent in late stage melanoma (Senzer et al., 2009), or in combination with chemoradiotherapy in head and neck squamous cell cancer (Harrington et al., 2010). Based on promising results, a phase III trial study was recently completed on 436 patients with advanced melanoma, and T-VEC demonstrated a significant improvement in the durable response rate (DRR) vs subcutaneous GM-CSF, with a tolerable safety profile (Kaufman et al., 2014). T-VEC has recently become the

first oncolytic virus approved by the US Food and Drug Administration (FDA) (Dolgin, 2015).

Ad5-D24-GMCSF and Ad5-RGD-D24-GMCSF are serotype 5 oncolytic adenoviruses with GM-CSF inserted in the E3 region, and the 24-base pair deletion in E1A for tumor-selectivity (Cerullo et al., 2010, Pesonen et al., 2012a). Ad5-RGD-D24-GMCSF also contains a RGD-4C modification of the fiber to improve virus transduction in cancer cells which frequently express high levels of αβ integrins on their surface. These viruses have been used in the ATAP program (discussed in section 3.6.1) to treat 20 and 7 patients, respectively, with advanced solid tumors. Results were promising, although ATAP is not a clinical trial, showing good tolerability, virus replication, GM-CSF production restricted to the tumor and low systemic GM-CSF levels. Furthermore both antiviral and antitumor T cell responses were detected (Cerullo et al., 2010, Pesonen et al., 2012a).

Ad5/3-E2F-D24-GMCSF is a quadruple modified oncolytic adenovirus expressing GM-CSF (Hemminki et al., 2015). It contains a tumor specific E2F1 promoter driving the viral E1A gene, which is deleted at the Rb-protein binding site. The fiber features a knob from serotype 3 to improve virus transduction to tumor cells. The virus showed promising results *in vitro* and *in vivo*, and thus 13 advanced cancer patients were treated in the ATAP, with good tolerability and evidence suggesting induction of antitumor immune responses, signs of antitumor efficacy, and accumulation of immunological cells, especially T-cells, to tumors after treatment (Hemminki et al., 2015).

Ad5/3-D24-GMCSF

Ad5/3-D24-GMCSF is a 5/3 capsid chimeric and p16/Rb pathway-selective oncolytic adenovirus armed with human GM-CSF (Koski et al., 2010). This virus is

expected to have a threefold mechanism of action: 1) transduction of and replication in cancer cells, with subsequent tumor cell lysis and release of tumor-associated antigens (TAAs) and danger signals at the tumor site; 2) local production of GM-CSF, which recruits and activates DCs, with subsequent presentation of TAAs to CD4+ and CD8+ T-cells in the local lymph node, thus activating an antitumor immune response; 3) local GM-CSF recruits NK cells to the tumor site, which directly kill tumor cells (Dranoff, 2003). In preclinical testing, Ad5/3-D24-GMCSF showed good oncolytic potential and production of functionally active human GM-CSF *in vitro* (Koski et al., 2010). Furthermore, the virus was effective in inhibiting tumor growth of syngeneic pancreatic tumors in immunocompetent Syrian hamsters, and it was more effective when combined with low-dose CP (Koski et al., 2010), due to CP effect on regulatory T-cells (Ghiringhelli et al., 2007, Cerullo et al., 2011). Selectivity of replication and local replication-linked production of GM-CSF in tumors were also demonstrated (Koski et al., 2010). Following preclinical testing, early clinical data from 21 advanced cancer patients treated in the ATAP showed good tolerability, with promising signs of antitumor immunity (Koski et al., 2010). In cancer patients, Ad5/3-D24-GMCSF also showed increased tumor cell autophagy, induction of antitumor immune responses and promising safety and efficacy when combined with temozolomide and low-dose CP (Liikanen et al., 2013). Although Ad5/3-D24-GMCSF was detected in patient serum for long periods even after a single dose, multiple injections of the virus showed improved tumor transduction and enhanced antitumor immunity, without increasing adverse reactions (Kanerva et al., 2013). A phase I clinical trial featuring Ad5/3-D24-GMCSF has also been completed in 2013, but the data has not yet been published, and phase I/II trials in several solid tumors are in planning (Oncos Therapeutics Ltd.).

2. AIMS OF THE STUDY

The aim of this thesis is to study the utility of Ad5/3-D24-GMCSF in the treatment of sarcoma, melanoma and breast cancer, as a single agent or in combination with chemotherapeutics, and to observe feasibility and safety in patients.

Specific aims

- To assess the efficacy of Ad5/3-D24-GMCSF for treatment of sarcoma, melanoma and breast cancer in preclinical models.
- To study the effects of virally-expressed GM-CSF on cells of the immune system.
- To analyze the safety, efficacy and survival of cancer patients treated in an Advanced Therapy Access Program (ATAP).
- To improve antitumor efficacy by combining Ad5/3-D24-GMCSF with chemotherapeutics that possess immunogenic properties (doxorubicin and ifosfamide) and that selectively reduce circulating regulatory T-cells (cyclophosphamide).

3. MATERIALS AND METHODS

Materials and methods are described in more detail in the original publications.

3.1 Cell lines

Characteristics of the cell lines used in the studies are described in **Table 2**.

Table 2. Description of the cell lines used in the studies

Cell line	Description	Source	Study
A549	Human lung adenocarcinoma cells	ATCC[1]	I-IV
293	Transformed human embryonic kidney cells	ATCC[1]	I-IV
SK-LMS-1	Human leiomyosarcoma cells	ATCC[1]	I, II
HT-1080	Human fibrosarcoma cells	ATCC[1]	I, II
RD	Human rhabdomyosarcoma cells	ATCC[1]	I, II
SW872	Human liposarcoma cells	ATCC[1]	I, II
SW982	Human synovial sarcoma cells	ATCC[1]	II
DDT1-MF2	Syrian hamster leiomyosarcoma cells	provided by Prof. Wold[2]	I, II
SK-MEL-28	Human melanoma cells	ATCC[1]	III
C8161	Human melanoma cells	provided by Prof. Welch[3]	III
A375M	Human melanoma cells	provided by Prof. Fidler[4]	III
Mel888	Human melanoma cells	provided by Dr. Schlom[5]	III
Mel624	Human melanoma cells	provided by Dr. Schlom[5]	III
pMelL	Human low-passage melanoma cells	provided by Dr. Nettelbeck[6]	III
RPMI 1846	Syrian hamster melanoma cells	ATCC[1]	III
B16-OVA	Mouse melanoma cells	provided by Prof. Vile[7]	II
MDA-MB-436	Human triple-negative breast cancer cells	ATCC[1]	IV

[1] American Type Culture Collection (Manassas, VA, USA)
[2] Prof. William S.M. Wold (St. Louis University, School of Medicine, MO, USA)
[3] Prof. Danny R. Welch (University of Alabama, Birmingham, AL, USA)
[4] Prof. Isaiah J. Fidler (University of Texas MD Anderson Cancer Center, Houston, TX, USA)
[5] Dr. Jeffrey Schlom, (National Cancer Institute, Bethesda, MD, USA)
[6] Dr. Dirk M. Nettelbeck (German Cancer Research Center, DKFZ, Heidelberg, Germany)
[7] Prof. Richard Vile (Mayo Clinic, Rochester, MN, USA)

Human lung adenocarcinoma cells A549 and transformed human embryonic kidney cells 293 were used for virus production. All cell lines were cultured under the recommended conditions.

3.2 Adenoviruses

Viruses were propagated on A549 (oncolytic viruses) or 293 (replication-deficient viruses) cells and purified on cesium chloride gradients. Virus particle concentration was determined by measuring the absorbance at 260 nm, and the number of infectious particles per ml (pfu/ml) was assessed by tissue culture infectious dose 50 assay ($TCID_{50}$) on 293 cells. Virus constructs were checked by polymerase chain reaction (PCR) for the presence of transgenes and genetic modifications, as well as for the absence of wild-type virus contamination. Details on virus cloning are described in the original publications or references.

3.2.1 Replication-deficient vectors

Replication-deficient vectors used in the studies are listed and described in **Table 3**. All replication-deficient constructs are deleted for E1A gene, and luciferase transgene is inserted in the deleted E1A region under cytomegalovirus (CMV) promoter. The Ad5/3 chimera was generated through replacement of the Ad5 fiber knob with the Ad3 knob, to increase transductional targeting. This is associated with high expression of serotype 3 adenovirus-receptor desmoglein-2 (DSG-2) in cancer cells (Wang et al., 2011). RGD-4C modification in the fiber HI loop enhances binding to cellular integrins and to cell-surface molecules containing heparan sulfate.

Table 3. Description of the replication-deficient viruses used in the studies

Virus name	Description	Source/reference(s)	Study
Ad5luc1	Serotype 5 wild type with luciferase in E1A	(Kanerva et al., 2002); (Krasnykh et al., 2001)	I, III, IV
Ad5/3luc1	Chimeric 5/3 fiber with luciferase in E1A	(Kanerva et al., 2002)	I, III, IV
Ad5lucRGD	Serotype 5 wild type, RGD-4C motif in HI loop, luciferase in E1A	(Dmitriev et al., 1998)	I, III, IV
Ad3CMV-luciferase	Serotype 3 with luciferase in E1A under CMV promoter	(Fleischli et al., 2007)	I, III, IV

3.2.2 Conditionally replicating vectors

Conditionally replicating viruses used in the studies are described in **Table 4**. Ad5wt is an unmodified wild-type serotype 5 adenovirus Ad300wt, purchased from ATCC. All the other oncolytic adenoviruses have a 24-base pair deletion in the Rb-binding site in the constant region CR2 of the E1A gene. As a result, these viruses replicate only in cells with Rb/p16 pathway defects, including most human tumor types (Fueyo et al., 2000). Ad5/3-D24-GMCSF (Koski et al., 2010) has the human granulocyte-macrophage colony-stimulating factor (GM-CSF) in the E3 gene, to stimulate antitumor immunity (Cerullo et al., 2010).

Table 4. Description of the oncolytic viruses used in the studies

Virus name	Description	Reference(s)	Study
Ad5wt	Serotype 5 wild type adenovirus Ad300wt	ATCC	I, III, IV
Ad5/3-D24	Chimeric 5/3 fiber, 24-base pair deletion in E1A	(Kanerva et al., 2003)	I-IV
Ad5/3-D24-GMCSF	Chimeric 5/3 fiber, 24-base pair deletion in E1A, human GM-CSF in E3	(Koski et al., 2010)	I-IV

3.3 Chemotherapeutic agents

Chemotherapeutics used in the studies are described in **Table 5**. Doxorubicin (Medac GmbH, Wedel, Germany) was diluted in 0.9% sodium chloride (NaCl) solution (B. Braun, Melsungen, Germany). Ifosfamide (Holoxan®, Baxter, Deerfield, IL) and 4-hydroperoxyifosfamide powder (Niomech GmbH, Bielefeld, Germany) were reconstituted in sterile water. Cyclophosphamide (Sendoxan, Baxter) was diluted in 0.9% NaCl solution. 4-hydroperoxycyclophosphamide (Niomech GmbH, Bielefeld, Germany) was diluted in sterile water.

Table 5. Description of the chemotherapeutic agents used in the studies

Name	Description	Source	Study
Doxorubicin	Anthracycline, interacts with DNA by intercalation	Medac GmbH, Wedel, Germany	II
Ifosfamide	Alkylating agent, interferes with DNA replication by forming DNA crosslinks	Holoxan®, Baxter, Deerfield, IL	II
4-hydroperoxyifosfamide	Pre-activated form of ifosfamide	Niomech GmbH, Bielefeld, Germany	II
Cyclophosphamide	Alkylating agent, interferes with DNA replication by forming DNA crosslinks	Sendoxan, Baxter	III, IV
4-hydroperoxycyclophosphamide	First active metabolite of cyclophosphamide	Niomech GmbH, Bielefeld, Germany	IV

3.4 *In vitro* studies

3.4.1 Transduction assays (I, III)

Cells were seeded on 24-well plates and infected in triplicates with Ad5luc1, Ad5/3luc1, Ad5lucRGD or Ad3CMV-luciferase at doses of 1, 10, 100 and 1000 viral particles (VP)/cell for 2h at 37°C in 200 µl of growth medium (GM) containing 2% of fetal bovine serum (FBS). Cells were then washed and 10% GM added. After 24h, cells were lysed by incubation with Luciferase Cell Culture Lysis Reagent for 20 minutes at room temperature. Cell lysates were analyzed for luciferase expression using Luciferase Assay System (Promega, Madison, WI, USA) and TopCount plate reader luminometer.

3.4.2 Cytotoxicity and combination assays (I-IV)

Cells were seeded at 5000-10 000 cells/well in GM containing 2-5% FBS. After overnight incubation, cells were infected in triplicates with tenfold dilutions of viruses, from 1000 VP/cell to 1 VP/cell. 24h post-infection, 100 µl of fresh 10% GM was added to each well, and cells were incubated at 37°C until cytotoxicity was measured. Cell viability was measured using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega) when a cytopathic effect (CPE) was observed in cells infected with the highest dilution of one of the viruses. In study II, cells were infected with 1000 VP/cell of virus alone or in combination with 5 ng/ml of doxorubicin and 150 ng/ml of 4-hydroperoxyifosfamide. Cells were incubated at 37°C, and cell viability was measured after 14 days. In study III, cells were infected in quadruplicates with tenfold dilutions from 100 VP/cell to 0.01 VP/cell, and 100 µl of 10% GM was added after 18h. In study IV, cells were infected with tenfold dilutions, from 1000 VP/cell to 1 VP/cell of virus alone or in combination with cyclophosphamide (0.0075, 0.05 mg/ml) or 4-hydroperoxycyclophosphamide

(0.001, 0.0025, 0.005 mg/ml). Cell viability was measured 5 days post-infection. Cell viability is expressed relative to uninfected control cells, whose mean absorbance is set as 100% viability.

3.4.3 Virus replication in Syrian hamster and human sarcoma cells (I, II)

In study I, we checked if Ad5/3-D24-GMCSF virus replicated in hamster leiomyosarcoma DDT1-MF2 cells. Cells were plated at 10 000 cells/well on two 96-well plates and infected with Ad5/3-D24-GMCSF in 10 replicates at 0.0001–1000 VP/cell in 100 mL of 2% GM. CPE was followed for 12 days, and the amount of infectious particles was calculated daily, based on the standard $TCID_{50}$ assay. In study II, adenoviral replication was assessed in Syrian hamsters DDT1-MF2 cells and in human sarcoma cells. Cell culture monolayers were harvested at selected time points and total DNA was extracted with QIAamp DNA Mini Kit (Qiagen, Valencia, CA). In hamster DDT1-MF2 cells, adenoviral E4 copy number was measured with quantitative PCR (qPCR), 72 h after infection of cells with 1000 VP/cell of Ad5/3-D24-GMCSF with or without chemotherapy (doxorubicin and 4-hydroperoxyifosfamide), and normalized to hamster housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). In human sarcoma cells adenoviral E4 copy number was measured with qPCR, 24, 48, 72 and 96 h after infection with 10 VP/cell of Ad5/3-D24-GMCSF alone or in combination with doxorubicin (50 ng/ml), and normalized to human beta-actin gene. Details on the primers and probes are in the original publication or references.

3.4.4 Effects of virally-expressed GM-CSF on human monocytes (III)

To study the effects of GM-CSF encoded by Ad5/3-D24-GMCSF on human monocytes, we infected human melanoma SK-MEL-28 cells with 10 multiplicity of infection (MOI) of either Ad5/3-D24 or Ad5/3-D24-GMCSF, or left the cells

uninfected. After 48 h, the cells were collected and the supernatants were filtered with 0.22 µm filter (Millex® - GP, Millipore, Ireland) and with 100K spin filter (Amicon® Ultra 0.5 ml, Millipore, Ireland). Human peripheral blood-derived monocytes were isolated from leukocyte-rich buffy coats from three different healthy blood donors (The Finnish Red Cross Blood Transfusion Service). Details on the protocol are described in the original publication (study III) and references. Monocytes were stimulated 24 h post-plating with filtered supernatant from Ad5/3-D24-GMCSF-infected or Ad5/3-D24-infected SK-MEL-28 melanoma cells. GM (Macrophage-SFM media, Gibco) was changed every other day. Commercial human recombinant GM-CSF (10 ng/ml, Immunotools), supernatant from uninfected SK-MEL-28 cells, Ad5/3-D24-GMCSF and Ad5/3-D24 viruses were used as controls. Four and seven days post-stimulation, we extracted RNA using RNeasy Mini Kit, and QIAcube (Qiagen), including a DNase digestion with RNase-free DNase set (Qiagen). cDNA was synthesized using High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Real-time PCR analysis of *PU.1*, *FCGR1A*, *CD163* and *GAPDH* genes was performed using FAST SYBRgreen master mix and 7500 FAST Real-Time PCR system (Applied Biosystems). SYBRgreen oligonucleotides and primers for analysis of M-CSF, GM-CSF and IFIT2 expression are described in the original publication (study III). Comparative threshold-cycles (Ct) method was used to calculate relative expression of target genes, after normalization to GAPDH.

3.4.5 Immunogenicity of cell death (II)

In study II, immunogenic cell death was assessed by measuring the exposure of calreticulin on cell surface, and the amount of adenosine triphosphate (ATP) and high-mobility group box 1 (HMGB1) protein released in the supernatant (Diaconu et al., 2012). Cells were seeded on 6-well plates at 500 000 cells/well and infected with 100-1000 VP/cell of virus (depending on the cell line) and/or treated with

chemotherapeutic (doxorubicin and 4-hydroperoxyifosfamide). Calreticulin exposure on cell surface was measured 12h post-infection, after incubation with anti-calreticulin antibody (Abcam, Cambridge, UK) and detection with Alexa-Fluor® 488 conjugated secondary antibody (Invitrogen, San Diego, CA). BD Accuri C6 (BD Biosciences, San Jose, CA) was used for flow cytometric analyses. For ATP and HMGB1 analyses, cell culture supernatants were collected 24 or 48 h post virus and/or chemotherapy treatment, and analyzed with ATP Determination Kit (Molecular Probes, Invitrogen) and HMGB1 ELISA Kit (IBL International, Hamburg, Germany).

3.5 Preclinical *in vivo* studies

Animal protocols were reviewed and approved by the Experimental Animal Committee of the University of Helsinki and the Provincial Government of Southern Finland. All animals were quarantined for at least one week, and their health status was monitored daily. Animals were euthanized according to local animal care rules and to humane end-point guidelines. Mice were anesthetized prior to any procedures either in isoflurane gas or with 100 µl (i.p.) of dexmedetomidine (Dexdomitor®, Orion Pharma, Espoo, Finland) and ketamine (Ketalar®, Pfizer, Helsinki, Finland) mix: 2 ml Ketalar® (50 mg/ml) + 1 ml Dexdomitor® (0,5 mg/ml) + 7 ml 0.9 % NaCl. After procedures, the sedative effects were reversed with 50 µl (i.p.) of atipamezole (Antisedan® 5 mg/ml, Orion Pharma), diluted 1:10 in NaCl. Hamsters were anesthetized with 250 µl (i.p.) of ketamine – dexmedetomidine mix: 50 mg/kg Ketalar® + 150 µg/kg Dexdomitor®.

3.5.1 Immunodeficient animal models (I-IV)

Female Nude/NMRI mice (3-4 weeks old) were provided by Harlan Laboratories (Indianapolis, IN, USA). In study I, mice were injected with 5 million SK-LMS-1,

HT-1080, RD or SW872 cells subcutaneously in both flanks. When tumors reached the size of approximately 5 mm diameter, viruses (Ad5/3-D24-GMCSF, Ad5/3-D24 or Ad5wt) diluted in 0.9% NaCl were injected intratumorally at 7 x 10^9 VP/tumor (2 tumors/mouse) on days 1, 4, 8 and 15. NaCl 0.9% was used as mock treatment. In study II, mice were injected with 5 million HT-1080 cells into both flanks, and treated with intratumoral injections of Ad5/3-D24-GMCSF (1 x 10^8 VP/tumor injected on days 1, 4, 8 and 15), intraperitoneal injections of doxorubicin (2 mg/kg injected on days 1, 8 and 15), or a combination of both. In study III, mice received 5 million SK-MEL-28 cells subcutaneously into both flanks. Tumors were treated with viruses (Ad5/3-D24-GMCSF, Ad5/3-D24, Ad5wt or Ad5/3luc1) injected intratumorally at 7 x 10^9 VP/tumor (2 tumors/mouse) on days 1, 4, 8 and 15. Concomitant low-dose CP (20 mg/kg in saline) was injected intraperitoneally the day after the first virus injection and every 3 days thereafter (Koski et al., 2010, Liikanen et al., 2013). In study IV, MDA-MB-436 cells (5 five million cells/tumor) were injected orthotopically in two different mammary fat pad sites. Mice were randomized into 9 groups and viruses (Ad5/3-D24-GMCSF, Ad5/3-D24, Ad5wt or Ad5/3luc1) were injected at 7 x 10^9 VP/tumor on days 1, 4, 8, 15, 29 and 43. Concomitant low-dose CP was administered as in study III. In all the studies, width and length of the tumors were measured every 2-3 days with digital calipers, and tumor volumes were calculated using the formula "0.5 x length x $width^2$".

3.5.2 Immunocompetent animal models (I, II)

Male Syrian hamsters (*Mesocricetus auratus*) were purchased from Harlan Laboratories at 5–6 weeks of age. In study I, hamsters were injected subcutaneously at four different sites with 5 million DDT1-MF2 cells/site and randomized into 12 groups. Ten groups of animals were treated intratumorally with Ad5/3-D24-GMCSF, Ad5/3-D24 or Ad5/3luc1 at three different doses: low (2.1 x 10^8 VP/tumor), medium (2.1 x 10^9 VP/tumor) or high dose (2.1 x 10^{10} VP/tumor),

or mock-treated with 0.9% NaCl. Hamsters received a single injection of the indicated viruses on day 1. Two groups of animals received multiple injections, either of 0.9% NaCl or of Ad5/3-D24-GMCSF in low dose on days 1, 4 and 8. One of the four tumors growing in each hamster was left without injection, to see if intratumoral injection of Ad5/3-D24-GMCSF leads to systemic viral spread and infection of uninjected tumors. In study II, hamsters received 5 million DDT1-MF2 cells subcutaneously into both flanks. In the first experiment, Ad5/3-D24-GMCSF was injected at 2.25 x 10^8 VP/tumor every 3 days. In addition, mice who received a combination treatment were injected with also doxorubicin (1 mg/kg) and ifosfamide (30 mg/kg) intraperitoneally every 3 days. In the second experiment, hamsters received the same amount of virus every other day, and doxorubicin dose was increased to 1.25 mg/kg, injected intraperitoneally every other day. Ifosfamide was not used. In study II, to perform mechanistic immunological analyses not possible in Syrian hamsters for lack of reagents, an immunocompetent mouse model was used. Female C57BL/6 mice (3-4 weeks old) were purchased from Harlan Laboratories. Animals were injected with 2.5 million B16-OVA melanoma cells into both flanks. Mice were treated every 3 days with 4.5 x 10^9 VP/tumor of Ad5/3-D24-GMCSF alone, doxorubicin/ifosfamide alone (1 and 30 mg/kg, respectively) or in combination.

3.5.3 Histological and immunohistological analyses (I, II)

In study I and II, to assess potential local or systemic pathological effects of the viruses, the tumors and relevant organs (heart, liver, spleen and kidney in both studies; lung and brain were also collected in study I) were collected and fixed in 10% buffered formalin for 24 h, followed by storage in 70% EtOH. Tissues were then trimmed and routinely embedded in paraffin wax. Sections (3–5 mm) were prepared and stained with hematoxylin-eosin (HE) for histological evaluation. In study I, we also performed immunohistological analyses to evaluate the presence of

T and B cells/plasma cells in the tumors. Cross-reacting antibodies against CD3 (rabbit anti-human CD3; Dako, Glostrup, Denmark) and CD79a (rat anti-human CD79acg; Dako) were used. Analysis was done using the streptavidin peroxidase method with heat pretreatment (citrate buffer pH 6.0) for antigen retrieval and diaminobenzidin as chromogen. Details on the protocol are in the original publication or references.

3.5.4 Quantitative PCR analyses (I, II)

Viral DNA load in tumors (I, II), organs (I), blood clots (I) and serum samples (I) was measured by qPCR. Tumors and organs were collected following euthanasia and stored at -80°C. Blood samples were collected into sterile tubes: serum and blood clots were separated by coagulation at room temperature and centrifugation at room temperature for 10 min at 1000 rpm. Serum samples were stored at -20^{o}C and the blood clots at -80^{o}C. Tumor tissues or blood clots (approximately 25 mg) were digested overnight with proteinase K in tissue lysis buffer ATL (Qiagen). For serum samples, 6 mL of poly(d)A carrier DNA (Roche) was added to 200 mL of the diluted serum sample (1:1 of serum and PBS). Total DNA was extracted using the QIAamp DNA Mini Kit (Qiagen) and QIAcube machine, according to the manufacturer's instructions. qPCR for the adenoviral *E4* gene was performed, and adenoviral E4 copy number was normalized to the hamster *GAPDH* gene. Details on the primers and probes are in the original publication or references.

3.5.5 Flow cytometry (II)

In study II, tumors and spleens from euthanized C57BL/6 mice were passed through a 40-mm cell strainer and cultured overnight at 37^{o}C. Cell suspensions were collected the next day, frozen and stored at -140 ^{o}C. Flow cytometric analyses were performed using BD Accuri C6 (BD Biosciences), with the following

antibodies purchased from BD Biosciences: anti-mouse CD11c FITC (clone HL3), anti-mouse CD3e APC (clone 145-2C11), anti-mouse CD80 PerCP-CyTM5.5 (clone 16-10A1), anti-mouse CD86 PE (clone GL1) and anti-mouse CD8a PE (clone 53-6.7).

3.6 Treatment of patients with oncolytic adenoviruses (I, III, IV)

3.6.1 Advanced Therapy Access Program (ATAP)

Between 2007 and 2012, a total of 290 patients with advanced solid tumors refractory to conventional therapies were treated with 10 different oncolytic adenoviruses at the Docrates Hospital in Helsinki, Finland, in the context of an Advanced Therapy Access Program (ATAP). ATAP is not a clinical trial but a personalized treatment program (Dnro 475/E6/06), with the purpose of offering experimental therapies to patients with metastatic solid tumors refractory to conventional treatment modalities. ATAP is operated under the 'Hospital Exemption' clause defined in the Advanced Therapy Directive (EU/1394/2007) and is regulated by the Finnish Medicines Agency (FIMEA) as determined by the Directive. ATAP was in compliance with European Union and Finnish Regulations, and was evaluated by The Gene Technology Board and Medicolegal Department of the Finnish Ministry of Social Affairs and Health. Patients signed a written informed consent, to confirm the understanding of the experimental approach of oncolytic adenovirus treatment instead of clinical trial, and treatments were administered according to Good Clinical Practice and the Declaration of Helsinki of World Medical Association, based on Article 35. Each patient was evaluated before treatment decisions, which were based on individual characteristics of the patients, tumor type, size and location. The treatment was offered to patients with solid tumors refractory to conventional therapies,

progressive disease, WHO performance score ≤3, and no major organ function deficiencies. The treatment was not offered if patients had organ transplant, brain metastasis, elevated bilirubin alanine transaminase (ALT) or aspartate transaminase (AST) (over 3-fold upper limit of normal), HIV or other immunosuppression, severe thrombocytopenia, or other severe disease or organ malfunction. FIMEA required the report of treatment results, adverse reactions and overall survival. Analysis of patient samples for retrospective studies has been approved by Helsinki University Central Hospital (HUCH) operative ethics committee (HUS 62/13/03/02/2013).

Of the 290 patients treated in ATAP, 115 patients received Ad5/3-D24-GMCSF during their treatments. Of these, all the patients with chemotherapy-refractory sarcoma (15/115), melanoma (9/115) and breast cancer (16/115) are included in this thesis (tot. 40 patients; studies I, III and IV). In addition to Ad5/3-D24-GMCSF treatments, patients were free to receive other cancer therapies, including additional virus treatments.

3.6.2 Treatment protocols and follow-up

In ATAP, patients received oncolytic adenoviruses intratumorally into the primary tumor and/or any injectable metastases. Injections were performed in ultrasound or CT guidance (10 mL volume injected intratumorally with 10 needle tracts). In case of intrapleural or intraperitoneal disease, injections were given into the relevant cavity. In this thesis, all treatments were given with Ad5/3-D24-GMCSF, with doses of up to 4×10^{11} VP. Patients received Ad5/3-D24-GMCSF as a single treatment or a serial treatment of three consecutive virus injections at 3-4 week intervals. Typically, at least one fifth of the dose was given intravenously (as a 2.5 mL bolus after intratumoral injection), to reach uninjectable lesions. To reduce regulatory T-cells, concurrent low-dose metronomic CP was administered either

per orally (50 mg daily starting one week before virus treatment), or by a single intravenous bolus of 100 mg on the day of the virus treatment, or a combination of these (Cerullo et al., 2011, Koski et al., 2010). Some patients received other concomitant therapies, such as low-dose pulse temozolomide (100 mg/day administered according to three different dosing schedules), to induce oncolytic autophagy (Liikanen et al., 2013), and/or verapamil (200 mg twice daily orally one day after virus treatment and continued for at least 4 weeks) to enhance virus replication (Koski et al., 2012). None of these "virus sensitizers" were expected to yield antitumor efficacy on their own; instead they were used to increase the effects of the virus.

Patients were monitored for 24 hours in the hospital and 4 weeks as outpatients. Adverse reactions (AR) were monitored for 28 days and recorded according to Common Terminology Criteria for Adverse Events (CTCAE) v3.0:

- Grade 1: mild AR
- Grade 2: moderate AR
- Grade 3: severe AR
- Grade 4: life-threatening or disabling AR
- Grade 5: AR leading to death

ARs were further classified as Serious Adverse Events (SAEs) if leading to patient hospitalization, prolongation of hospitalization, malformation, or death. Pre-existing symptoms were not listed unless they worsened. Transient lymphocytopenia in the peripheral blood (frequently observed in association with oncolytic adenovirus treatments) was not considered as an AR, as there is evidence indicating possibly redistribution of lymphocytes (i.e. trafficking of lymphocytes to tumors), thus contributing to treatment efficacy (Reid et al., 2002, Brahmer et al., 2010, Kanerva et al., 2013, Hemminki et al., 2015).

3.6.3 Detection of viral DNA in patient serum samples

Total DNA was extracted with QIAamp mini kit (Qiagen, Hilden, Germany) from serum of patients, whose blood was collected before and after virus treatment. DNA concentration was measured spectrophotometrically, and analyzed at multiple timepoints by qPCR, to determine viral titer and signs of viral replication. Details on primers, probes and protocol are described in the original publications and references (Cerullo et al., 2010, Koski et al., 2010, Escutenaire et al., 2011).

3.6.4 Neutralizing antibody titer determination

Neutralizing antibody titer was measured from patient serum samples using a non-replicating adenovirus with the same virus capsid of the virus that the patient had received as treatment. In case of Ad5/3-D24-GMCSF treatments, Ad5/3luc1 was used. Details on the protocol are described in the original publication and references (Cerullo et al., 2010, Koski et al., 2010).

3.6.5 Analysis of treatment efficacy and overall survival

Tumor size was assessed by contrast-enhanced computed tomography (CT), magnetic resonance imaging (MRI), or [18F]-fluorodeoxyglucose positron emission tomography with a low resolution CT scan (PET-CT) before treatment and after a median of 4.5 weeks from the latest virus treatment. In case of a serial treatment, the radiological evaluation was performed after the third virus injection (the last of the serial treatment). Maximum tumor diameters were calculated according to modified Response Evaluation Criteria in Solid Tumors (RECIST) v1.1 (Eisenhauer et al., 2009, Koski et al., 2013), and/or PET criteria (Koski et al., 2013), including injected and uninjected lesions. Modified RECIST criteria were:

- Complete Response (CR = tumor completely undetectable after treatment)

- Partial Response (PR ≥ 30% reduction in the sum of tumor diameters)
- Minor Response (MR = 10-29% reduction in the size of lesions)
- Stable Disease (SD ≤ 9% reduction or < 20% increase)
- Progressive Disease (PD ≥ 20% increase or new lesions)

PET criteria (using summed SUV_{max} from the 5 most active lesions in the PET images) were:

- Complete Metabolic Response (CMR = disappearance of all metabolically active tumors)
- Partial Metabolic Response (PMR ≥ 30% decline in summed SUV_{max})
- Minor Metabolic Response (MMR = 10-29% decline in summed SUV_{max})
- Stable Metabolic Disease (SMD ≤ 9% reduction or < 30% increase)
- Progressive Metabolic Disease (PMD ≥ 30% increase or new metastatic lesions with ≥ 2 cm diameter and/or sufficient ^{18}F-FDG uptake)

In study IV, tumor markers were also evaluated when elevated at baseline, by comparing baseline values to the best or worst response, and using the same percentages listed above.

Overall survival was calculated both from the day of the first virus treatment and from the day of the first Ad5/3-D24-GMCSF treatment.

3.7 Statistics (I-IV)

Statistical analyses were performed using SPSS statistics software version 18-21 (SPSS, Chicago, IL), GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA), and Microsoft Excel 2007 (Microsoft, Redmond, WA). Two-tailed Student's *t* test for independent samples was used for *in vitro* data (I-IV) and to assess tumor volume for nude mice experiment in study III. Type III Tests of Fixed Effects and repeated measures ANOVA on log-transformed data were used for animal experiments in study I and II. Mann-Whitney U test with Bonferroni correction was used to assess tumor volume for nude mice experiment in study IV. *p* values of <0.05 were considered significant. In study II, therapeutic synergy was assessed using the fractional tumor volume (FTV) method (Yokoyama et al., 2000, Xu et al., 2011). The expected FTV (i.e., the product of FTV values for monotherapies) of the combination therapy is divided by the observed FTV of the combination, and the obtained ratio indicates the nature of the interaction (ratio >1 indicates synergy and ratio <1 indicates a less than additive effect). For each experimental group, FTV is obtained by dividing the mean tumor volume of the experimental group by the mean tumor volume of the control group. Kaplan–Meier method with Log-Rank tests was used to process patient survival data (I, III, IV).

4. RESULTS AND DISCUSSION

4.1 Preclinical *in vitro* results

4.1.1 Effects of capsid modifications on tumor transduction (I, III)

Serotype 5 adenoviruses have been used most frequently in gene therapy. Because their primary receptor (coxsackie-adenovirus receptor, CAR) is expressed on cancer cells at variable and often low levels (Bauerschmitz et al., 2002), the efficacy of gene therapy vectors based on serotype 5 adenoviruses might be reduced. To overcome this limitation and to increase gene delivery and antitumor efficacy, the fiber knob region of Ad5 can be replaced with the Ad3 knob, enhancing cancer cell transduction and antitumor efficacy in preclinical testing (Kanerva et al., 2003, Kanerva and Hemminki, 2004). This is associated with high expression of desmoglein-2 (DSG-2) (Wang et al., 2011), the receptor for serotype 3 adenoviruses, on tumor cells (Kanerva et al., 2002, Tuve et al., 2006).

In this thesis, we assessed the transduction of human sarcoma cell lines and Syrian hamster sarcoma (**Study I**) and melanoma cells (**Study III**) by adenoviruses with different capsid modifications. As shown also previously (Kanerva et al., 2003), transduction in human cells was improved with a chimeric 5/3 fiber. In contrast, transduction of the hamster cells was most efficient with the serotype 5 capsid (**Study I, Fig. 1d; Study III, Suppl. Fig. S2b**). Ad3CMV-luciferase failed to transduce the cell line at any VP number/cell. While human cells are permissive to human adenoviruses, transduction of Syrian hamster cells is not enhanced by 5/3 chimerism. Syrian hamster cells might express lower levels of the receptor(s) relevant for Ad5/3 than human cells, or hamster DSG-2 might not allow binding to the human Ad3 fiber (Trinh et al., 2012). In conclusion, 5/3 chimerism enhanced the transduction of human but not of hamster cancer cells, suggesting that other

mechanisms may be responsible for Ad5/3 entry into Syrian hamster cells. Indeed, Ad5/3 shares only the fiber knob with Ad3, and the length of the fiber shaft may be a determinant of adenoviral attachment strategy (Ambriovic-Ristov et al., 2003).

4.1.2 Ad5/3-D24-GMCSF *in vitro* efficacy, as single agent or in combination with chemotherapy (I-IV)

Preclinical reports with GM-CSF-encoding viruses have indicated promising antitumor efficacy in the treatment of advanced solid tumors (Cerullo et al., 2010, Koski et al., 2010, Burke, 2010, Pesonen et al., 2012a). Ad5/3-D24-GMCSF has shown good tolerability and efficacy *in vitro*, in animal models and in cancer patients (Koski et al., 2010, Kanerva et al., 2013). We studied the susceptibility of human and Syrian hamster cancer cells to oncolysis by Ad5/3-D24-GMCSF. All the human sarcoma and melanoma cell lines were permissive and susceptible to Ad5/3-D24-GMCSF (**Study I, Fig. 1; Study III, Fig.1**). Cell-killing efficacy of Ad5/3-D24-GMCSF was superior to that of Ad5wt and comparable to that of Ad5/3-D24, as expected, since GM-CSF does not add efficacy *in vitro*. Interestingly, in human melanoma pMelL cells which do not express CAR, both Ad5/3-D24 and Ad5/3-D24-GMCSF viruses induced greater cell killing than Ad5wt (**Study III, Fig. 1g**).

In hamster sarcoma cells, despite their permissiveness to Ad5/3-D24-GMCSF replication (**Study I, Suppl. Fig. S1**), Ad5wt virus was slightly more effective than Ad5/3-D24-GMCSF and Ad5/3-D24, while hamster melanoma cells were resistant to both Ad5/3D24 and wild-type adenoviruses (**Study III, Suppl. Fig. S2a**), as previously reported also by others (Zhang et al., 1996). These results suggest that possible additional mechanisms limit the adenoviral replication-cycle in these hamster melanoma cells. Certain cell lines from species other than humans support the full replication cycle of human adenoviruses, although at reduced efficacy

(Jogler et al., 2006). In rodent and rabbit cells, restriction of productive human adenovirus replication acts primarily at a post-entry step, and thus, some of the late steps of replication may be compromised also in Syrian hamsters (Jogler et al., 2006).

Preclinical and clinical studies have shown that oncolytic viruses can be combined with conventional anticancer treatments, such as chemotherapy, enhancing the efficacy of the viral treatment without increasing side effects (Cerullo et al., 2011, Liikanen et al., 2013, Ottolino-Perry et al., 2010). In our *in vitro* combination studies (**Study II and IV**) we saw an additional cell killing when Ad5/3-D24-GMCSF was combined with doxorubicin (with or without ifosfamide) in human and Syrian hamster STS cells (**Study II, Fig. 1e, Fig. 4**), and with low-dose CP or 4-HP-CP in human TNBC cells (**Study IV, Fig. 1**). Since CP is a prodrug, and thus it is not expected to be active in cells *in vitro* which lack cytochrome P450 enzymes (Sladek, 1972), our result shows that pathways other than liver enzymes can also activate CP, which is in line with a previous publication (Ginsberg et al., 1977). In conclusion, Ad5/3-D24-GMCSF was able to enter into and replicate in human and hamster STS cells, and in human melanoma and breast cancer cells, with subsequent oncolysis. The addition of chemotherapeutic agents *in vitro* resulted in additional cell killing both in human and Syrian hamster STS cells, and in human TNBC cells. Next, we sought to find out the mechanisms underlying the combination effect between virus and chemotherapy. Possible reasons could be related to altered replication kinetics and induction of immunogenic tumor cell death, as reported also previously (Raki et al., 2005, Liikanen et al., 2013).

4.1.3 Adenoviral replication in absence or presence of doxorubicin and/or 4-hydroperoxyifosfamide (I, II)

Based on our transduction results, to better understand if Syrian hamster is permissive to replication of 5/3 chimeric viruses, we studied Ad5/3-D24-GMCSF replication in the Syrian hamster DDT1-MF2 leiomyosarcoma cell line, and the effect of chemotherapy on adenoviral replication *in vitro* (**Studies I and II**). In a progressive infectivity assay, oncolysis of DDT1-MF2 cells developed overtime, suggesting productive replication (**Study I, Suppl. Fig. S1**). The addition of doxorubicin and 4-hydroperoxyifosfamide led to an increase in adenoviral replication compared to the virus alone (p = 0.0033; **Study II, Fig. 1d**). Also in human STS cells, viral genome copy number was significantly higher in the presence of doxorubicin in 4 out of 5 analyzed cell lines (**Study II, Fig. 4**). We conclude that Ad5/3-D24-GMCSF replicates in Syrian hamster STS cells and that chemotherapy enhances adenoviral replication, both in human and hamster STS cells. These results are in line with previous reports suggesting increased viral replication in the presence of other chemotherapeutic agents such as gemcitabine (Raki et al., 2005), paclitaxel and docetaxel (Yu et al., 2001), as well as increased replication of oncolytic measles virus mediated by doxorubicin-induced senescence (Weiland et al., 2014).

4.1.4 Immunogenicity of cell death induced by the combination of Ad5/3-D24-GMCSF and chemotherapy (II)

Given the immunogenic properties of doxorubicin and ifosfamide, we studied the induction of immunogenic cell death (ICD) after infection with Ad5/3-D24-GMCSF (an intrinsically immunogenic agent) and doxorubicin/4-hydroperoxyifosfamide in the Syrian hamster DDT1-MF2 leiomyosarcoma cells (**Study II**). The combination of virus with the two chemotherapeutic drugs

resulted in increased calreticulin exposure in comparison to control cells and a trend for increase over single agents (**Study II, Fig. 1a**). Furthermore, the cells treated with the combination regimen released more HMGB1 in the supernatant, compared to control cells and single agents (**Study II, Fig. 1b**), and the amount of extracellular ATP was significantly higher compared to virus alone (**Study II, Fig. 1c**). In the human fibrosarcoma cell line HT-1080, the combination of virus and doxorubicin resulted in significantly increased calreticulin exposure over control cells and virus alone, and trends for increased amount of released HMGB1 and ATP (**Study II, Suppl. Fig. S2a-c**). As a conclusion, the combination of Ad5/3-D24-GMCSF with chemotherapeutic agents with immunogenic properties enhances induction of ICD both in human and Syrian hamster STS cells. This is in line with previous observations of enhanced ICD following combination of Ad5/3-D24-GMCSF with low-dose CP and temozolomide (Liikanen et al., 2013)

4.2 Preclinical *in vivo* results

4.2.1 Ad5/3-D24-GMCSF antitumor efficacy in immunodeficient animal models, as single agent or in combination with chemotherapy (I-IV)

Efficacy studies *in vivo* using human cells require the use of xenograft models. This model does not allow the study of GM-CSF-mediated effects, since human GM-CSF is not bioactive in mice due to species-specificity (Shanafelt et al., 1991). Despite this limitation, we used the model to confirm the oncolytic *in vivo* antitumor potency of Ad5/3-D24-GMCSF (**Studies I-IV**). As a single agent, Ad5/3-D24-GMCSF was more effective than mock and Ad5wt-treated animals in a STS xenograft model (**Study I, Fig. 2**). The effect of the treatment was enhanced when the virus was combined with doxorubicin, in the same fibrosarcoma xenograft model, with improved survival and evidence of synergism (**Study II,**

Fig. 5a-c). In studies III and IV, the virus was combined with low-dose CP, in a melanoma and a TNBC xenografts models. In these models, low-dose CP alone did not show any effect, while in combination with Ad5/3-D24-GMCSF, led to a significant enhancement of tumor growth inhibition, compared to CP and virus only treatments (**Study III, Fig. 2a, Suppl. Fig. S1; Study IV, Fig. 2, Suppl. Fig. S1**). In particular, in the melanoma xenograft model, the mice treated with this combination exhibited complete tumor regression (**Study III, Fig. 2a, Suppl. Fig. S1**). As expected, in this model where human GM-CSF is not bioactive, Ad5/3-D24 was as efficient as Ad5/3-D24-GMCSF, as a single agent and in combination with low-dose CP (**Study III, Fig. 2b, Suppl. Fig. S1; Study IV, Suppl. Fig. S1**). These results obtained from immunodeficient animals (lacking T-cells) suggest that low-dose CP has possible mechanisms of action other than reduction of regulatory T-cells (Koski et al., 2010, Cerullo et al., 2011). Indeed, previous publications report CP as an anti-angiogenic drug able to modify tumor microenvironment, when administered at low continuous dosage (Man et al., 2002, Wang et al., 2012b, Loven et al., 2013, Nicolini et al., 2004). In conclusion, efficacy studies in xenograft models showed superior antitumor potency of Ad5/3-D24-GMCSF over controls. Furthermore, when chemotherapy was added, the efficacy of the treatment was improved in all three tumor types, suggesting useful combination effects with the virus.

4.2.2 Ad5/3-D24-GMCSF antitumor efficacy in immunocompetent animal models, as single agent or in combination with chemotherapy (I, II)

To study the efficacy of Ad5/3-D24-GMCSF in an immunocompetent animal model in which also human GM-CSF is bioactive (Cohen et al., 1988, Cerullo et al., 2010, Koski et al., 2010), we performed two *in vivo* experiments in Syrian hamsters, reported also semi-permissive for human adenovirus replication (Thomas et al., 2006). First, we attempted to study Ad5/3-D24-GMCSF as a single agent, in

a dose-escalation efficacy study (**Study I**). Syrian hamsters carrying four DDT1-MF2 leiomyosarcoma tumors were treated with a single intratumoral injection of Ad5/3-D24-GMCSF or control viruses (Ad5/3-D24, Ad5/3luc1) at three different doses: low (2.1 x 10^8 VP/tumor), medium (2.1 x 10^9 VP/tumor) or high dose (2.1 x 10^{10} VP/tumor). To study the effect of a multiple administration of the virus, two groups of animals received three injections of Ad5/3-D24-GMCSF (or NaCl as mock treatment) in low dose on days 1, 4 and 8. Doses and treatment days were selected according to previous studies (Koski et al., 2010) and treatment schedule of human patients. Treatment with a single injection of medium or high dose of Ad5/3-D24-GMCSF resulted in statistically significant tumor growth inhibition compared to mock-treated animals (**Study I, Suppl. Fig. S2a**), whereas a single low-dose injection of any viruses resulted in no difference in tumor growth (**Study I, Suppl. Fig. S2b**). Similarly, a single injection of medium or high dose of Ad5/3-D24 resulted in statistically significant slower tumor growth, compared to mock animals, despite the absence of GM-CSF (**Study I, Suppl. Fig. S2c-d**). In this experiment, GM-CSF-expressing virus did not enhance antitumor efficacy, as measured by tumor growth, in consequence of possible GM-CSF-mediated inflammatory swelling (Kanerva et al., 2013), which could have affected tumor size measurements. In contrast to the single low-dose treatment, multiple injections of Ad5/3-D24-GMCSF in low dose resulted in statistically significant antitumor activity compared to mock treatment (**Study I, Suppl. Fig. S2e,f**).

Treatment outcome can benefit from systemic effects, which are important also for the treatment of metastatic diseases. Intratumorally injected oncolytic viruses could release virus progeny into the blood, leading to transduction of metastases. Furthermore, locally administered viruses could induce a systemic antitumor immune response. To assess if Ad5/3-D24-GMCSF treatment has systemic effects, we left one of the four tumors in each hamster without virus injection. In Ad5/3-D24-GMCSF treatments, we observed no difference in the size of injected and

uninjected tumors (**Study I, Fig. 3a**). Interestingly, uninjected tumors in Ad5/3-D24-treated animals grew faster compared to the uninjected tumors in Ad5/3-D24-GMCSF-treated animals, suggesting GM-CSF-mediated systemic effects (**Study I, Fig. 3a,b**). Furthermore, virus genomes were recovered from both injected and uninjcted tumors, blood clots and cell-free serum, suggesting virus dissemination into the blood and transduction of metastases (**Study I, Fig. 3c,d; Suppl. Fig. S3, S4**). To assess if the presence of virus DNA in uninjected tumors is required for antitumor effects in distant metastases, we compared the size of uninjected tumors that contained Ad5/3-D24-GMCSF genomes versus uninjected tumors that did not contain Ad5/3-D24-GMCSF DNA. We observed no difference, suggesting that GM-CSF produced by the virus can arouse immunological effects in distant metastases also without the presence of virus DNA.

GM-CSF systemic concentration is an important aspect, since GM-CSF is known to cause systemic toxicity and to recruit myeloid-derived suppressor cells (MDSC), leading to reduction of antitumor immune responses (Serafini et al., 2004). Our results show the ability of intratumorally injected Ad5/3-D24-GMCSF to reach distant uninjected tumors (in the same animal) through the blood stream, leading to systemic effects useful for the treatment of metastatic cancer. However, as previously shown, intratumoral injections of GM-CSF support local production of GM-CSF at the tumor site, without increasing systemic concentration to the "threshold level" above which GM-CSF results in tumor immunosuppression (Koski et al., 2010). In conclusion, multiple dosing with Ad5/3-D24-GMCSF emerged as more efficient than a single injection. Furthemore, intratumorally injected virus can spread to non-injected tumors of the same animal, and virally-produced GM-CSF can mediate immunological effects in distant metastases.

To improve the overall treatment outcome, in **Study II** we evaluated the combination of Ad5/3-D24-GMCSF with doxorubicin and ifosfamide in the same immunocompetent Syrian hamster animal model, based on promising *in vitro* results (**Study II, Fig. 1e**). The animals, implanted with DDT1-MF2 leiomyosarcoma cells, received intraperitoneal injection of doxorubicin (1 mg/kg) and ifosfamide (30 mg/kg) and/or intratumoral injection of Ad5/3-D24-GMCSF (4.5×10^9 VP/kg) every 3 days. Both single treatments (Ad5/3-D24-GMCSF and chemotherapy alone) inhibited tumor growth compared to mock-treated animals, while the combination treatment was the most effective in controlling tumor growth and improving the survival of the animals (**Study II, Fig. 2a,b**). Furthermore, the FTV method to assess combination effects revealed therapeutic synergy, with an odds ratio > 1 (**Study II, Fig. 2c**). We also studied the combination of Ad5/3-D24-GMCSF with doxorubicin without ifosfamide in the same animal model (**Study II, Fig. 3**), based on the results of a randomized phase III trial for patients with advanced STS (van der Graaf et al., 2012) which showed no additional effects of doxorubicin+ifosfamide treatment compared to doxorubicin alone. In this experiment, hamsters received intratumoral virus injections (4.5×10^9 VP/kg) and/or intraperitoneal doxorubicin injections (1.25 mg/kg) every other day. With the increased doses and administration frequency of doxorubicin and virus, Ad5/3-D24-GMCSF treatment did not result in better antitumor efficacy than the chemotherapeutic treatment alone. Both doxorubicin alone and the combination treatment reduced tumor growth significantly compared to mock treatment, but the difference between the two groups was not significant (**Study II, Fig. 3a**). Interestingly, less efficacy was observed when Ad5/3-D24-GMCSF administration was more frequent (**Study II, Fig. 3a)**, probably attributable to inflammatory swelling in response to adenovirus injection (Reid et al., 2005, Koski et al., 2013), result obtained also in **Study I**. The degree of permissivity of Syrian hamsters to the chimeric 5/3 fiber is not clear. According to our findings (*in vitro* transduction

assay and *in vivo* experiments), Syrian hamsters may be less permissive to 5/3 chimeric viruses than to Ad5wt (**Study I and II**), and thus it is not optimal for the evaluation of Ad5/3-D24-GMCSF oncolytic efficiency. We conclude that combination of virus and chemotherapeutic drugs results into therapeutic synergy in the Syrian hamster STS model, improving also the survival of the animals.

Of note, apart from the neoplastic process, necrosis and acute hemorrhage in tumor tissues, none of the hamsters (treated and untreated) exhibited any significant pathological changes in any of the analyzed normal organs, suggesting lack of local or systemic pathological effects of the virus treatment (**Study I, II**).

4.2.3 Effects of chemotherapy on adenoviral replication *in vivo* (II)

Based on our *in vitro* results showing significant increase in adenoviral replication in DDT1-MF2 cells infected with Ad5/3-D24-GMCSF and doxorubicin/4-hydroperoxyifosfamide (**Study II, Fig. 1d**), we collected DDT1-MF2 tumors from Syrian hamsters treated with intratumoral injections of Ad5/3-D24-GMCSF and/or intraperitoneal injections of doxorubicin, to analyze adenoviral *E4* copy number. A significant increase in copy number was observed in combination-treated tumors compared to tumors treated with virus alone, suggesting increased adenoviral replication in the presence of doxorubicin (**Study II, Fig. 3b**). Similar results were obtained by previous studies which reported the combination of viruses with different chemotherapeutic drugs (Raki et al., 2005, Yu et al., 2001, Weiland et al., 2014). In conclusion, adenoviral replication was increased by the presence of chemotherapeutic drugs both *in vitro* and *in vivo* in Syrian hamster STS tumors treated with combination regimens.

4.2.4 *In vivo* immunological effects of Ad5/3-D24-GMCSF alone or in combination with chemotherapy (I, II)

Given the potent immunostimulatory capacity of GM-CSF, we studied the immunological effects of the virus in immunocompetent Syrian hamsters (**Study I**) and C57BL/6 mice (**Study II**). Since GM-CSF has a major role in stimulating DCs, which can result in T-cell attack on tumors (Dranoff, 2003), in Study I we analyzed the tumors collected from Syrian hamsters for the degree of T-cell (CD3+) and B cell/plasma cell (CD79a+) infiltration. We observed a trend for more T-cells in injected and uninjected tumors of virus-treated animals compared to tumors of mock-treated animals (**Study I, Suppl. Fig. S5a**). In particular, the trend was highest in the animals that were treated with Ad5/3-D24-GMCSF-repeated low-dose (**Study I, Suppl. Fig. S5a, S6**). No difference was seen in B-cell and plasma cell infiltration, which were also often observed in tumor periphery (**Study I, Suppl. Fig. S5b**). Some tumors, in particular tumors treated with low-dose Ad5/3-D24-GMCSF, also displayed heterophil (equivalent of neutrophils in other species) infiltration, which could be a local effect of GM-CSF (Khajah et al., 2011). Immunological studies in Syrian hamsters are limited by the lack of reagents. Because there are currently no antibodies that would allow the study of different T-cell subsets, our result (obtained with a cross-reacting antibody against CD3) does not separate between cytotoxic and regulatory T-cells. For this reason, the mechanistic immunological effects of Ad5/3-D24-GMCSF and doxorubicin/ifosfamide combination therapy were studied in immunocompetent C57BL/6 mice carrying B16-OVA melanoma tumors (**Study II**), with the limitation that human adenovirus does not induce oncolysis of mouse cells. Tumors and spleens of mice treated with Ad5/3-D24-GMCSF and/or doxorubicin/ifosfamide chemotherapy every 3 days were stained with antibodies against key markers of DC maturation (CD80 and CD86). We observed no difference in tumor samples between groups, but a statically significant increase in

DC maturation in spleens of combination-treated mice compared to those treated with virus only (**Study II, Suppl. Fig. S1**). We also analyzed CD8+ cytotoxic T-cell in tumors and spleens, but we saw no significant differences between treatment groups.

With regard to human immune cells, we showed that GM-CSF encoded by the virus and produced from infected human melanoma cells, led to differentiation of human primary monocytes into macrophages (**Study III, Fig. 3 and Suppl. Fig. S3, S4**), which is an important step in the induction of immune responses against the tumor. These results suggest that virally encoded GM-CSF can achieve important immunological effects (induction of monocyte-macrophage differentiation, DC maturation, and possible recruitment of immune cells at the tumor site), but further studies are required to clarify these points.

4.2.5 Mechanisms of synergy between Ad5/3-D24-GMCSF and doxorubicin-based chemotherapy (II)

In Study II, we concluded that Ad5/3-D24-GMCSF combined with doxorubicin or doxorubicin plus ifosfamide is an effective treatment modality for STS in a fully immunocompetent system, with evidence of therapeutic synergy. But what is the mechanism of synergy between virus and chemotherapy? Our results, obtained from different animal models (each with its limitations), revealed that:

1) The combination of doxorubicin and ifosfamide is immunogenic (**Study II, Fig. 1a-c**). Immunogenicity of cell death is an important aspect for stimulation of DCs, which can consequently take up tumor-associated antigens (TAAs) and present them to CD4+ and CD8+ T-cells in the local lymph node, for activation of adaptive immune response (Smyth et al., 2001). In this context, the role of Ad5/3-D24-GMCSF replication is to mediate tumor cell lysis, with

consequent release of TAAs and increase of “danger signals” at the tumor site. In the meanwhile, GM-CSF produced by the virus further recruits and activates DCs.

2) Ifosfamide is an alkylating agent, analog of cyclophosphamide. Like cyclophosphamide, it can downregulate circulating regulatory T-cells in immunocompetent hosts (**Study II, Fig. 2**), with following suppression of their inhibitory functions on cytotoxic T-cells and NK- cells (Ghiringhelli et al., 2007). Since tumors recruit immunosuppressive regulatory T-cells to evade antitumor T-cell responses, the use of ifosfamide and cyclophosphamide in cancer therapy can lead to a restoration of peripheral T-cell proliferation. When combined with oncolytic adenovirus treatment, cyclophosphamide (and ifosfamide) mediates similar effects (Koski et al., 2010, Cerullo et al., 2011).

3) Doxorubicin enhances adenovirus replication (**Study II, Fig. 3,4**), particularly in human cells fully permissive to chimeric 5/3 adenoviruses (**Study II, Fig. 4**). Increased virus replication leads to the immunological events described in point number 1, resulting in an adaptive immune response.

4.3 Clinical results

Based on previous promising published results (Koski et al., 2010, Liikanen et al., 2013, Kanerva et al., 2013), we hypothesized that treatments with Ad5/3-D24-GMCSF could particularly benefit patients with advanced sarcoma, melanoma and breast cancer. To corroborate this hypothesis, using registry research techniques, we collected the available preliminary human data from the ATAP for each specific tumor type, in terms of safety, biological virus activity, possible signs of efficacy and overall survival.

4.3.1 Characteristics of patients and treatments (I, III, IV)

In **Studies I, III and IV**, 15 patients with chemotherapy-refractory STS (13/15) and primary bone sarcomas (2/15), 9 patients with advanced melanoma and 16 with late stage breast cancer, respectively, received treatments with Ad5/3-D24-GMCSF. Of the 16 patients with breast cancer, 4 had a TNBC. Patients received single injections of Ad5/3-D24-GMCSF (single treatment) and/or 3 injections with Ad5/3-D24-GMCSF within 10 weeks (serial treatment). In some of the serial treatments, Ad5/3-D24-GMCSF was one of the viruses (out of 3) included in the treatment. In ATAP, in addition to Ad5/3-D24-GMCSF treatments, patients were free to receive other cancer therapies, including additional virus treatments. Details on patients and treatments are available in **Study I, Suppl. Materials and Suppl. Table S1**; **Study III, Suppl. Materials and Suppl. Table S1, Study IV, Suppl. Materials and Suppl. Table S1.**

4.3.2 Safety of treatments and adverse reactions (I, III, IV)

Adverse reactions were monitored and recorded in every patient who was treated in ATAP. Ad5/3-D24-GMCSF treatments were overall well-tolerated, with mostly grade 1-2 adverse reactions (fever, fatigue, nausea, flu-like symptoms, pain and hematological disturbances). Grade 3 ARs were reported in 5/15 sarcoma patients (**Study I, Table 1**), 3/9 melanoma patients (**Study III, Suppl. Table S2**), and 3/16 breast cancer patients (**Study IV, Suppl. Table S2**), but none were classified as a SAE. Grade 4 ARs were encountered in 1/15 sarcoma patients, 1/9 melanoma patients, and 1/16 breast cancer patients. Of these, only the grade 4 thrombocytopenia reported in sarcoma patient S281 was classified as SAE, since the patient was hospitalized for a thrombocyte infusion 4 weeks after the last Ad5/3-D24-GMCSF treatment. Of note, the patient had also received radiotherapy and dexamethasone which may also have contributed to the low thrombocyte

number. Grade 4 dyspnea and pericardium/pleural fluid reported in melanoma patient I266, and grade 4 ketoacidosis reported in breast cancer patient R317 were caused respectively by disease progression and pre-existing diabetes. Transient lymphopenia in the peripheral blood was observed in almost all the patients. This is not unexpected, since viral infections are known to reduce lymphocyte numbers in blood (Wen et al., 2011). Thus, this phenomenon can be frequently encountered also in association with oncolytic virus treatments, and likely to reflect redistribution of lymphocytes from blood to the sites of infection (Reid et al., 2002, Brahmer et al., 2010, Kanerva et al., 2013, Hemminki et al., 2015). Further studies on tumor biopsies are required to determine the relevance of this phenomenon.

4.3.3 Neutralizing antibody responses and virus titers in patient serum after treatment (I, III, IV)

To study the activity of the virus in human patients, we assessed neutralizing antibody titer and the presence of adenovirus genomes in serum before and after viral treatments. At baseline, neutralizing antibodies against Ad5/3 were detectable in low to intermediate range in 9/12 evaluable sarcoma patients (**Study I, Table 2**), 3/6 melanoma patients (**Study III, Table 1**), and 4/5 evaluable breast cancer patients (**Study IV, Suppl. Table S3**). After treatment, the titer increased or remained stable in all the evaluable patients. Prolonged presence of viral genomes in serum is indicative of virus replication, since injected virus is rapidly cleared from the blood stream (Galanis et al., 2005). Overall, there was no viral DNA in serum at baseline, while we frequently observed measurable viral DNA levels at one day after treatment, and increase of the viral DNA load beyond day 2, suggesting virus replication (**Study I, Table 2; Study III, Table 1; Study IV, Suppl. Table S3**). We did not observe a clear correlation between neutralizing antibody titers and viral DNA in serum, in line with other reports (Galanis et al., 2005, Nemunaitis et al., 2001).

4.3.4 Clinical responses and survival (I, III, IV)

The regulatory agency FIMEA required reporting data on safety, viral kinetics and treatment responses obtained from ATAP. Thus, we reported possible signs of efficacy of Ad5/3-D24-GMCSF treatments in sarcoma, melanoma and breast cancer patients. In **Study I**, the tumors of 9/15 patients were imaged with CT: tumors size after treatment was compared to pre-treatment CT scans, and the response was evaluated according to modified RECIST 1.1. criteria. A total of 12 evaluations were performed (3 patients were evaluated after each Ad5/3-D24-GMCSF injection), and treatments resulted in stable disease or better in 8/12 evaluations (**Study I, Table 2**). In **Study III**, 4/9 patients were evaluable for treatment response, resulting in disease control in 3/4 patients (**Study III**, **Table 1**). In **Study IV**, 13/16 patients were evaluable according to modified RECIST 1.1. and/or PET criteria. Overall, 1/14 had a MR/PMR, 2/14 a SD, 10/14 a PD/PMD (**Study IV, Table 1 and Fig. 3a,b**). Furthermore, serum breast cancer antigen 15-3 (Ca15-3) and carcinoembryonic antigen (CEA) levels were also followed-up as possible indicators of treatment response for 13 patients whom had had elevated marker levels before the start of the virus treatment (**Study IV, Table 1 and Fig. 3c**): 1/13 patient had a CR (with CEA), 1/13 had a PR (with Ca15-3), 5/13 had a MR (3 with Ca15-3 and 2 with CEA), 1/13 a SD (with Ca15-3) and 5/13 a PD (with Ca15-3). In one patient (R170), both Ca15-3 and CEA were measured, and different responses were obtained (129% increase of Ca15-3 and 10% reduction of CEA marker levels) (**Study IV, Table 1 and Fig. 3c**). TNBC patients (4/16) showed PD after treatment, underlining the aggressive nature of this breast cancer subgroup. Of note, one of these patients (R328) showed a 22% reduction in the size of injected lesions and 70% decrease of tumor marker CEA, suggesting antitumor activity of the virus. However, in the final RECIST analysis the patient showed PMD, due to two new metastases (**Study IV, Table 1 and Fig. 3).**

Overall survival data was also collected and, a total of 4 patients (one sarcoma patient, 2 melanoma patients and one breast cancer patient) were still alive at the time of submitting the original manuscripts (**Figure 2**). At the time of writing this thesis, survival was updated: the sarcoma patient, one melanoma patient and the breast cancer patient are still alive, over 6, 6.5 and 4 years after treatment respectively, while there is no information available regarding the second melanoma patient. Median survival was 170, 51 and 233 days after the first Ad5/3-D24-GMCSF treatment, respectively of all treated sarcoma (**Study I, Fig. 4**), melanoma (**Study III, Suppl. Fig. S5**) and breast cancer patients (**Study IV, Suppl. Fig. S2**).

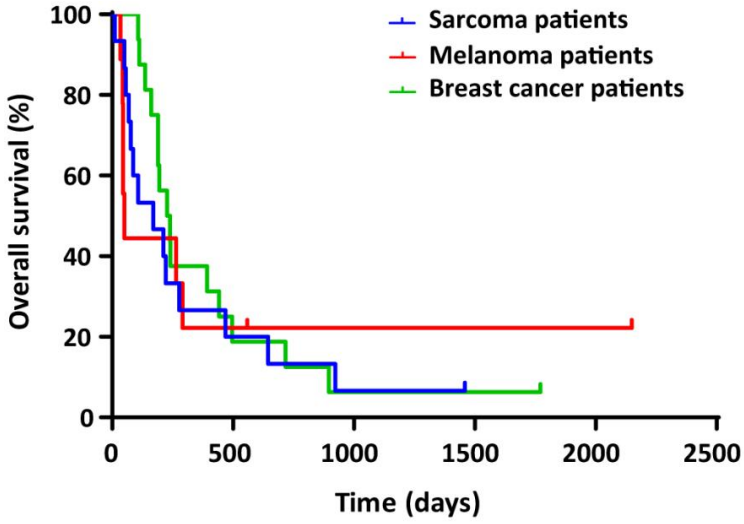

Figure 2. Overall survival of Ad5/3-D24-GMCSF-treated sarcoma, melanoma and breast cancer patients. Survival after the first treatment with Ad5/3-D24-GMCSF virus was analyzed by Kaplan-Meier method. Four patients (one sarcoma, two melanoma and one breast cancer patient) were alive at the time of submitting the original manuscripts. Sarcoma, N = 15; Melanoma, N = 9; Breast cancer, N = 16.

5. SUMMARY AND CONCLUSIONS

In this thesis, we investigated Ad5/3-D24-GMCSF for the treatment of sarcoma, melanoma and breast cancer, and we explored methods for improving the overall antitumor efficacy of the virus by combining oncolytic virus therapy with commonly used chemotherapeutic agents. We summarized and analyzed data from cancer patients treated in ATAP, providing preliminary information useful for optimal designing of potential future clinical trials. Our results are in line with previous publications on Ad5/3-D24-GMCSF, suggesting that this virus is a promising agent for treatment of advanced solid tumors.

With regard to *in vitro* results, in study I and III, we demonstrated that 5/3 chimerism enhanced the viral transduction of human sarcoma cell lines but not of hamster sarcoma and melanoma cells. These results, together with previous publications, suggest that Syrian hamsters are semi-permissive to human serotype 5 adenoviruses, but not at the same degree to 5/3 chimeric adenoviruses.

In vivo, we studied Ad5/3-D24-GMCSF as a single agent (study I) and in combination with doxorubicin and ifosfamide (study II), in the only hamster sarcoma model currently available. In study I, due to rapid tumor growth, assessment of tumor size did not indicate antitumor efficacy. Nevertheless, in study II we observed an efficient tumor-growth control when the virus was combined with doxorubicin with or without ifosfamide, and we demonstrated increased adenoviral replication in the presence of chemotherapy.

Based on previous studies showing advantages in using low-dose metronomic chemotherapy in combination with oncolytic immunotherapy, we studied the combination of Ad5/3-D24-GMCSF with low-dose CP in melanoma and breast cancer preclinical models (studies III and IV), and we observed improved antitumor efficacy in the combination treatment compared to single agents.

Our *in vivo* results in study I showed the ability of intratumorally injected Ad5/3-D24-GMCSF to reach distant uninjected tumors (in the same animal) through the blood stream, leading to systemic effects useful for the treatment of metastatic cancer. In addition, in study III we demonstrated that virally encoded GM-CSF, produced from tumor cells *in vitro*, can stimulate differentiation of human primary monocytes into macrophages, important for induction of immune responses.

Finally, our preliminary data from cancer patients treated in ATAP showed promising results regarding safety, signs of possible treatment benefits and patient survival. However, these results should be interpreted with caution as only clinical trials may ultimately determine if the safety and efficacy features obtained *in vitro* and *in vivo* experiments on animal models are retained in humans. Of note, the first phase I study of Ad5/3-D24-GMCSF in late-stage refractory cancer patients was recently successfully completed by Oncos Therapeutics Ltd., proving safety of the treatment and induction of innate, adaptive and antitumor immune responses, with stabilization of disease in 40% of evaluable patients after 3 months (Oncos Therapeutics Ltd., unpublished).

In conclusion, oncolytic immunotherapy combined with low-dose chemotherapy is a promising approach for treatment of cancer, where the antitumor effects of the virus overlap with the beneficial effects of low-dose chemotherapy, leading to oncolysis of cancer cells, strong pro-immunogenic signals, induction of innate and adaptive immune responses, while low-dose chemotherapy enhances viral replication, reduces tumor immunosuppression, and modifies tumor microenvironment. Phase I/II trials studying combination regimens including Ad5/3-D24-GMCSF are in the planning stages.

6. FUTURE PROSPECTS

Combination therapies

The rationale for combination therapies is to use treatments that work by different mechanisms, thereby increasing the chance of killing more cancer cells and to prevent the emergence of resistance. For some tumors, the best approach is a combination of surgery, radiotherapy, and chemotherapy. Radiotherapy and chemotherapy can be administered before surgery to shrink a tumor, thus improving the chance of complete surgical removal. If applied after surgery, radiotherapy and low-dose chemotherapy help to destroy remaining cancer cells and those that have spread to distant sites. While increasing the likelihood of a cure, combination therapies can also cause more side effects, and thus the goal is to find appropriate combination of treatments to achieve synergistic killing while avoiding additive toxicity (Al-Lazikani et al., 2012).

The rise of new treatment options, including oncolytic virotherapy, cancer immunotherapy and targeted therapy has increased the number of appealing combination approaches, mainly aiming at inhibiting molecular pathways that are crucial for tumor growth or destroying cancer cells *via* oncolysis, while modulating and stimulating host antitumor immune responses (Vanneman and Dranoff, 2012). Several clinical trials are underway to evaluate the optimal sequencing of different therapies (ClinicalTrials.gov). With regard to oncolytic virotherapy, oncolytic viruses have been combined with standard radiation therapy and chemotherapeutic agents, as well as with novel biologic therapies, including immunotherapy (Ottolino-Perry et al., 2010). With the discovery that radiotherapy enhances viral oncolysis, and that certain viral proteins sensitize cells to radiation therapy, the combination of these two treatments has been widely studied, with promising synergistic antitumor effects in preclinical models (Ottolino-Perry et al., 2010).

Oncolytic viruses have been investigated in combination with a multitude of standard chemotherapeutics with different mechanisms of action. Combination of oncolytic virotherapy with cyclophosphamide, doxorubicin and ifosfamide are described in this thesis. Other combination regimens which have been evaluated include cisplatin, 5-fluorouracil, paclitaxel, docetaxel, rapamycin (Ottolino-Perry et al., 2010), and other alkylating agents such as temozolomide (Liikanen et al., 2013).

With the idea that oncolytic viruses exert their effects through both direct lysis of tumor cells and through induction of an immune response, researchers have started to explore strategies to enhance viral-induced antitumoral immunity. Among these, the insertion of immunostimulatory molecules such as GM-CSF in the virus genome has been described in section 1.8. Furthermore, an emerging strategy to enhance tumor killing is the combination of oncolytic viruses with adoptive cell therapy. In this setting, viral oncolysis may be able to enhance the efficacious effects of adoptively transferred T cells.

In summary, the future of cancer treatment is moving towards new combination treatments, including oncolytic virotherapy, standard therapies (chemotherapy, radiation therapy), and novel immunotherapeutics, aiming both at understanding the interplay between the agents of choice and providing the best possible clinical outcome. As forms of therapy such as checkpoint-inhibiting antibodies, targeted therapy and adoptive T-cell therapy are gaining more and more attention during the years, it is easy to envision many other combination strategies which may ultimately reach standard care in the treatment of many advanced tumors.

7. ACKNOWLEDGEMENTS

This work was carried out during the years 2011 – 2015 in the Cancer Gene Therapy Group (CGTG), which was previously part of the Molecular Cancer Biology Program in Biomedicum Helsinki and currently affiliated to Department of Pathology, Faculty of Medicine, University of Helsinki.

I want to thank the Dean of the Faculty of Medicine, Professor Risto Renkonen, the head of the Haartman Institute, Professor Tom Böhling, the former and present directors of the Molecular Cancer Biology Program, Professors Kari Alitalo, Jorma Keski-Oja and Marikki Laihio, as well as the head of Biomedicum and Faculty Research Programs, Professor Olli A. Jänne, for providing excellent research facilities. I want to express my sincere gratitude to Professor Eivind Per Thor Straten for accepting the tough role of thesis opponent, making my dissertation possible.

I wish to thank the Doctoral Programme in Biomedicine and the Doctoral Programme in Clinical Research for the excellent graduate student programs, continuous guidance and education, and for providing financial support. These studies were financially supported by Doctoral Programmes in Biomedicine and in Clinical Research, European Research Council, American Society of Clinical Oncology (ASCO) Foundation, Helsinki University Central Hospital (HUCH) Research Funds (EVO), K. Albin Johansson Foundation, Oncos Therapeutics Ltd., Sigrid Juselius Foundation, Academy of Finland, Biocentrum Helsinki, Biocenter Finland, Cancer Organizations and University of Helsinki.

I want to express my sincere gratitude for my thesis committee members Docents Satu Mustjoki and Hanna Jarva for providing valuable advice and encouragement, as well as Docent Hanna Jarva and Professor Kimmo Taari for the time you spent in reviewing my thesis.

I am deeply grateful to my supervisor and the leader of CGTG, Professor Akseli Hemminki for believing in me and giving me the opportunity to join your research group. You always had time to discuss my research problems and suggest the right solution. I admire your optimism, enthusiasm and determination.

I am sincerely grateful to my two post-doc supervisors, Sari and Anniina: thank you for all your help with experiments, writing and revisions, and for providing professional advice for my projects. I also want to thank all the senior researchers of CGTG, especially Vincenzo, Lotta and Markus for advising me with study designs and suggesting new ideas.

I thank all the current and previous members of CGTG: Suvi, my first hands-on mentor, for introducing me to the CGTG research world; Siri, my mamma (even though she doesn´t cook like my real mamma does), for all your advices regarding life and for listening to me every time I had an ongoing drama in my life; Mikko, my buddy, for being the "clown" of the office and for always making me laugh. Thank you for all the chocolate you bought for me and for trying to cheer me up every time I was sad. I will never forget the first thing you said when we met in Biomedicum ("Prosciutto!") and the second thing ("I have 100 hamsters, can you help me?"). But with you, even to deal with 100 hamsters was funny; Minna, our scientific officer, thank you for all your help with endless practical matters, without you I would have been completely lost 90% of the time. I am also grateful to all the other CGTG members, including Ilkka, Riikka, Kristian, Otto, Mari, Marko, Petri, Sadia, Karo, and talented technicians Aila, Kikka, Suski, Maiju, Saila, Saija. I thank all the co-authors and collaborators from Finland and outside Finland for making this thesis possible.

Special thanks to all the Finnish rock and metal bands for making my life better, cheerful, and full of gigs. HIM, Negative, Amorphis, Sonata Arctica, Sturm und Drang, Sunrise Avenue, 69Eyes, Poets of the Fall: without you I would have

probably never ended up in this country and I would have missed a great experience.

I thank all my friends outside the lab and the people I met during these years in Finland, who contributed to make my life happier: my former band members Andrea, Jani, Jussi and Eero for all the fun we had at our rehearsals place and during live performances: sharing the stage with Prana, Jason, Jude and Vivian has been a great honor for me; the “Italian gang” Gabriele, Sebastiano, Beppe and Ivan for always reminding me about Italy, Italian food and lifestyle and for all the great parties at Borg Soundlab; Marco, for always reserving time to listen to my problems; my numerous flatmates Elisa, Cristina, Francesca and, again, Gabriele, for making my evenings at home so enjoyable. Special thanks to Francesco and Morgan for being with me at the very beginning of this adventure, supporting me during my pre-interview anxiety.

Finally, I want to express my deepest gratitude for Eduard, who stands me every day (I know I haven´t been the easiest person in the world during the last 5 months) and for my beloved family: mamma Giovanna and papá Ettore, to whom I dedicate this thesis, for all your love and support despite the distance; seeing you so seldom is not easy; my brother Pietro and my sister Susanna for all the funny times we have every time we see each other, it´s always like being kids playing together; my cute little nephew Samuel, he´s only 2 years old and he already rocks!

Helsinki, 17.7.2015

Simona Bramante

8. REFERENCES

AL-LAZIKANI, B., BANERJI, U. & WORKMAN, P. 2012. Combinatorial drug therapy for cancer in the post-genomic era. *Nat Biotechnol,* 30, 679-92.

ALBA, R., BOSCH, A. & CHILLON, M. 2005. Gutless adenovirus: last-generation adenovirus for gene therapy. *Gene Ther,* 12 Suppl 1, S18-27.

ALEMANY, R. 2008. A smart move against cancer for vaccinia virus. *Lancet Oncol,* 9, 507-8.

AMBRIOVIC-RISTOV, A., MERCIER, S. & ELOIT, M. 2003. Shortening adenovirus type 5 fiber shaft decreases the efficiency of postbinding steps in CAR-expressing and nonexpressing cells. *Virology,* 312, 425-33.

AMENDOLA, B. E., AMENDOLA, M. A., MCCLATCHEY, K. D. & MILLER, C. H., JR. 1989. Radiation-associated sarcoma: a review of 23 patients with postradiation sarcoma over a 50-year period. *Am J Clin Oncol,* 12, 411-5.

ANDRE, F. & ZIELINSKI, C. C. 2012. Optimal strategies for the treatment of metastatic triple-negative breast cancer with currently approved agents. *Ann Oncol,* 23 Suppl 6, vi46-51.

ANDTBACKA, R. H., KAUFMAN, H. L., COLLICHIO, F., AMATRUDA, T., SENZER, N., CHESNEY, J., DELMAN, K. A., SPITLER, L. E., PUZANOV, I., AGARWALA, S. S., MILHEM, M., CRANMER, L., CURTI, B., LEWIS, K., ROSS, M., GUTHRIE, T., LINETTE, G. P., DANIELS, G. A., HARRINGTON, K., MIDDLETON, M. R., MILLER, W. H., JR., ZAGER, J. S., YE, Y., YAO, B., LI, A., DOLEMAN, S., VANDERWALDE, A., GANSERT, J. & COFFIN, R. 2015. Talimogene Laherparepvec Improves Durable Response Rate in Patients With Advanced Melanoma. *J Clin Oncol*.

ARANDA, F., VACCHELLI, E., EGGERMONT, A., GALON, J., SAUTES-FRIDMAN, C., TARTOUR, E., ZITVOGEL, L., KROEMER, G. & GALLUZZI, L. 2013. Trial Watch: Peptide vaccines in cancer therapy. *Oncoimmunology,* 2, e26621.

ARELLANO, M. & LONIAL, S. 2008. Clinical uses of GM-CSF, a critical appraisal and update. *Biologics,* 2, 13-27.

ATKINS, M. B., LOTZE, M. T., DUTCHER, J. P., FISHER, R. I., WEISS, G., MARGOLIN, K., ABRAMS, J., SZNOL, M., PARKINSON, D., HAWKINS, M., PARADISE, C., KUNKEL, L. & ROSENBERG, S. A. 1999. High-dose recombinant interleukin 2 therapy for patients with metastatic melanoma: analysis of 270 patients treated between 1985 and 1993. *J Clin Oncol,* 17, 2105-16.

AWADA, A., GIL, T., SALES, F., DUBUISSON, M., VEREECKEN, P., KLASTERSKY, J., MOERMAN, C., DE VALERIOLA, D. & PICCART, M. J. 2004. Prolonged schedule of temozolomide (Temodal) plus liposomal doxorubicin (Caelyx) in advanced solid cancers. *Anticancer Drugs,* 15, 499-502.

BARNETT, B. G., TILLMAN, B. W., CURIEL, D. T. & DOUGLAS, J. T. 2002. Dual targeting of adenoviral vectors at the levels of transduction and transcription enhances the specificity of gene expression in cancer cells. *Mol Ther,* 6, 377-85.

BASKAR, R., LEE, K. A., YEO, R. & YEOH, K. W. 2012. Cancer and radiation therapy: current advances and future directions. *Int J Med Sci,* 9, 193-9.

BAUERSCHMITZ, G. J., BARKER, S. D. & HEMMINKI, A. 2002. Adenoviral gene therapy for cancer: from vectors to targeted and replication competent agents (review). *Int J Oncol,* 21, 1161-74.

BAUERSCHMITZ, G. J., GUSE, K., KANERVA, A., MENZEL, A., HERRMANN, I., DESMOND, R. A., YAMAMOTO, M., NETTELBECK, D. M., HAKKARAINEN, T., DALL, P., CURIEL, D. T. & HEMMINKI, A. 2006. Triple-targeted oncolytic adenoviruses featuring the cox2 promoter, E1A transcomplementation, and serotype chimerism for enhanced selectivity for ovarian cancer cells. *Mol Ther,* 14, 164-74.

BEATTY, M. S. & CURIEL, D. T. 2012. Chapter two--Adenovirus strategies for tissue-specific targeting. *Adv Cancer Res,* 115, 39-67.

BERGELSON, J. M., CUNNINGHAM, J. A., DROGUETT, G., KURT-JONES, E. A., KRITHIVAS, A., HONG, J. S., HORWITZ, M. S., CROWELL, R. L. & FINBERG, R. W. 1997. Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. *Science,* 275, 1320-3.

BERK, A. J. 2005. Recent lessons in gene expression, cell cycle control, and cell biology from adenovirus. *Oncogene,* 24, 7673-85.

BETT, A. J., PREVEC, L. & GRAHAM, F. L. 1993. Packaging capacity and stability of human adenovirus type 5 vectors. *J Virol,* 67, 5911-21.

BHATIA, S., TYKODI, S. S. & THOMPSON, J. A. 2009. Treatment of Metastatic Melanoma: An Overview. *Oncology (Williston Park, N.Y.),* 23, 488-496.

BISCHOFF, J. R., KIRN, D. H., WILLIAMS, A., HEISE, C., HORN, S., MUNA, M., NG, L., NYE, J. A., SAMPSON-JOHANNES, A., FATTAEY, A. & MCCORMICK, F. 1996. An adenovirus mutant that replicates selectively in p53-deficient human tumor cells. *Science,* 274, 373-6.

BRADLEY, R. R., LYNCH, D. M., IAMPIETRO, M. J., BORDUCCHI, E. N. & BAROUCH, D. H. 2012. Adenovirus serotype 5 neutralizing antibodies target both hexon and fiber following vaccination and natural infection. *J Virol,* 86, 625-9.

BRAHMER, J. R., DRAKE, C. G., WOLLNER, I., POWDERLY, J. D., PICUS, J., SHARFMAN, W. H., STANKEVICH, E., PONS, A., SALAY, T. M., MCMILLER, T. L., GILSON, M. M., WANG, C., SELBY, M., TAUBE, J. M., ANDERS, R., CHEN, L., KORMAN, A. J., PARDOLL, D. M., LOWY, I. & TOPALIAN, S. L. 2010. Phase I study of single-agent anti-programmed death-1 (MDX-1106) in refractory solid tumors: safety, clinical activity, pharmacodynamics, and immunologic correlates. *J Clin Oncol,* 28, 3167-75.

BURKE, J. M. 2010. GM-CSF-armed, replication-competent viruses for cancer. *Cytokine Growth Factor Rev,* 21, 149-51.

BURKE, J. M., LAMM, D. L., MENG, M. V., NEMUNAITIS, J. J., STEPHENSON, J. J., ARSENEAU, J. C., AIMI, J., LERNER, S., YEUNG, A. W., KAZARIAN, T., MASLYAR, D. J. & MCKIERNAN, J. M. 2012. A first in human phase 1 study of CG0070, a GM-CSF expressing oncolytic adenovirus, for the treatment of nonmuscle invasive bladder cancer. *J Urol,* 188, 2391-7.

BURNINGHAM, Z., HASHIBE, M., SPECTOR, L. & SCHIFFMAN, J. D. 2012. The epidemiology of sarcoma. *Clin Sarcoma Res,* 2, 14.

BUZDAR, A. U. 2009. Role of biologic therapy and chemotherapy in hormone receptor- and HER2-positive breast cancer. *Ann Oncol,* 20, 993-9.

CAMPOS, S. K. & BARRY, M. A. 2007. Current advances and future challenges in Adenoviral vector biology and targeting. *Curr Gene Ther,* 7, 189-204.

CASARES, N., PEQUIGNOT, M. O., TESNIERE, A., GHIRINGHELLI, F., ROUX, S., CHAPUT, N., SCHMITT, E., HAMAI, A., HERVAS-STUBBS, S., OBEID, M., COUTANT, F., METIVIER, D., PICHARD, E., AUCOUTURIER, P., PIERRON, G., GARRIDO, C., ZITVOGEL, L. & KROEMER, G. 2005. Caspase-dependent immunogenicity of doxorubicin-induced tumor cell death. *J Exp Med,* 202, 1691-701.

CERULLO, V., DIACONU, I., KANGASNIEMI, L., RAJECKI, M., ESCUTENAIRE, S., KOSKI, A., ROMANO, V., ROUVINEN, N., TUUMINEN, T., LAASONEN, L., PARTANEN, K., KAUPPINEN, S., JOENSUU, T., OKSANEN, M., HOLM, S. L., HAAVISTO, E., KARIOJA-KALLIO, A., KANERVA, A., PESONEN, S., ARSTILA, P. T. & HEMMINKI, A. 2011. Immunological effects of low-dose cyclophosphamide in cancer patients treated with oncolytic adenovirus. *Mol Ther,* 19, 1737-46.

CERULLO, V., PESONEN, S., DIACONU, I., ESCUTENAIRE, S., ARSTILA, P. T., UGOLINI, M., NOKISALMI, P., RAKI, M., LAASONEN, L., SÄRKIOJA, M., RAJECKI, M., KANGASNIEMI, L., GUSE, K., HELMINEN, A., AHTIAINEN, L., RISTIMÄKI, A., RÄISÄNEN-SOKOLOWSKI, A., HAAVISTO, E., OKSANEN, M., KARLI, E., KARIOJA-KALLIO, A., HOLM, S. L., KOURI, M., JOENSUU, T., KANERVA, A. & HEMMINKI, A. 2010. Oncolytic adenovirus coding for granulocyte macrophage colony-stimulating factor induces antitumoral immunity in cancer patients. *Cancer Res,* 70, 4297-309.

CERULLO, V., SEILER, M. P., MANE, V., BRUNETTI-PIERRI, N., CLARKE, C., BERTIN, T. K., RODGERS, J. R. & LEE, B. 2007. Toll-like receptor 9 triggers an innate immune response to helper-dependent adenoviral vectors. *Mol Ther,* 15, 378-85.

CHANG, D. Z., LOMAZOW, W., JOY SOMBERG, C., STAN, R. & PERALES, M. A. 2004. Granulocyte-macrophage colony stimulating factor: an adjuvant for cancer vaccines. *Hematology,* 9, 207-15.

CHANG, J., ZHAO, X., WU, X., GUO, Y., GUO, H., CAO, J., LOU, D., YU, D. & LI, J. 2009. A Phase I study of KH901, a conditionally replicating granulocyte-macrophage colony-stimulating factor: armed oncolytic adenovirus for the treatment of head and neck cancers. *Cancer Biol Ther,* 8, 676-82.

CHU, Y., HEISTAD, D., CYBULSKY, M. I. & DAVIDSON, B. L. 2001. Vascular cell adhesion molecule-1 augments adenovirus-mediated gene transfer. *Arterioscler Thromb Vasc Biol,* 21, 238-42.

CLINICALTRIALS.GOV. https://clinicaltrials.gov/ct2/show/NCT01438112. [Accessed 17th June 2015].

COGLIANO, V. J., BAAN, R., STRAIF, K., GROSSE, Y., LAUBY-SECRETAN, B., EL GHISSASSI, F., BOUVARD, V., BENBRAHIM-TALLAA, L., GUHA, N., FREEMAN, C., GALICHET, L. & WILD, C. P. 2011. Preventable exposures associated with human cancers. *J Natl Cancer Inst,* 103, 1827-39.

COHEN, A. M., HINES, D. K., KORACH, E. S. & RATZKIN, B. J. 1988. In vivo activation of neutrophil function in hamsters by recombinant human granulocyte colony-stimulating factor. *Infect Immun,* 56, 2861-5.

COHEN, C. J., SHIEH, J. T., PICKLES, R. J., OKEGAWA, T., HSIEH, J. T. & BERGELSON, J. M. 2001. The coxsackievirus and adenovirus receptor is a transmembrane component of the tight junction. *Proc Natl Acad Sci U S A,* 98, 15191-6.

D'ANGELO, S. P., TAP, W. D., SCHWARTZ, G. K. & CARVAJAL, R. D. 2014. *Sarcoma Immunotherapy: Past Approaches and Future Directions*.

DANAEI, G., VANDER HOORN, S., LOPEZ, A. D., MURRAY, C. J. & EZZATI, M. 2005. Causes of cancer in the world: comparative risk assessment of nine behavioural and environmental risk factors. *Lancet,* 366, 1784-93.

DANTHINNE, X. & IMPERIALE, M. J. 2000. Production of first generation adenovirus vectors: a review. *Gene Ther,* 7, 1707-14.

DECHECCHI, M. C., MELOTTI, P., BONIZZATO, A., SANTACATTERINA, M., CHILOSI, M. & CABRINI, G. 2001. Heparan sulfate glycosaminoglycans are receptors sufficient to mediate the initial binding of adenovirus types 2 and 5. *J Virol,* 75, 8772-80.

DIACONU, I., CERULLO, V., HIRVINEN, M. L., ESCUTENAIRE, S., UGOLINI, M., PESONEN, S. K., BRAMANTE, S., PARVIAINEN, S., KANERVA, A., LOSKOG, A. S., ELIOPOULOS, A. G., PESONEN, S. & HEMMINKI, A. 2012. Immune response is an important aspect of the antitumor effect produced by a CD40L-encoding oncolytic adenovirus. *Cancer Res,* 72, 2327-38.

DIX, B. R., EDWARDS, S. J. & BRAITHWAITE, A. W. 2001. Does the antitumor adenovirus ONYX-015/dl1520 selectively target cells defective in the p53 pathway? *J Virol,* 75, 5443-7.

DMITRIEV, I., KRASNYKH, V., MILLER, C. R., WANG, M., KASHENTSEVA, E., MIKHEEVA, G., BELOUSOVA, N. & CURIEL, D. T. 1998. An adenovirus vector with genetically modified fibers demonstrates expanded tropism via utilization of a coxsackievirus and adenovirus receptor-independent cell entry mechanism. *J Virol,* 72, 9706-13.

DMITRIEV, I. P., KASHENTSEVA, E. A. & CURIEL, D. T. 2002. Engineering of adenovirus vectors containing heterologous peptide sequences in the C terminus of capsid protein IX. *J Virol,* 76, 6893-9.

DOBBELSTEIN, M. 2004. Replicating adenoviruses in cancer therapy. *Curr Top Microbiol Immunol,* 273, 291-334.

DOLGIN, E. 2015. Oncolytic viruses get a boost with first FDA-approval recommendation. *Nat Rev Drug Discov,* 14, 369-371.

DRANOFF, G. 2003. GM-CSF-secreting melanoma vaccines. *Oncogene,* 22, 3188-92.

DUCIMETIERE, F., LURKIN, A., RANCHERE-VINCE, D., DECOUVELAERE, A. V., ISAAC, S., CLARET-TOURNIER, C., SUIGNARD, Y., SALAMEIRE, D., CELLIER, D., ALBERTI, L., BRINGUIER, P. P., BLAY, J. Y. & RAY-COQUARD, I. 2010. [Incidence rate, epidemiology of sarcoma and molecular biology. Preliminary results from EMS study in the Rhone-Alpes region]. *Bull Cancer,* 97, 629-41.

EISENHAUER, E. A., THERASSE, P., BOGAERTS, J., SCHWARTZ, L. H., SARGENT, D., FORD, R., DANCEY, J., ARBUCK, S., GWYTHER, S., MOONEY, M., RUBINSTEIN, L., SHANKAR,

L., DODD, L., KAPLAN, R., LACOMBE, D. & VERWEIJ, J. 2009. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). *Eur J Cancer,* 45, 228-47.

ELWOOD, J. M. & JOPSON, J. 1997. Melanoma and sun exposure: an overview of published studies. *Int J Cancer,* 73, 198-203.

ESCUTENAIRE, S., CERULLO, V., DIACONU, I., AHTIAINEN, L., HANNUKSELA, P., OKSANEN, M., HAAVISTO, E., KARIOJA-KALLIO, A., HOLM, S. L., KANGASNIEMI, L., RIBACKA, C., KAUPPINEN, S., JOENSUU, T., ARSTILA, T. P., PESONEN, S., KANERVA, A. & HEMMINKI, A. 2011. In vivo and in vitro distribution of type 5 and fiber-modified oncolytic adenoviruses in human blood compartments. *Ann Med,* 43, 151-63.

FERLAY, J., SHIN, H. R., BRAY, F., FORMAN, D., MATHERS, C. & PARKIN, D. M. 2010. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer,* 127, 2893-917.

FINKELSTEIN, S. E., FISHMAN, M., CONLEY, A. P., GABRILOVICH, D., ANTONIA, S. & CHIAPPORI, A. 2012. Cellular immunotherapy for soft tissue sarcomas. *Immunotherapy,* 4, 283-90.

FUEYO, J., GOMEZ-MANZANO, C., ALEMANY, R., LEE, P. S., MCDONNELL, T. J., MITLIANGA, P., SHI, Y. X., LEVIN, V. A., YUNG, W. K. & KYRITSIS, A. P. 2000. A mutant oncolytic adenovirus targeting the Rb pathway produces anti-glioma effect in vivo. *Oncogene,* 19, 2-12.

GAGGAR, A., SHAYAKHMETOV, D. M. & LIEBER, A. 2003. CD46 is a cellular receptor for group B adenoviruses. *Nat Med,* 9, 1408-12.

GALANIS, E., OKUNO, S. H., NASCIMENTO, A. G., LEWIS, B. D., LEE, R. A., OLIVEIRA, A. M., SLOAN, J. A., ATHERTON, P., EDMONSON, J. H., ERLICHMAN, C., RANDLEV, B., WANG, Q., FREEMAN, S. & RUBIN, J. 2005. Phase I-II trial of ONYX-015 in combination with MAP chemotherapy in patients with advanced sarcomas. *Gene Ther,* 12, 437-45.

GALLUCCI, J. M. 2008. Who deserves the credit for discovering ether's use as a surgical anesthetic? *J Hist Dent,* 56, 38-43.

GALLUZZI, L., SENOVILLA, L., ZITVOGEL, L. & KROEMER, G. 2012. The secret ally: immunostimulation by anticancer drugs. *Nat Rev Drug Discov,* 11, 215-33.

GARBER, K. 2006. China approves world's first oncolytic virus therapy for cancer treatment. *J Natl Cancer Inst,* 98, 298-300.

GHIRINGHELLI, F., LARMONIER, N., SCHMITT, E., PARCELLIER, A., CATHELIN, D., GARRIDO, C., CHAUFFERT, B., SOLARY, E., BONNOTTE, B. & MARTIN, F. 2004. CD4+CD25+ regulatory T cells suppress tumor immunity but are sensitive to cyclophosphamide which allows immunotherapy of established tumors to be curative. *Eur J Immunol,* 34, 336-44.

GHIRINGHELLI, F., MENARD, C., PUIG, P. E., LADOIRE, S., ROUX, S., MARTIN, F., SOLARY, E., LE CESNE, A., ZITVOGEL, L. & CHAUFFERT, B. 2007. Metronomic cyclophosphamide regimen selectively depletes CD4+CD25+ regulatory T cells and restores T and NK effector functions in end stage cancer patients. *Cancer Immunol Immunother,* 56, 641-8.

GINN, S. L., ALEXANDER, I. E., EDELSTEIN, M. L., ABEDI, M. R. & WIXON, J. 2013. Gene therapy clinical trials worldwide to 2012 - an update. *J Gene Med,* 15, 65-77.

GINSBERG, A. H., MONTE, W. T. & JOHNSON, K. P. 1977. Effect of cyclophosphamide in vitro and on vaccinia virus replication in tissue culture. *J Virol,* 21, 277-83.

GOODMAN, L. S., WINTROBE, M. M., DAMESHEK, W., GOODMAN, M. J., GILMAN, A. & MCLENNAN, M. T. 1984. Landmark article Sept. 21, 1946: Nitrogen mustard therapy. Use of methyl-bis(beta-chloroethyl)amine hydrochloride and tris(beta-chloroethyl)amine hydrochloride for Hodgkin's disease, lymphosarcoma, leukemia and certain allied and miscellaneous disorders. By Louis S. Goodman, Maxwell M. Wintrobe, William Dameshek, Morton J. Goodman, Alfred Gilman and Margaret T. McLennan. *JAMA,* 251, 2255-61.

GORDON, Y. J., ROMANOWSKI, E. & ARAULLO-CRUZ, T. 1992. An ocular model of adenovirus type 5 infection in the NZ rabbit. *Invest Ophthalmol Vis Sci,* 33, 574-80.

GOTTLIEB, J. A., BAKER, L. H., QUAGLIANA, J. M., LUCE, J. K., WHITECAR, J. P., JR., SINKOVICS, J. G., RIVKIN, S. E., BROWNLEE, R. & FREI, E., 3RD 1972. Chemotherapy of sarcomas with a combination of adriamycin and dimethyl triazeno imidazole carboxamide. *Cancer,* 30, 1632-8.

GREBER, U. F., SUOMALAINEN, M., STIDWILL, R. P., BOUCKE, K., EBERSOLD, M. W. & HELENIUS, A. 1997. The role of the nuclear pore complex in adenovirus DNA entry. *EMBO J,* 16, 5998-6007.

GUO, J. & XIN, H. 2006. Chinese gene therapy. Splicing out the West? *Science,* 314, 1232-5.

GUSE, K., RANKI, T., ALA-OPAS, M., BONO, P., SÄRKIOJA, M., RAJECKI, M., KANERVA, A., HAKKARAINEN, T. & HEMMINKI, A. 2007. Treatment of metastatic renal cancer with capsid-modified oncolytic adenoviruses. *Mol Cancer Ther,* 6, 2728-36.

HAISMA, H. J., BOESJES, M., BEERENS, A. M., VAN DER STRATE, B. W., CURIEL, D. T., PLUDDEMANN, A., GORDON, S. & BELLU, A. R. 2009. Scavenger receptor A: a new route for adenovirus 5. *Mol Pharm,* 6, 366-74.

HAKKARAINEN, T., RAJECKI, M., SARPARANTA, M., TENHUNEN, M., AIRAKSINEN, A. J., DESMOND, R. A., KAIREMO, K. & HEMMINKI, A. 2009. Targeted radiotherapy for prostate cancer with an oncolytic adenovirus coding for human sodium iodide symporter. *Clin Cancer Res,* 15, 5396-403.

HALL, K., BLAIR ZAJDEL, M. E. & BLAIR, G. E. 2010. Unity and diversity in the human adenoviruses: exploiting alternative entry pathways for gene therapy. *Biochem J,* 431, 321-36.

HARRINGTON, K. J., HINGORANI, M., TANAY, M. A., HICKEY, J., BHIDE, S. A., CLARKE, P. M., RENOUF, L. C., THWAY, K., SIBTAIN, A., MCNEISH, I. A., NEWBOLD, K. L., GOLDSWEIG, H., COFFIN, R. & NUTTING, C. M. 2010. Phase I/II study of oncolytic HSV GM-CSF in combination with radiotherapy and cisplatin in untreated stage III/IV squamous cell cancer of the head and neck. *Clin Cancer Res,* 16, 4005-15.

HAWKINS, M. M., DRAPER, G. J. & KINGSTON, J. E. 1987. Incidence of second primary tumours among childhood cancer survivors. *Br J Cancer,* 56, 339-47.

HEMMINKI, O., BAUERSCHMITZ, G., HEMMI, S., LAVILLA-ALONSO, S., DIACONU, I., GUSE, K., KOSKI, A., DESMOND, R. A., LAPPALAINEN, M., KANERVA, A., CERULLO, V., PESONEN, S. & HEMMINKI, A. 2011. Oncolytic adenovirus based on serotype 3. *Cancer Gene Ther,* 18, 288-96.

HEMMINKI, O., DIACONU, I., CERULLO, V., PESONEN, S. K., KANERVA, A., JOENSUU, T., KAIREMO, K., LAASONEN, L., PARTANEN, K., KANGASNIEMI, L., LIEBER, A., PESONEN, S. & HEMMINKI, A. 2012. Ad3-hTERT-E1A, a fully serotype 3 oncolytic adenovirus, in patients with chemotherapy refractory cancer. *Mol Ther,* 20, 1821-30.

HEMMINKI, O., PARVIAINEN, S., JUHILA, J., TURKKI, R., LINDER, N., LUNDIN, J., KANKAINEN, M., RISTIMÄKI, A., KOSKI, A., LIIKANEN, I., OKSANEN, M., NETTELBECK, D. M., KAIREMO, K., PARTANEN, K., JOENSUU, T., KANERVA, A. & HEMMINKI, A. 2015. Immunological data from cancer patients treated with Ad5/3 E2F Delta24 GMCSF suggests utility for tumor immunotherapy. *Oncotarget,* 6, 4467-81.

HENDRICKX, R., STICHLING, N., KOELEN, J., KURYK, L., LIPIEC, A. & GREBER, U. F. 2014. Innate immunity to adenovirus. *Hum Gene Ther,* 25, 265-84.

HEO, J., REID, T., RUO, L., BREITBACH, C. J., ROSE, S., BLOOMSTON, M., CHO, M., LIM, H. Y., CHUNG, H. C., KIM, C. W., BURKE, J., LENCIONI, R., HICKMAN, T., MOON, A., LEE, Y. S., KIM, M. K., DANESHMAND, M., DUBOIS, K., LONGPRE, L., NGO, M., ROONEY, C., BELL, J. C., RHEE, B. G., PATT, R., HWANG, T. H. & KIRN, D. H. 2013. Randomized dose-finding clinical trial of oncolytic immunotherapeutic vaccinia JX-594 in liver cancer. *Nat Med,* 19, 329-36.

HINRICHS, C. S. & ROSENBERG, S. A. 2014. Exploiting the curative potential of adoptive T-cell therapy for cancer. *Immunol Rev,* 257, 56-71.

HODI, F. S., O'DAY, S. J., MCDERMOTT, D. F., WEBER, R. W., SOSMAN, J. A., HAANEN, J. B., GONZALEZ, R., ROBERT, C., SCHADENDORF, D., HASSEL, J. C., AKERLEY, W., VAN DEN EERTWEGH, A. J., LUTZKY, J., LORIGAN, P., VAUBEL, J. M., LINETTE, G. P., HOGG, D., OTTENSMEIER, C. H., LEBBE, C., PESCHEL, C., QUIRT, I., CLARK, J. I., WOLCHOK, J. D., WEBER, J. S., TIAN, J., YELLIN, M. J., NICHOL, G. M., HOOS, A. & URBA, W. J. 2010. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med,* 363, 711-23.

HONG, S. S., KARAYAN, L., TOURNIER, J., CURIEL, D. T. & BOULANGER, P. A. 1997. Adenovirus type 5 fiber knob binds to MHC class I alpha2 domain at the surface of human epithelial and B lymphoblastoid cells. *EMBO J,* 16, 2294-306.

HU, J. C., COFFIN, R. S., DAVIS, C. J., GRAHAM, N. J., GROVES, N., GUEST, P. J., HARRINGTON, K. J., JAMES, N. D., LOVE, C. A., MCNEISH, I., MEDLEY, L. C., MICHAEL, A., NUTTING, C. M., PANDHA, H. S., SHORROCK, C. A., SIMPSON, J., STEINER, J., STEVEN, N. M., WRIGHT, D. & COOMBES, R. C. 2006. A phase I study of OncoVEXGM-CSF, a second-generation oncolytic herpes simplex virus expressing granulocyte macrophage colony-stimulating factor. *Clin Cancer Res,* 12, 6737-47.

HUANG, X. & YANG, Y. 2009. Innate immune recognition of viruses and viral vectors. *Hum Gene Ther,* 20, 293-301.

HUTTUNEN, K. M., RAUNIO, H. & RAUTIO, J. 2011. Prodrugs--from serendipity to rational design. *Pharmacol Rev,* 63, 750-71.

HWANG, T. H., MOON, A., BURKE, J., RIBAS, A., STEPHENSON, J., BREITBACH, C. J., DANESHMAND, M., DE SILVA, N., PARATO, K., DIALLO, J. S., LEE, Y. S., LIU, T. C., BELL, J. C. & KIRN, D. H. 2011. A mechanistic proof-of-concept clinical trial with JX-594, a targeted multi-mechanistic oncolytic poxvirus, in patients with metastatic melanoma. *Mol Ther,* 19, 1913-22.

IM, S. A., KIM, J. S., GOMEZ-MANZANO, C., FUEYO, J., LIU, T. J., CHO, M. S., SEONG, C. M., LEE, S. N., HONG, Y. K. & YUNG, W. K. 2001. Inhibition of breast cancer growth in vivo by antiangiogenesis gene therapy with adenovirus-mediated antisense-VEGF. *Br J Cancer,* 84, 1252-7.

ITO, H., AOKI, H., KUHNEL, F., KONDO, Y., KUBICKA, S., WIRTH, T., IWADO, E., IWAMARU, A., FUJIWARA, K., HESS, K. R., LANG, F. F., SAWAYA, R. & KONDO, S. 2006. Autophagic cell death of malignant glioma cells induced by a conditionally replicating adenovirus. *J Natl Cancer Inst,* 98, 625-36.

JIANG, M., SHI, W., ZHANG, Q., WANG, X., GUO, M., CUI, Z., SU, C., YANG, Q., LI, Y., SHAM, J., LIU, X., WU, M. & QIAN, Q. 2006. Gene therapy using adenovirus-mediated full-length anti-HER-2 antibody for HER-2 overexpression cancers. *Clin Cancer Res,* 12, 6179-85.

JOGLER, C., HOFFMANN, D., THEEGARTEN, D., GRUNWALD, T., UBERLA, K. & WILDNER, O. 2006. Replication properties of human adenovirus in vivo and in cultures of primary cells from different animal species. *J Virol,* 80, 3549-58.

JOOSS, K. & CHIRMULE, N. 2003. Immunity to adenovirus and adeno-associated viral vectors: implications for gene therapy. *Gene Ther,* 10, 955-63.

JOUNAIDI, Y., DOLOFF, J. C. & WAXMAN, D. J. 2007. Conditionally replicating adenoviruses for cancer treatment. *Curr Cancer Drug Targets,* 7, 285-301.

JUDSON, I., VERWEIJ, J., GELDERBLOM, H., HARTMANN, J. T., SCHOFFSKI, P., BLAY, J. Y., KERST, J. M., SUFLIARSKY, J., WHELAN, J., HOHENBERGER, P., KRARUP-HANSEN, A., ALCINDOR, T., MARREAUD, S., LITIERE, S., HERMANS, C., FISHER, C., HOGENDOORN, P. C., DEI TOS, A. P. & VAN DER GRAAF, W. T. 2014. Doxorubicin alone versus intensified doxorubicin plus ifosfamide for first-line treatment of advanced or metastatic soft-tissue sarcoma: a randomised controlled phase 3 trial. *Lancet Oncol,* 15, 415-23.

KANERVA, A., BAUERSCHMITZ, G. J., YAMAMOTO, M., LAM, J. T., ALVAREZ, R. D., SIEGAL, G. P., CURIEL, D. T. & HEMMINKI, A. 2004. A cyclooxygenase-2 promoter-based conditionally replicating adenovirus with enhanced infectivity for treatment of ovarian adenocarcinoma. *Gene Ther,* 11, 552-9.

KANERVA, A. & HEMMINKI, A. 2004. Modified adenoviruses for cancer gene therapy. *Int J Cancer,* 110, 475-80.

KANERVA, A., LAVILLA-ALONSO, S., RAKI, M., KANGASNIEMI, L., BAUERSCHMITZ, G. J., TAKAYAMA, K., RISTIMÄKI, A., DESMOND, R. A. & HEMMINKI, A. 2008. Systemic

therapy for cervical cancer with potentially regulatable oncolytic adenoviruses. *PLoS One,* 3, e2917.

KANERVA, A., MIKHEEVA, G. V., KRASNYKH, V., COOLIDGE, C. J., LAM, J. T., MAHASRESHTI, P. J., BARKER, S. D., STRAUGHN, M., BARNES, M. N., ALVAREZ, R. D., HEMMINKI, A. & CURIEL, D. T. 2002. Targeting adenovirus to the serotype 3 receptor increases gene transfer efficiency to ovarian cancer cells. *Clin Cancer Res,* 8, 275-80.

KANERVA, A., NOKISALMI, P., DIACONU, I., KOSKI, A., CERULLO, V., LIIKANEN, I., TÄHTINEN, S., OKSANEN, M., HEISKANEN, R., PESONEN, S., JOENSUU, T., ALANKO, T., PARTANEN, K., LAASONEN, L., KAIREMO, K., KANGASNIEMI, L. & HEMMINKI, A. 2013. Antiviral and antitumor T-cell immunity in patients treated with GM-CSF-coding oncolytic adenovirus. *Clin Cancer Res,* 19, 2734-44.

KANERVA, A., ZINN, K. R., CHAUDHURI, T. R., LAM, J. T., SUZUKI, K., UIL, T. G., HAKKARAINEN, T., BAUERSCHMITZ, G. J., WANG, M., LIU, B., CAO, Z., ALVAREZ, R. D., CURIEL, D. T. & HEMMINKI, A. 2003. Enhanced therapeutic efficacy for ovarian cancer with a serotype 3 receptor-targeted oncolytic adenovirus. *Mol Ther,* 8, 449-58.

KANGASNIEMI, L., KIVILUOTO, T., KANERVA, A., RAKI, M., RANKI, T., SARKIOJA, M., WU, H., MARINI, F., HÖCKERSTEDT, K., ISONIEMI, H., ALFTHAN, H., STENMAN, U. H., CURIEL, D. T. & HEMMINKI, A. 2006. Infectivity-enhanced adenoviruses deliver efficacy in clinical samples and orthotopic models of disseminated gastric cancer. *Clin Cancer Res,* 12, 3137-44.

KANTOFF, P. W., HIGANO, C. S., SHORE, N. D., BERGER, E. R., SMALL, E. J., PENSON, D. F., REDFERN, C. H., FERRARI, A. C., DREICER, R., SIMS, R. B., XU, Y., FROHLICH, M. W. & SCHELLHAMMER, P. F. 2010. Sipuleucel-T immunotherapy for castration-resistant prostate cancer. *N Engl J Med,* 363, 411-22.

KAUFMAN, H. L., ANDTBACKA, R. H. I., COLLICHIO, F. A., AMATRUDA, T., SENZE, R. N. N., CHESNEY, J., DELMAN, K. A., SPITLER, L. E., PUZANOV, I., YE, Y., LI, A., GANSERT, J. L., COFFIN, R. & ROSS, M. I. 2014. Primary overall survival (OS) from OPTiM, a randomized phase III trial of talimogene laherparepvec (T-VEC) versus subcutaneous (SC) granulocyte-macrophage colony-stimulating factor (GM-CSF) for the treatment (tx) of unresected stage IIIB/C and IV melanoma. *J Clin Oncol* 32:5s, ASCO 2014 (suppl; abstr 9008a).

KELLY, E. & RUSSELL, S. J. 2007. History of oncolytic viruses: genesis to genetic engineering. *Mol Ther,* 15, 651-9.

KERN, P., KALISCH, A., KOLBERG, H. C., KIMMIG, R., OTTERBACH, F., VON MINCKWITZ, G., SIKOV, W. M., POTT, D. & KURBACHER, C. 2013. Neoadjuvant, anthracycline-free chemotherapy with carboplatin and docetaxel in triple-negative, early-stage breast cancer: a multicentric analysis of feasibility and rates of pathologic complete response. *Chemotherapy,* 59, 387-94.

KHAJAH, M., MILLEN, B., CARA, D. C., WATERHOUSE, C. & MCCAFFERTY, D. M. 2011. Granulocyte-macrophage colony-stimulating factor (GM-CSF): a chemoattractive agent for murine leukocytes in vivo. *J Leukoc Biol,* 89, 945-53.

KHARE, R., CHEN, C. Y., WEAVER, E. A. & BARRY, M. A. 2011. Advances and future challenges in adenoviral vector pharmacology and targeting. *Curr Gene Ther,* 11, 241-58.

KIM, J., KIM, P. H., YOO, J. Y., YOON, A. R., CHOI, H. J., SEONG, J., KIM, I. W., KIM, J. H. & YUN, C. O. 2009. Double E1B 19 kDa- and E1B 55 kDa-deleted oncolytic adenovirus in combination with radiotherapy elicits an enhanced anti-tumor effect. *Gene Ther,* 16, 1111-21.

KIM, J., LEE, B., KIM, J. S., YUN, C. O., KIM, J. H., LEE, Y. J., JOO, C. H. & LEE, H. 2002. Antitumoral effects of recombinant adenovirus YKL-1001, conditionally replicating in alpha-fetoprotein-producing human liver cancer cells. *Cancer Lett,* 180, 23-32.

KOSKI, A., AHTINEN, H., LILJENBACK, H., ROIVAINEN, A., KOSKELA, A., OKSANEN, M., PARTANEN, K., LAASONEN, L., KAIREMO, K., JOENSUU, T. & HEMMINKI, A. 2013. [(18)F]-fluorodeoxyglucose positron emission tomography and computed tomography in response evaluation of oncolytic adenovirus treatments of patients with advanced cancer. *Hum Gene Ther,* 24, 1029-41.

KOSKI, A., KANGASNIEMI, L., ESCUTENAIRE, S., PESONEN, S., CERULLO, V., DIACONU, I., NOKISALMI, P., RAKI, M., RAJECKI, M., GUSE, K., RANKI, T., OKSANEN, M., HOLM, S. L., HAAVISTO, E., KARIOJA-KALLIO, A., LAASONEN, L., PARTANEN, K., UGOLINI, M., HELMINEN, A., KARLI, E., HANNUKSELA, P., JOENSUU, T., KANERVA, A. & HEMMINKI, A. 2010. Treatment of cancer patients with a serotype 5/3 chimeric oncolytic adenovirus expressing GMCSF. *Mol Ther,* 18, 1874-84.

KOSKI, A., RAKI, M., NOKISALMI, P., LIIKANEN, I., KANGASNIEMI, L., JOENSUU, T., KANERVA, A., PESONEN, S., ALEMANY, R. & HEMMINKI, A. 2012. Verapamil results in increased blood levels of oncolytic adenovirus in treatment of patients with advanced cancer. *Mol Ther,* 20, 221-9.

KRASNYKH, V., BELOUSOVA, N., KOROKHOV, N., MIKHEEVA, G. & CURIEL, D. T. 2001. Genetic targeting of an adenovirus vector via replacement of the fiber protein with the phage T4 fibritin. *J Virol,* 75, 4176-83.

KUNZ, A. N. & OTTOLINI, M. 2010. The role of adenovirus in respiratory tract infections. *Curr Infect Dis Rep,* 12, 81-7.

LACEY, J. V., JR., KREIMER, A. R., BUYS, S. S., MARCUS, P. M., CHANG, S. C., LEITZMANN, M. F., HOOVER, R. N., PROROK, P. C., BERG, C. D. & HARTGE, P. 2009. Breast cancer epidemiology according to recognized breast cancer risk factors in the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial Cohort. *BMC Cancer,* 9, 84.

LAI, C. M., LAI, Y. K. & RAKOCZY, P. E. 2002. Adenovirus and adeno-associated virus vectors. *DNA Cell Biol,* 21, 895-913.

LAWRENCE, T. S., TEN HAKEN, R. K. & GIACCIA, A. 2008. Principles of Radiation Oncology *In: DeVita VT Jr, Lawrence TS, Rosenberg SA, editors. Cancer: Principles and Practice of Oncology. 8th ed. Philadelphia: Lippincott Williams and Wilkins.*

LEE, S. & MARGOLIN, K. 2011. Cytokines in cancer immunotherapy. *Cancers (Basel),* 3, 3856-93.

LEI, N., SHEN, F. B., CHANG, J. H., WANG, L., LI, H., YANG, C., LI, J. & YU, D. C. 2009. An oncolytic adenovirus expressing granulocyte macrophage colony-stimulating factor shows improved specificity and efficacy for treating human solid tumors. *Cancer Gene Ther,* 16, 33-43.

LEJEUNE, F. J., RUEGG, C. & LIENARD, D. 1998. Clinical applications of TNF-alpha in cancer. *Curr Opin Immunol,* 10, 573-80.

LETTIERI, C. K., HINGORANI, P. & KOLB, E. A. 2012. Progress of oncolytic viruses in sarcomas. *Expert Rev Anticancer Ther,* 12, 229-42.

LI, C. Y., HUANG, Q. & KUNG, H. F. 2005. Cytokine and immuno-gene therapy for solid tumors. *Cell Mol Immunol,* 2, 81-91.

LI, J., ZENG, W., HUANG, Y., ZHANG, Q., HU, P., RABKIN, S. D. & LIU, R. 2012. Treatment of breast cancer stem cells with oncolytic herpes simplex virus. *Cancer Gene Ther,* 19, 707-14.

LI, Y., CHEN, Y., DILLEY, J., ARROYO, T., KO, D., WORKING, P. & YU, D. C. 2003. Carcinoembryonic antigen-producing cell-specific oncolytic adenovirus, OV798, for colorectal cancer therapy. *Mol Cancer Ther,* 2, 1003-9.

LIEDTKE, C., MAZOUNI, C., HESS, K. R., ANDRE, F., TORDAI, A., MEJIA, J. A., SYMMANS, W. F., GONZALEZ-ANGULO, A. M., HENNESSY, B., GREEN, M., CRISTOFANILLI, M., HORTOBAGYI, G. N. & PUSZTAI, L. 2008. Response to neoadjuvant therapy and long-term survival in patients with triple-negative breast cancer. *J Clin Oncol,* 26, 1275-81.

LIEN, K., GEORGSDOTTIR, S., SIVANATHAN, L., CHAN, K. & EMMENEGGER, U. 2013. Low-dose metronomic chemotherapy: a systematic literature analysis. *Eur J Cancer,* 49, 3387-95.

LIIKANEN, I., AHTIAINEN, L., HIRVINEN, M. L., BRAMANTE, S., CERULLO, V., NOKISALMI, P., HEMMINKI, O., DIACONU, I., PESONEN, S., KOSKI, A., KANGASNIEMI, L., PESONEN, S. K., OKSANEN, M., LAASONEN, L., PARTANEN, K., JOENSUU, T., ZHAO, F., KANERVA, A. & HEMMINKI, A. 2013. Oncolytic adenovirus with temozolomide induces autophagy and antitumor immune responses in cancer patients. *Mol Ther,* 21, 1212-23.

LIIKANEN, I., DIAS, J. D., NOKISALMI, P., SLONIECKA, M., KANGASNIEMI, L., RAJECKI, M., DOBNER, T., TENHUNEN, M., KANERVA, A., PESONEN, S., AHTIAINEN, L. & HEMMINKI, A. 2010. Adenoviral E4orf3 and E4orf6 proteins, but not E1B55K, increase killing of cancer cells by radiotherapy in vivo. *Int J Radiat Oncol Biol Phys,* 78, 1201-9.

LIND, M. J. 2008. Principles of cytotoxic chemotherapy. *Medicine,* 36, 19-23.

LIU, E. B., WADFORD, D. A., SETO, J., VU, M., HUDSON, N. R., THRASHER, L., TORRES, S., DYER, D. W., CHODOSH, J., SETO, D. & JONES, M. S. 2012. Computational and serologic analysis of novel and known viruses in species human adenovirus D in which serology and genomics do not correlate. *PLoS One,* 7, e33212.

LOVEN, D., HASNIS, E., BERTOLINI, F. & SHAKED, Y. 2013. Low-dose metronomic chemotherapy: from past experience to new paradigms in the treatment of cancer. *Drug Discov Today,* 18, 193-201.

LUTSIAK, M. E., SEMNANI, R. T., DE PASCALIS, R., KASHMIRI, S. V., SCHLOM, J. & SABZEVARI, H. 2005. Inhibition of CD4(+)25+ T regulatory cell function implicated in enhanced immune response by low-dose cyclophosphamide. *Blood,* 105, 2862-8.

MALHOTRA, V. & PERRY, M. C. 2003. Classical chemotherapy: mechanisms, toxicities and the therapeutic window. *Cancer Biol Ther,* 2, S2-4.

MAN, S., BOCCI, G., FRANCIA, G., GREEN, S. K., JOTHY, S., HANAHAN, D., BOHLEN, P., HICKLIN, D. J., BERGERS, G. & KERBEL, R. S. 2002. Antitumor effects in mice of low-dose (metronomic) cyclophosphamide administered continuously through the drinking water. *Cancer Res,* 62, 2731-5.

MANALO, D., MUFSON, M. A., ZOLLAR, L. M. & MANKAD, V. N. 1971. Adenovirus infection in acute hemorrhagic cystitis. A study in 25 children. *Am J Dis Child,* 121, 281-5.

MARTIN, M. E. & BERK, A. J. 1998. Adenovirus E1B 55K represses p53 activation in vitro. *J Virol,* 72, 3146-54.

MASTRANGELO, M. J., MAGUIRE, H. C., JR., EISENLOHR, L. C., LAUGHLIN, C. E., MONKEN, C. E., MCCUE, P. A., KOVATICH, A. J. & LATTIME, E. C. 1999. Intratumoral recombinant GM-CSF-encoding virus as gene therapy in patients with cutaneous melanoma. *Cancer Gene Ther,* 6, 409-22.

MATHIAS, P., GALLENO, M. & NEMEROW, G. R. 1998. Interactions of soluble recombinant integrin alphav beta5 with human adenoviruses. *J Virol,* 72, 8669-75.

MCCLAY, E. F. 1989. Epidemiology of bone and soft tissue sarcomas. *Semin Oncol,* 16, 264-72.

MOCELLIN, S., ROSSI, C. R., PILATI, P. & NITTI, D. 2005. Tumor necrosis factor, cancer and anticancer therapy. *Cytokine Growth Factor Rev,* 16, 35-53.

MURUVE, D. A. 2004. The innate immune response to adenovirus vectors. *Hum Gene Ther,* 15, 1157-66.

NAGY, B., MUCSI, I., MOLNAR, J. & THURZO, L. 2002. Combined effect of cisplatin and 5-fluorouracil with irradiation on tumor cells in vitro. *Anticancer Res,* 22, 135-8.

NAYAK, S. & HERZOG, R. W. 2010. Progress and prospects: immune responses to viral vectors. *Gene Ther,* 17, 295-304.

NEMUNAITIS, J., KHURI, F., GANLY, I., ARSENEAU, J., POSNER, M., VOKES, E., KUHN, J., MCCARTY, T., LANDERS, S., BLACKBURN, A., ROMEL, L., RANDLEV, B., KAYE, S. & KIRN, D. 2001. Phase II trial of intratumoral administration of ONYX-015, a replication-selective adenovirus, in patients with refractory head and neck cancer. *J Clin Oncol,* 19, 289-98.

NICKLIN, S. A., WU, E., NEMEROW, G. R. & BAKER, A. H. 2005. The influence of adenovirus fiber structure and function on vector development for gene therapy. *Mol Ther,* 12, 384-93.

NICOLINI, A., MANCINI, P., FERRARI, P., ANSELMI, L., TARTARELLI, G., BONAZZI, V., CARPI, A. & GIARDINO, R. 2004. Oral low-dose cyclophosphamide in metastatic hormone refractory prostate cancer (MHRPC). *Biomed Pharmacother,* 58, 447-50.

NIELSEN, L. L., GURNANI, M., SYED, J., DELL, J., HARTMAN, B., CARTWRIGHT, M. & JOHNSON, R. C. 1998. Recombinant E1-deleted adenovirus-mediated gene

therapy for cancer: efficacy studies with p53 tumor suppressor gene and liver histology in tumor xenograft models. *Hum Gene Ther,* 9, 681-94.

O'SHEA, C. C., JOHNSON, L., BAGUS, B., CHOI, S., NICHOLAS, C., SHEN, A., BOYLE, L., PANDEY, K., SORIA, C., KUNICH, J., SHEN, Y., HABETS, G., GINZINGER, D. & MCCORMICK, F. 2004. Late viral RNA export, rather than p53 inactivation, determines ONYX-015 tumor selectivity. *Cancer Cell,* 6, 611-23.

OBEID, M., TESNIERE, A., GHIRINGHELLI, F., FIMIA, G. M., APETOH, L., PERFETTINI, J. L., CASTEDO, M., MIGNOT, G., PANARETAKIS, T., CASARES, N., METIVIER, D., LAROCHETTE, N., VAN ENDERT, P., CICCOSANTI, F., PIACENTINI, M., ZITVOGEL, L. & KROEMER, G. 2007. Calreticulin exposure dictates the immunogenicity of cancer cell death. *Nat Med,* 13, 54-61.

ONCOS THERAPEUTICS LTD. www.oncos.com. [Accessed 16th June 2015].

OTTOLINO-PERRY, K., DIALLO, J. S., LICHTY, B. D., BELL, J. C. & MCCART, J. A. 2010. Intelligent design: combination therapy with oncolytic viruses. *Mol Ther,* 18, 251-63.

OVCARICEK, T., FRKOVIC, S. G., MATOS, E., MOZINA, B. & BORSTNAR, S. 2011. Triple negative breast cancer - prognostic factors and survival. *Radiol Oncol,* 45, 46-52.

OZBAY HOSNUT, F., CANAN, O., OZCAY, F. & BILEZIKCI, B. 2008. Adenovirus infection as possible cause of acute liver failure in a healthy child: a case report. *Turk J Gastroenterol,* 19, 281-3.

PACINI, D. L., DUBOVI, E. J. & CLYDE, W. A., JR. 1984. A new animal model for human respiratory tract disease due to adenovirus. *J Infect Dis,* 150, 92-7.

PARK, B. H., HWANG, T., LIU, T. C., SZE, D. Y., KIM, J. S., KWON, H. C., OH, S. Y., HAN, S. Y., YOON, J. H., HONG, S. H., MOON, A., SPETH, K., PARK, C., AHN, Y. J., DANESHMAND, M., RHEE, B. G., PINEDO, H. M., BELL, J. C. & KIRN, D. H. 2008. Use of a targeted oncolytic poxvirus, JX-594, in patients with refractory primary or metastatic liver cancer: a phase I trial. *Lancet Oncol,* 9, 533-42.

PEARSON, S., JIA, H. & KANDACHI, K. 2004. China approves first gene therapy. *Nat Biotechnol,* 22, 3-4.

PESONEN, S., DIACONU, I., CERULLO, V., ESCUTENAIRE, S., RAKI, M., KANGASNIEMI, L., NOKISALMI, P., DOTTI, G., GUSE, K., LAASONEN, L., PARTANEN, K., KARLI, E., HAAVISTO, E., OKSANEN, M., KARIOJA-KALLIO, A., HANNUKSELA, P., HOLM, S. L., KAUPPINEN, S., JOENSUU, T., KANERVA, A. & HEMMINKI, A. 2012a. Integrin targeted oncolytic adenoviruses Ad5-D24-RGD and Ad5-RGD-D24-GMCSF for treatment of patients with advanced chemotherapy refractory solid tumors. *Int J Cancer,* 130, 1937-47.

PESONEN, S., DIACONU, I., KANGASNIEMI, L., RANKI, T., KANERVA, A., PESONEN, S. K., GERDEMANN, U., LEEN, A. M., KAIREMO, K., OKSANEN, M., HAAVISTO, E., HOLM, S. L., KARIOJA-KALLIO, A., KAUPPINEN, S., PARTANEN, K. P., LAASONEN, L., JOENSUU, T., ALANKO, T., CERULLO, V. & HEMMINKI, A. 2012b. Oncolytic immunotherapy of advanced solid tumors with a CD40L-expressing replicating adenovirus: assessment of safety and immunologic responses in patients. *Cancer Res,* 72, 1621-31.

PESONEN, S., NOKISALMI, P., ESCUTENAIRE, S., SÄRKIOJA, M., RAKI, M., CERULLO, V., KANGASNIEMI, L., LAASONEN, L., RIBACKA, C., GUSE, K., HAAVISTO, E., OKSANEN, M., RAJECKI, M., HELMINEN, A., RISTIMÄKI, A., KARIOJA-KALLIO, A., KARLI, E., KANTOLA, T., BAUERSCHMITZ, G., KANERVA, A., JOENSUU, T. & HEMMINKI, A. 2010. Prolonged systemic circulation of chimeric oncolytic adenovirus Ad5/3-Cox2L-D24 in patients with metastatic and refractory solid tumors. *Gene Ther,* 17, 892-904.

QUESADA, J. R., HERSH, E. M., MANNING, J., REUBEN, J., KEATING, M., SCHNIPPER, E., ITRI, L. & GUTTERMAN, J. U. 1986. Treatment of hairy cell leukemia with recombinant alpha-interferon. *Blood,* 68, 493-7.

RAJECKI, M., AF HÄLLSTRÖM, T., HAKKARAINEN, T., NOKISALMI, P., HAUTANIEMI, S., NIEMINEN, A. I., TENHUNEN, M., RANTANEN, V., DESMOND, R. A., CHEN, D. T., GUSE, K., STENMAN, U. H., GARGINI, R., KAPANEN, M., KLEFSTRÖM, J., KANERVA, A., PESONEN, S., AHTIAINEN, L. & HEMMINKI, A. 2009. Mre11 inhibition by oncolytic adenovirus associates with autophagy and underlies synergy with ionizing radiation. *Int J Cancer,* 125, 2441-9.

RAKI, M., KANERVA, A., RISTIMAKI, A., DESMOND, R. A., CHEN, D. T., RANKI, T., SARKIOJA, M., KANGASNIEMI, L. & HEMMINKI, A. 2005. Combination of gemcitabine and Ad5/3-Delta24, a tropism modified conditionally replicating adenovirus, for the treatment of ovarian cancer. *Gene Ther,* 12, 1198-205.

RAMESH, N., GE, Y., ENNIST, D. L., ZHU, M., MINA, M., GANESH, S., REDDY, P. S. & YU, D. C. 2006. CG0070, a conditionally replicating granulocyte macrophage colony-stimulating factor--armed oncolytic adenovirus for the treatment of bladder cancer. *Clin Cancer Res,* 12, 305-13.

RANKI, T., SÄRKIOJA, M., HAKKARAINEN, T., VON SMITTEN, K., KANERVA, A. & HEMMINKI, A. 2007. Systemic efficacy of oncolytic adenoviruses in imagable orthotopic models of hormone refractory metastatic breast cancer. *Int J Cancer,* 121, 165-74.

RAVIRAJ, J., BOKKASAM, V. K., KUMAR, V. S., REDDY, U. S. & SUMAN, V. 2014. Radiosensitizers, radioprotectors, and radiation mitigators. *Indian J Dent Res,* 25, 83-90.

REED, D. & ALTIOK, S. 2011. Metastatic soft tissue sarcoma chemotherapy: an opportunity for personalized medicine. *Cancer Control,* 18, 188-95.

REID, T., WARREN, R. & KIRN, D. 2002. Intravascular adenoviral agents in cancer patients: lessons from clinical trials. *Cancer Gene Ther,* 9, 979-86.

REID, T. R., FREEMAN, S., POST, L., MCCORMICK, F. & SZE, D. Y. 2005. Effects of Onyx-015 among metastatic colorectal cancer patients that have failed prior treatment with 5-FU/leucovorin. *Cancer Gene Ther,* 12, 673-81.

REKOSH, D. M., RUSSELL, W. C., BELLET, A. J. & ROBINSON, A. J. 1977. Identification of a protein linked to the ends of adenovirus DNA. *Cell,* 11, 283-95.

ROBINSON, M. H. 2008. Radiotherapy: technical aspects. *Medicine,* 36, 9-14.

ROBSON, T. & HIRST, D. G. 2003. Transcriptional Targeting in Cancer Gene Therapy. *J Biomed Biotechnol,* 2003, 110-137.

RODRIGUEZ, R., SCHUUR, E. R., LIM, H. Y., HENDERSON, G. A., SIMONS, J. W. & HENDERSON, D. R. 1997. Prostate attenuated replication competent adenovirus (ARCA) CN706: a selective cytotoxic for prostate-specific antigen-positive prostate cancer cells. *Cancer Res,* 57, 2559-63.

ROELVINK, P. W., LIZONOVA, A., LEE, J. G., LI, Y., BERGELSON, J. M., FINBERG, R. W., BROUGH, D. E., KOVESDI, I. & WICKHAM, T. J. 1998. The coxsackievirus-adenovirus receptor protein can function as a cellular attachment protein for adenovirus serotypes from subgroups A, C, D, E, and F. *J Virol,* 72, 7909-15.

ROJAS, J. J., CASCALLO, M., GUEDAN, S., GROS, A., MARTINEZ-QUINTANILLA, J., HEMMINKI, A. & ALEMANY, R. 2009. A modified E2F-1 promoter improves the efficacy to toxicity ratio of oncolytic adenoviruses. *Gene Ther,* 16, 1441-51.

ROSENBERG, S. A., AEBERSOLD, P., CORNETTA, K., KASID, A., MORGAN, R. A., MOEN, R., KARSON, E. M., LOTZE, M. T., YANG, J. C., TOPALIAN, S. L. & ET AL. 1990. Gene transfer into humans--immunotherapy of patients with advanced melanoma, using tumor-infiltrating lymphocytes modified by retroviral gene transduction. *N Engl J Med,* 323, 570-8.

ROWE, W. P., HUEBNER, R. J., GILMORE, L. K., PARROTT, R. H. & WARD, T. G. 1953. Isolation of a cytopathogenic agent from human adenoids undergoing spontaneous degeneration in tissue culture. *Proc Soc Exp Biol Med,* 84, 570-3.

RUSSELL, W. C. 2000. Update on adenovirus and its vectors. *J Gen Virol,* 81, 2573-604.

RUSSELL, W. C. 2009. Adenoviruses: update on structure and function. *J Gen Virol,* 90, 1-20.

SANDHU, R., PARKER, J. S., JONES, W. D., LIVASY, C. A. & COLEMAN, W. B. 2010. Microarray-Based Gene Expression Profiling for Molecular Classification of Breast Cancer and Identification of New Targets for Therapy. *Laboratory Medicine,* 41, 364-372.

SEILER, M. P., CERULLO, V. & LEE, B. 2007. Immune response to helper dependent adenoviral mediated liver gene therapy: challenges and prospects. *Curr Gene Ther,* 7, 297-305.

SENZER, N. N., KAUFMAN, H. L., AMATRUDA, T., NEMUNAITIS, M., REID, T., DANIELS, G., GONZALEZ, R., GLASPY, J., WHITMAN, E., HARRINGTON, K., GOLDSWEIG, H., MARSHALL, T., LOVE, C., COFFIN, R. & NEMUNAITIS, J. J. 2009. Phase II clinical trial of a granulocyte-macrophage colony-stimulating factor-encoding, second-generation oncolytic herpesvirus in patients with unresectable metastatic melanoma. *J Clin Oncol,* 27, 5763-71.

SERAFINI, P., CARBLEY, R., NOONAN, K. A., TAN, G., BRONTE, V. & BORRELLO, I. 2004. High-dose granulocyte-macrophage colony-stimulating factor-producing vaccines impair the immune response through the recruitment of myeloid suppressor cells. *Cancer Res,* 64, 6337-43.

SHANAFELT, A. B., JOHNSON, K. E. & KASTELEIN, R. A. 1991. Identification of critical amino acid residues in human and mouse granulocyte-macrophage colony-stimulating factor and their involvement in species specificity. *J Biol Chem,* 266, 13804-10.

SHANAFELT, T. D., LIN, T., GEYER, S. M., ZENT, C. S., LEUNG, N., KABAT, B., BOWEN, D., GREVER, M. R., BYRD, J. C. & KAY, N. E. 2007. Pentostatin, cyclophosphamide, and rituximab regimen in older patients with chronic lymphocytic leukemia. *Cancer,* 109, 2291-8.

SHERR, C. J. & MCCORMICK, F. 2002. The RB and p53 pathways in cancer. *Cancer Cell,* 2, 103-12.

SHORT, J. J., PEREBOEV, A. V., KAWAKAMI, Y., VASU, C., HOLTERMAN, M. J. & CURIEL, D. T. 2004. Adenovirus serotype 3 utilizes CD80 (B7.1) and CD86 (B7.2) as cellular attachment receptors. *Virology,* 322, 349-59.

SKUBITZ, K. M. & D'ADAMO, D. R. 2007. Sarcoma. *Mayo Clin Proc,* 82, 1409-32.

SLADEK, N. E. 1972. Therapeutic efficacy of cyclophosphamide as a function of inhibition of its metabolism. *Cancer Res,* 32, 1848-54.

SMITH, J. G., WIETHOFF, C. M., STEWART, P. L. & NEMEROW, G. R. 2010. Adenovirus. *Curr Top Microbiol Immunol,* 343, 195-224.

SMYTH, M. J., GODFREY, D. I. & TRAPANI, J. A. 2001. A fresh look at tumor immunosurveillance and immunotherapy. *Nat Immunol,* 2, 293-9.

SUNG, M. W., CHEN, S. H., THUNG, S. N., ZHANG, D. Y., HUANG, T. G., MANDELI, J. P. & WOO, S. L. 2002. Intratumoral delivery of adenovirus-mediated interleukin-12 gene in mice with metastatic cancer in the liver. *Hum Gene Ther,* 13, 731-43.

SWAIKA, A., CROZIER, J. A. & JOSEPH, R. W. 2014. Vemurafenib: an evidence-based review of its clinical utility in the treatment of metastatic melanoma. *Drug Des Devel Ther,* 8, 775-87.

TAMANINI, A., NICOLIS, E., BONIZZATO, A., BEZZERRI, V., MELOTTI, P., ASSAEL, B. M. & CABRINI, G. 2006. Interaction of adenovirus type 5 fiber with the coxsackievirus and adenovirus receptor activates inflammatory response in human respiratory cells. *J Virol,* 80, 11241-54.

THOMAS, M. A., SPENCER, J. F., LA REGINA, M. C., DHAR, D., TOLLEFSON, A. E., TOTH, K. & WOLD, W. S. 2006. Syrian hamster as a permissive immunocompetent animal model for the study of oncolytic adenovirus vectors. *Cancer Res,* 66, 1270-6.

TOPALIAN, S. L., HODI, F. S., BRAHMER, J. R., GETTINGER, S. N., SMITH, D. C., MCDERMOTT, D. F., POWDERLY, J. D., CARVAJAL, R. D., SOSMAN, J. A., ATKINS, M. B., LEMING, P. D., SPIGEL, D. R., ANTONIA, S. J., HORN, L., DRAKE, C. G., PARDOLL, D. M., CHEN, L., SHARFMAN, W. H., ANDERS, R. A., TAUBE, J. M., MCMILLER, T. L., XU, H., KORMAN, A. J., JURE-KUNKEL, M., AGRAWAL, S., MCDONALD, D., KOLLIA, G. D., GUPTA, A., WIGGINTON, J. M. & SZNOL, M. 2012. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med,* 366, 2443-54.

TRINH, H. V., LESAGE, G., CHENNAMPARAMPIL, V., VOLLENWEIDER, B., BURCKHARDT, C. J., SCHAUER, S., HAVENGA, M., GREBER, U. F. & HEMMI, S. 2012. Avidity binding of human adenovirus serotypes 3 and 7 to the membrane cofactor CD46 triggers infection. *J Virol,* 86, 1623-37.

TUVE, S., LIU, Y., TRAGOOLPUA, K., JACOBS, J. D., YUMUL, R. C., LI, Z. Y., STRAUSS, R., HELLSTROM, K. E., DISIS, M. L., ROFFLER, S. & LIEBER, A. 2009. In situ adenovirus vaccination engages T effector cells against cancer. *Vaccine,* 27, 4225-39.

TUVE, S., WANG, H., WARE, C., LIU, Y., GAGGAR, A., BERNT, K., SHAYAKHMETOV, D., LI, Z., STRAUSS, R., STONE, D. & LIEBER, A. 2006. A new group B adenovirus receptor is expressed at high levels on human stem and tumor cells. *J Virol,* 80, 12109-20.

TYYNELÄ, K., SANDMAIR, A. M., TURUNEN, M., VANNINEN, R., VAINIO, P., KAUPPINEN, R., JOHANSSON, R., VAPALAHTI, M. & YLÄ-HERTTUALA, S. 2002. Adenovirus-mediated herpes simplex virus thymidine kinase gene therapy in BT4C rat glioma model. *Cancer Gene Ther,* 9, 917-24.

VAN DER BIJ, G. J., OOSTERLING, S. J., BEELEN, R. H., MEIJER, S., COFFEY, J. C. & VAN EGMOND, M. 2009. The perioperative period is an underutilized window of therapeutic opportunity in patients with colorectal cancer. *Ann Surg,* 249, 727-34.

VAN DER GRAAF, W., JUDSON, I. & VERWEIJ, J. 2012. Results of a randomized phase III trial (EORTC 62012) of single agent doxorubicin versus doxorubicin plus ifosfamide as first line chemotherapy for patients with advanced or metastatic soft tissue sarcoma: A survival study by the EORTC Soft Tissue and Bone Sarcoma Group. *ESMO Congress. Abstract LBA7. Presented October 1, 2012.*

VANNEMAN, M. & DRANOFF, G. 2012. Combining immunotherapy and targeted therapies in cancer treatment. *Nat Rev Cancer,* 12, 237-51.

VATNER, R. E., COOPER, B. T., VANPOUILLE-BOX, C., DEMARIA, S. & FORMENTI, S. C. 2014. Combinations of immunotherapy and radiation in cancer therapy. *Front Oncol,* 4, 325.

VIGNE, E., MAHFOUZ, I., DEDIEU, J. F., BRIE, A., PERRICAUDET, M. & YEH, P. 1999. RGD inclusion in the hexon monomer provides adenovirus type 5-based vectors with a fiber knob-independent pathway for infection. *J Virol,* 73, 5156-61.

VOLK, A. L., RIVERA, A. A., KANERVA, A., BAUERSCHMITZ, G., DMITRIEV, I., NETTELBECK, D. M. & CURIEL, D. T. 2003. Enhanced adenovirus infection of melanoma cells by fiber-modification: incorporation of RGD peptide or Ad5/3 chimerism. *Cancer Biol Ther,* 2, 511-5.

VOLPERS, C. & KOCHANEK, S. 2004. Adenoviral vectors for gene transfer and therapy. *J Gene Med,* 6 Suppl 1, S164-71.

WALTERS, R. W., FREIMUTH, P., MONINGER, T. O., GANSKE, I., ZABNER, J. & WELSH, M. J. 2002. Adenovirus fiber disrupts CAR-mediated intercellular adhesion allowing virus escape. *Cell,* 110, 789-99.

WANG, H., LI, Z. Y., LIU, Y., PERSSON, J., BEYER, I., MOLLER, T., KOYUNCU, D., DRESCHER, M. R., STRAUSS, R., ZHANG, X. B., WAHL, J. K., 3RD, URBAN, N., DRESCHER, C., HEMMINKI, A., FENDER, P. & LIEBER, A. 2011. Desmoglein 2 is a receptor for adenovirus serotypes 3, 7, 11 and 14. *Nat Med,* 17, 96-104.

WANG, J., HU, P., ZENG, M., RABKIN, S. D. & LIU, R. 2012a. Oncolytic herpes simplex virus treatment of metastatic breast cancer. *Int J Oncol,* 40, 757-63.

WANG, K., HUANG, S., KAPOOR-MUNSHI, A. & NEMEROW, G. 1998. Adenovirus internalization and infection require dynamin. *J Virol,* 72, 3455-8.

WANG, R., QIN, S., CHEN, Y., LI, Y., CHEN, C., WANG, Z., ZHENG, R. & WU, Q. 2012b. Enhanced anti-tumor and anti-angiogenic effects of metronomic

cyclophosphamide combined with Endostar in a xenograft model of human lung cancer. *Oncol Rep,* 28, 439-45.

WARDE, P. 2008. Radiotherapy: practical applications and clinical aspects. *Medicine,* 36, 15-18.

WEILAND, T., LAMPE, J., ESSMANN, F., VENTURELLI, S., BERGER, A., BOSSOW, S., BERCHTOLD, S., SCHULZE-OSTHOFF, K., LAUER, U. M. & BITZER, M. 2014. Enhanced killing of therapy-induced senescent tumor cells by oncolytic measles vaccine viruses. *Int J Cancer,* 134, 235-43.

WHITBY, D., HOWARD, M. R., TENANT-FLOWERS, M., BRINK, N. S., COPAS, A., BOSHOFF, C., HATZIOANNOU, T., SUGGETT, F. E., ALDAM, D. M., DENTON, A. S. & ET AL. 1995. Detection of Kaposi sarcoma associated herpesvirus in peripheral blood of HIV-infected individuals and progression to Kaposi's sarcoma. *Lancet,* 346, 799-802.

WHYTE, P., BUCHKOVICH, K. J., HOROWITZ, J. M., FRIEND, S. H., RAYBUCK, M., WEINBERG, R. A. & HARLOW, E. 1988. Association between an oncogene and an anti-oncogene: the adenovirus E1A proteins bind to the retinoblastoma gene product. *Nature,* 334, 124-9.

WICKHAM, T. J., CARRION, M. E. & KOVESDI, I. 1995. Targeting of adenovirus penton base to new receptors through replacement of its RGD motif with other receptor-specific peptide motifs. *Gene Ther,* 2, 750-6.

WICKHAM, T. J., ROELVINK, P. W., BROUGH, D. E. & KOVESDI, I. 1996. Adenovirus targeted to heparan-containing receptors increases its gene delivery efficiency to multiple cell types. *Nat Biotechnol,* 14, 1570-3.

WOLCHOK, J. D., KLUGER, H., CALLAHAN, M. K., POSTOW, M. A., RIZVI, N. A., LESOKHIN, A. M., SEGAL, N. H., ARIYAN, C. E., GORDON, R. A., REED, K., BURKE, M. M., CALDWELL, A., KRONENBERG, S. A., AGUNWAMBA, B. U., ZHANG, X., LOWY, I., INZUNZA, H. D., FEELY, W., HORAK, C. E., HONG, Q., KORMAN, A. J., WIGGINTON, J. M., GUPTA, A. & SZNOL, M. 2013. Nivolumab plus ipilimumab in advanced melanoma. *N Engl J Med,* 369, 122-33.

WOLCHOK, J. D., NEYNS, B., LINETTE, G., NEGRIER, S., LUTZKY, J., THOMAS, L., WATERFIELD, W., SCHADENDORF, D., SMYLIE, M., GUTHRIE, T., JR., GROB, J. J., CHESNEY, J., CHIN, K., CHEN, K., HOOS, A., O'DAY, S. J. & LEBBE, C. 2010. Ipilimumab monotherapy in patients with pretreated advanced melanoma: a randomised, double-blind, multicentre, phase 2, dose-ranging study. *Lancet Oncol,* 11, 155-64.

WOLD, W. S. M. & HORWITZ, M. S. 2007. Adenoviruses.

WORLD HEALTH ORGANIZATION. 2015. http://www.who.int/cancer/treatment/en/. [Accessed 2nd July 2015 2015].

XU, H., NIU, X., ZHANG, Q., HAO, L., DING, Y., LIU, W. & YAO, L. 2011. Synergistic antitumor efficacy by combining adriamycin with recombinant human endostatin in an osteosarcoma model. *Oncol Lett,* 2, 773-778.

YOKOYAMA, Y., DHANABAL, M., GRIFFIOEN, A. W., SUKHATME, V. P. & RAMAKRISHNAN, S. 2000. Synergy between angiostatin and endostatin: inhibition of ovarian cancer growth. *Cancer Res,* 60, 2190-6.

YOUNG, S. D., WHISSELL, M., NOBLE, J. C., CANO, P. O., LOPEZ, P. G. & GERMOND, C. J. 2006. Phase II clinical trial results involving treatment with low-dose daily oral cyclophosphamide, weekly vinblastine, and rofecoxib in patients with advanced solid tumors. *Clin Cancer Res,* 12, 3092-8.

YU, D. C., CHEN, Y., DILLEY, J., LI, Y., EMBRY, M., ZHANG, H., NGUYEN, N., AMIN, P., OH, J. & HENDERSON, D. R. 2001. Antitumor synergy of CV787, a prostate cancer-specific adenovirus, and paclitaxel and docetaxel. *Cancer Res,* 61, 517-25.

ZHANG, J. F., HU, C., GENG, Y., SELM, J., KLEIN, S. B., ORAZI, A. & TAYLOR, M. W. 1996. Treatment of a human breast cancer xenograft with an adenovirus vector containing an interferon gene results in rapid regression due to viral oncolysis and gene therapy. *Proc Natl Acad Sci U S A,* 93, 4513-8.

ZHANG, L., AKBULUT, H., TANG, Y., PENG, X., PIZZORNO, G., SAPI, E., MANEGOLD, S. & DEISSEROTH, A. 2002. Adenoviral vectors with E1A regulated by tumor-specific promoters are selectively cytolytic for breast cancer and melanoma. *Mol Ther,* 6, 386-93.

ZHENG, S., ULASOV, I. V., HAN, Y., TYLER, M. A., ZHU, Z. B. & LESNIAK, M. S. 2007. Fiber-knob modifications enhance adenoviral tropism and gene transfer in malignant glioma. *J Gene Med,* 9, 151-60.

PART C – ORIGINAL PUBLICATIONS