ASPECTS OF IMMUNO- AND SUICIDE GENE THERAPIES FOR CANCER - A COMBINATION WITH RADIATION THERAPY

Ph.D. thesis

Gergely Huszty, M.D.

Semmelweis University
School of Ph.D. Studies, Pathological Sciences
Chairman: Prof. László Kopper, M.D., Ph.D., D.Sc.

Tutor: Géza Sáfrány, M.D., Ph.D., D.Sc.

Official reviewers: Veronika Karcagi, M.D., Ph.D.
László Kóbori, M.D., Ph.D.

PhD final examination board

president: Prof. Anna Tompa, M.D., Ph.D., D.Sc.

members: András Lászik, M.D., Ph.D.
Péter Várady, M.D., Ph.D.
Zoltán Marcsek, M.D., Ph.D.

Budapest
2009

Dedicated to the memory of

Pálszabó Mária and
Serfőző László

Table of contents

Abbreviations

Ad	adenovirus
APC	antigen presenting cell
CAR	coxsackie and adenovirus receptor
CD (n)	cluster of differentiation (number)
cDNA	complementary DNA
CSF	colony stimulating factor
CTL	cytotoxic lymphocyte
dA	deoxyadenosine
DC	dendritic cell
dCK	deoxycitidine kinase
DDC	dermal dendritic cell
DDT	1,1,1-trichloro-2,2-bis(4-chlorophenyl) -ethane
DEPC	Diethylpyrocarbonat
dFdC	difluoro-deoxycytidine (gemcitabine)
DMF	dose modifying factor
DMSO	dimethylsulfoxid
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
EDTA	ethylenediaminetetraacetic acid
EGF(R)	epidermal growth factor (receptor)
EGD	esophagogastroduodenoscopy
EGTA	ethylene glycol-bis(2-aminoethylether) -N,N,N`N`-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EST	expressed sequence tag
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FL	FMS-like tyrosine kinase - ligand
Flt3	FMS-like tyrosine kinase
GDEPT	gene-directed enzyme-prodrug therapy
HRR	homologous recombination repair
HSV-TK	herpes simplex- derived thymidine kinase
IDC	interstitial dendritic cell
IFN-γ	interferon-gamma
IL (n)	interleukin (number)
IPTG	isopropyl-beta-D-1-thiogalactopyranosid
LPS	lipopolysacharide
MMLV	moloney-murine leukaemia virus
MOPS	3-(N-morpholino) propanesulphonic acid
NHEJ	nonhomologous end joining pathway
NK	natural killer cell
oligo(dT)	oligo-deoxythymidine
oligo(T)	oligo-thymidine
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
RACE	rapid amplification of cDNA ends
rFL	recombinant FL
RT	radiation therapy / room temperature
SCF	stem cell factor
SCID	severe combined immune deficiency
SDS	sodiumdodecylsulphate
TAA	tumor associated antigen
TCR	T cell receptor
TEMED	N, N, N', N'-tetramethylethylendiamin
TFRC	transferrin receptor
TGF(α,β)	transforming growth factor (α,β)
TiDC	tumor infiltrating dendritic cell
TK2	thymidine kinase 2
TNF-α	tumor necrosis factor - alpha
Treg	regulatory T cell
Tsup	suppressor T cell
UTR	untranslated region
VEGF	vascular endothelial growth factor
v/v	concentration - volume by volume
w/v	concentration - weight by volume
w/w	concentration - weight by weight

1. Introduction

Tumor cells are derived from normal cells through a number of genomic changes that lead to uncontrolled mitogenic activity. Tumors may escape apoptotic and immunological defense mechanisms of the host resulting in intractable growth. Despite of recent advances, surgery remains the main approach to treat (solid) tumors in general, being involved in the treatment of up to 80% of all malignancies[1]. Other conventional treatments include chemotherapy - that aims to specifically kill rapidly dividing cells or targets tumor cells (over)expressing specific molecules -, and radiotherapy. About half of cancer patients receive radiation therapy at some time during their treatment[2].

Alternative treatment strategies - most of them still experimental - may roughly be classified by action as oncolytic viral treatments (tumor lysis through selectively replicating viruses), specific gene therapies (aiming to restore/add or inhibit expression of a particular protein), antiangiogenic strategies (blockade of tumor vasculature), immunotherapies (tumor lysis through manipulation of the host immune system), stem cell therapies (graft-versus-tumor response induction through stem cell transfer) or the combination of these. Technically, gene therapy may serve as a tool of all of these approaches. Having very different range of side effects compared to other current treatments, gene therapy may be tolerably combined with them as an adjuvant[3].

The current status of cancer gene therapy in general and the ongoing clinical trials is constantly being reviewed[3]. Here, we focus on two major applications: immuno-gene- and (radiosensitizing) gene-directed enzyme-prodrug therapies. These modalities are especially promising from the wide palette of anticancer gene therapies: they may be less affected by the still unsolved problem of ineffective *in vivo* gene transfer.

First, we report our results concerning an immuno-gene-therapeutical approach against experimental pancreatic cancer - together with the description of a novel marker of malignancy in human pancreatic cancers with potential in diagnosis and targeting. Second, a novel gene-directed enzyme-prodrug strategy is proposed in experimental gliomas to improve the radiosensitizing effect of gemcitabine, a drug used in the treatment of both pancreatic and glial tumors in the clinic. The need to discover new treatment strategies against these malignancies is urgent: both of them are therapy resistant at the present state.

1.1. Pancreatic cancer - current concept of treatment

Pancreatic adenocarcinoma represents the tenth most common malignancy[4], and the sixth leading cause of cancer death in Europe[5]. Pancreatic cancer occurs more frequently in men than in women and is more common among blacks than whites. Roughly 80% of cases occur between 60 and 80 years of age. Other risk factors include a history of hereditary or chronic pancreatitis, cigarette smoking and occupational exposure to some carcinogens[6]. The majority (80-90%) of patients have adenocarcinoma with ductal differentiation. It is a tumor of extremely poor prognosis with mortality rates practically equal to its incidence rates. Overall five-year survival rate is less than 5% for all diagnosed patients[7, 8]. Patients usually present with advanced disease; two-thirds of them either have locally advanced or metastatic disease.

Up to now only early surgical therapy offers the opportunity of long-term survival. However, very few patients are suitable for surgery due to late diagnosis of disease caused by absence of clinical symptoms in early stage and the lack of screening tests[7, 8]. Even for these few selected 15% to 20% of patients who present with a resectable (Stage I-II) tumor by radiographic criteria and undergo surgery, the five-year survival rate is only 20% and it is even worse in node-positive patients[9]. For patients with unresectable tumors, the prognosis is dismal.

Despite tremendous efforts made in the development of useful chemotherapy regimens or newer approaches such as gene therapy, antiangiogenic strategies and inhibition of signal transduction[10], only minimal clinical improvements could be achieved. According to the current treatment standards, chemotherapy and radiation therapy offer modest benefits, although active clinical studies are underway in an attempt to improve the outcome. Recent clinical trials showed improvement in disease control placing gemcitabine alone or combined with radiotherapy instead of 5-FU into the forefront of palliative treatment of locally advanced pancreatic cancer[11, 12] together with the EGFR-receptor inhibitor erlotinib. For postoperative adjuvant treatment of resectable pancreatic cancer 5-FU with or without radiation remains the standard by now because there is still a lack of supporting data for other regimens. However, several chemortherapeutical regimens are being investigated as adjuvants, many of them utilizing combinations of different cytotoxic drugs (e.g. doxorubicin, mitomycin, gemcitabine, cisplatin, capecitabine, 5-FU) [11-13]. A very promising example for this is a

regimen consisting of cisplatin, interferon-α, and 5-FU with radiation[13-16]. This regimen has produced median survival of 45 months and an approximate 45% five-year overall survival, and is currently being tested in multi-institutional phase II[13] and III[17] trials. International controversy still exists as to what represents the optimum approach to the non-surgical therapy of pancreatic cancer at this time[18]. All agree, however, that any current approach is still far from ideal. Still, the latter study using interferon-α in combination may open new perspectives by demonstrating the special relevance of immunotherapeutic strategies for the treatment of pancreatic carcinoma.

1.2. Antitumoral immunity, immuno-gene therapies

In general, traditional methods used to treat cancer patients are usually traumatic – both physically and mentally, and frequently fail to prevent recurrences or cure metastases. As an alternative, cancer immunotherapy has been actively explored in the last decades. The term immunotherapy refers to any approach that seeks to manipulate the immune system of a patient for therapeutic benefit[19]. Its main concept is based on observations that cancer cells interact with the immune system. Early studies in mice already from 1953 showed that cells of the immune system can recognize and reject tumors[20]. The incidence of some cancers is increased in immunodeficient patients[21]. High incidence of malignancies was reported in patients receiving chronic immunosuppressive therapy after organ transplantation; including those that are not known to be virally associated[22]. High incidence of lymphomas and Kaposi's sarcomas, in patients with HIV-1 infection and AIDS were reported[23]. It has been shown that immune cells collect at tumor sites and specifically respond to tumor-associated antigens (TAAs)[24,25]. These observations highlighted the importance of the integrity of the immune system on tumor development and supported the scientific rationale for approaches to augment immune responses of the host against cancer. Recently, the increasing incidence of many cancers with age has been postulated to be caused by immunosenescence[26].

1.2.1. The effectors: T lymphocytes, NK and B cells

Hypothetically, effectors of both humoral[27] and cellular immunity have the potential to elicit anticancer immune responses, perhaps with the predominance of the cellular arm mediated by cytotoxic lymphocytes and NK cells[28]. The concept of immune surveillance that is based on the function of T-cells was initially formulated by Burnet[29]

in 1970. It has been later proven, that tumors express a variety of neoantigens that are frequently produced in excessive amounts or structurally aberrant[30]. Although these tumor antigens are mostly poor immunogens because they are mainly self-antigens, they may contain specific epitopes that can induce anti-tumor responses[31]. This response is mediated by cytotoxic CD8+ T lymphocytes.

Cytotoxic T lymphocytes may recognize tumor-derived peptides presented on the surface of the tumor cells by MHC class I molecules. Interaction of the T cell surface receptor CD28 with CD80 (B7.1) or CD86 (B7.2) on any of the antigen-presenting cells is necessary for generation of antigen-specific immune responsiveness ("two signal model")[32] -without this co-stimulation, antigen-specific T cells enter a state of tolerance and possibly apoptosis[33]. Since tumors will often, through a natural selection process, evolve to lose expression of MHC molecules[34] and key co-stimulatory molecules to avoid activating CTL, antigen presenting cells have special importance of inducing antigen specific T-cell responses. Pathological data corroborates with the CTL-hypothesis: lymphocyte infiltration of tumors has been shown to correlate with improved survival for a great variety of solid tumor types[23, 34, 35].

$CD4^+$ T lymphocytes are not direct effectors of antitumoral responses but they represent a key element in optimal activation of $CD8^+$ T cells and in the maintenance of immune memory. They secrete cytokines that promote the proliferation and activation of CTL and B cells. They too, recognize antigen in the context of peptide presented by MHC class II molecules via their TCR, but generally CD4+ cells are primed by DCs or B cells acting as APCs[28].

The "indiscriminately cytotoxic population of lymphocytes" against tumor cells were first isolated from blood of healthy individuals by Sinkovics et al.[36] in 1969. They were later named as natural killer (NK) cells that mediate innate immunity against pathogens and tumors[37]. They comprise up to 10-15% of peripheral blood lymphocytes. NK cells use the same killing mechanisms as CTLs and can secrete similar cytokines[28]. However, they do not express a single, invariant receptor molecule, but express multiple activating and inhibitory receptors. It is the synthesis of these signals that determines the functional response of the NK cell[37]. Target cell specificity is not provided by the activating receptors on NK cells that may be stimulated by a variety of signals, but rather by an array of inhibitory receptors that recognize MHC class I[28]. NK cells have

been shown to be capable of killing certain tumors *in vivo* and *in vitro*, and they may also play a particularly important role in controlling the development of tumors that downregulate MHC I expression[37, 28]. NKT cells are a special recently discovered subset of lymphocytes that express both T cell and NK cell markers[38]. They recognize glycolipid antigen in the context of the MHC-like molecule CD1d via their semi-invariant surface receptor. NKT cells are an area of intense research and are thought to play a role in biasing the immune response toward Th1 or Th2 imbalance, are implicated in immune surveillance and protection against carcinogenesis, and conversely: a regulatory or immunosuppressive subset of NKT cells has also recently been reported. Although the number of NKT cells is small, they produce large amounts of IL-4 or IFN-gamma, cytokines that promote both humoral and cellular immunity.

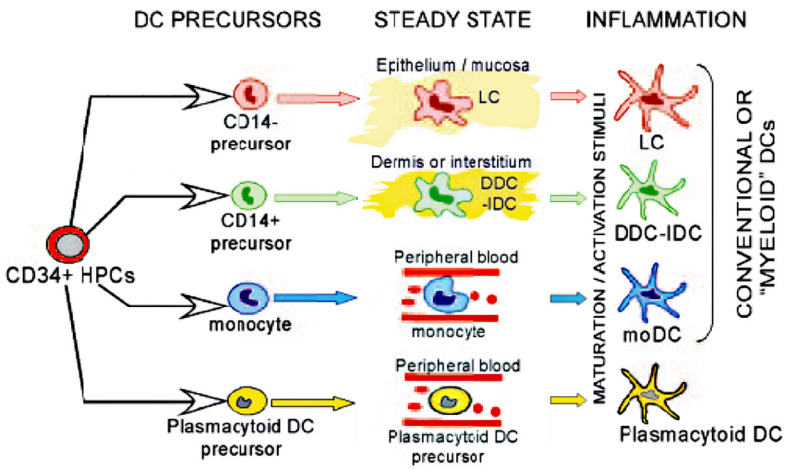
B cells can theoretically recognize tumor antigen via the B cell receptor, a monospecific immunoglobulin or antibody expressed on their surface. After binding and subsequent cytokine stimulation mainly from T helper cells, B cells get activated and recruit T and B cells to the site of inflammation, become an APC itself and can differentiate into plasma cell secreting large amounts of antibody[32]. Theoretically, IgG1 and IgG3 can direct the killing of tumor cells by both antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). ADCC is triggered by the interaction between the Fc region of an antibody and its receptors on neutrophils, NK cells, or macrophages[28, 32] resulting in phagocytosis or lysis. In CDC, recruitment of the complement component C1q by IgG bound to the tumor cell surface activates complement cascade, or the tumor cell-bound C1q itself can bind to complement receptors such as C1qR, CR1 (CD35), and CR3 (CD11b/CD18) on neutrophils, macrophages, or NK cells resulting in cell destruction[28]. By screening a phage expression library with serum from cancer patients, a number of cancer testes antigens have been detected[39]. However, it is still unclear whether or not these antigens have *in vivo* relevance with respect to T cell–dependent immunity. Hypothetically, humoral immune responses are dependent on T-cell help, and therefore, it is conceivable that a T-cell response against these serologically defined antigens should be present[22].

1.2.2. The role of dendritic cells

Paul Langerhans first described dendritic cells in human skin but thought these were cutaneous nerve cells[40]. Steinman and Cohn[41] discovered these cells a century later in

mouse spleen and applied the term "dendritic cells" based on their unique morphology. Progress in the study of DC biology exploded in the 1990s when cytokine-driven methods were developed for expanding and differentiating DCs *ex vivo*[42-48]. It became obvious that DCs are the central players in the induction, regulation and coordination of adaptive immune responses, and also in the regulation of innate immunity[47, 49, 50]. DCs are potent immunogens under inflammatory conditions, yet are also critical to the induction and maintenance of self-tolerance in the steady state[51].

Figure 1. Development of human DCs. Resident populations of immature, non-activated DCs (middle section) are normally found in the steady state in the periphery and/or the circulation. „Dangerous insults" (usually provided by a microbial pathogen or product) to the steady state can lead to inflammation with full maturation and activation of each DC subset[55].



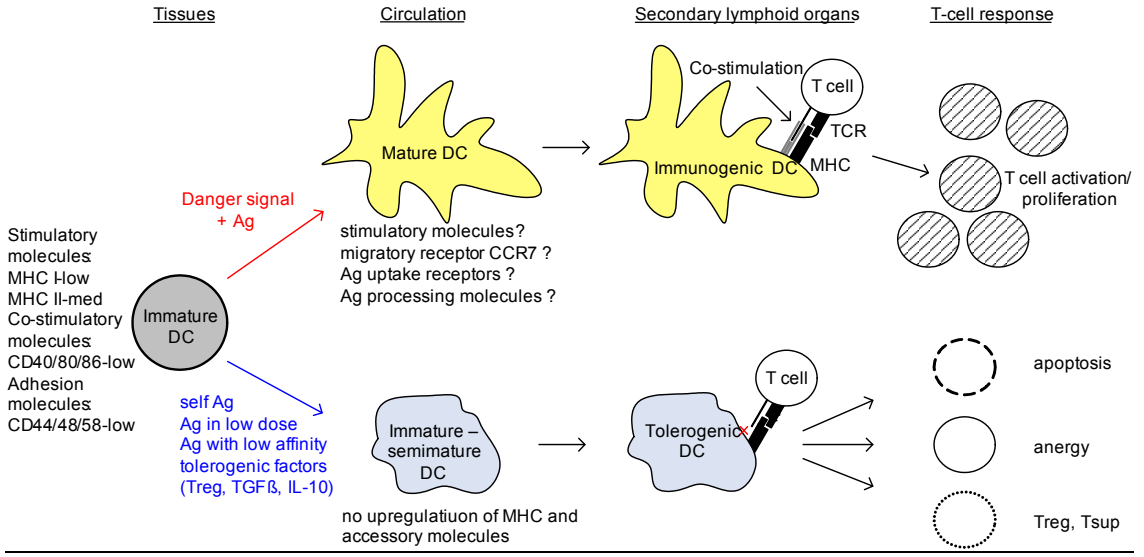
modified from Rossi M et al, Journal of Immunology - August 2005

DCs are all bone marrow-derived leukocytes[52]. They are distinct from follicular dendritic cells, which are not leukocytes, have stromal origin, and interact with B cells in the germinal center of lymph nodes, but do not influence immunity dependent on T, NK, or NKT cells[53]. DCs represent a heterogenous cell population. Different subsets can be found in various tissues including peripheral blood. Many of them reside in sites of interface with the environment (skin and mucosae), where they represent 1%–2% of the total cell numbers[49]. Precursors in blood and bone marrow can give rise to four types of DCs under cytokine-driven conditions *ex vivo* (Figure 1.) in humans. The cell subsets described according to this classification which is based on *ex vivo* development of DCs may be somewhat different from the *in vivo* situation[54], but this terminology is widely used in the literature. These subsets include the ones termed as conventional or "myeloid" DCs[46-48, 55]: CD14+ blood monocyte-derived DCs (moDCs), dermal DCs or

interstitial DCs (DDC-IDCs), Langerhans cells (LCs), and "lymphoid" or plasmacytoid DCs[56]. DCs in lymphoid tissues include populations termed conventional or myeloid, as well as plasmacytoid, the latter named because of their morphological resemblance to plasma cells[56]. Circulating blood monocytes can differentiate into moDCs under the influence of specific cytokines, and exhibit sensitivity to ambient cytokines that can drive their differentiation to DCs resembling either activated LCs or DDC-IDCs[47, 56]. This may provide biologic flexibility to monocyte precursors and moDCs as first responders to causes of inflammation. There are differences in murine and human DC development; murine DCs exhibit more complex differentiation patterns across lymphoid and myeloid precursors[47]. Human DCs are all CD8- and (especially the ones from the lymphoid lineage) may not express CD11c, a marker strongly expressed by mouse DCs[47]. The developmental pathway of DCs is still an intensively researched area. According to the widely accepted model, dendritic cells constitutively patrol through the blood, peripheral tissues, lymph and secondary lymphoid organs and capture various self and non-self antigens throughout the body[57]. The function of the developed immature DCs is to monitor the environment by capturing antigens. These cells are characterized by intermediate surface expression of MHC-class II and low level of costimulatory molecules, including CD80 (B7.1), CD86 (B7.2) and CD40[49, 50, 58-61]. Antigens are presented by both MHC I and II molecules in DCs. Constitutively, however, peripheral dendritic cells present antigens quite inefficiently[57] – except the immature DC population in lymphoid organs[33] – and can not activate T cells. To do so, a "danger signal" is required that induce dendritic cells to enter a developmental program: maturation[55]. The "danger signals" that induce dendritic cell maturation affect DCs mostly through direct interaction with specific surface receptors. These signals include necrotic cell compounds, bacterial compounds (LPS and double stranded RNA through Toll-like receptors 2,3,4), viral products, CD4+ T cells through CD40L-CD40 interaction, FcR activation as well as inflammatory cytokines (TNF-α, IL1β, PGE-2) whose secretion is induced by pathogens or mechanical stress[57]. As response to the immunogenic stimuli, upregulation of the co-stimulatory molecules CD80/86/40 occurs[49, 50, 57-61]. T lymphocytes and endothelial cells contribute to the final maturation of dendritic cells through direct cell-to-cell contact and the secretion of cytokines[60]. Maturation also induces massive migration of dendritic cells out of peripheral tissues[49] -

thus, dendritic cells become the most potent APCs, and the only ones capable of activating naïve T lymphocytes. In secondary lymphoid organs, DCs present antigens to naïve T cells, thereby inducing a cellular immune response that involves both $CD4^+$ T helper 1 cells and cytolytic $CD8^+$ T cells[57]. DCs may also activate naïve[62] and memory[63] B cells, NK cells[64-66] and NKT cells[67]. The class of immunity together with the effectors involved depends largely on the DC subset and on the cytokine environment during maturation. In general, mature DCs induce type 1 immunity – although type 2 immunity was also reported when under special cytokine environment *in vitro*[68].

Figure 2. Induction of T-cell responses by DCs. Semimature DCs continuously present self-Ags, and probably harmless non-self-Ags, in secondary lymphoid organs to maintain peripheral tolerance and anergy[51, 47]. In contrast, "danger signals" lead to inflammation with maturation and activation of DCs and T cell activation[55].



Although DCs may induce cytotoxic immune response, this function is dependent on maturational state (Figure 2). Inactivated, immature DCs seem to serve the paradoxical role of peripheral tolerance to antigens[33, 47, 51, 58, 59, 63, 70]. A rational of peripheral tolerance is the complementation of central tolerance, during which newly generated autoantigen-responsive T cells are deleted. Central tolerance is also dependent on (thymic) DCs[69], but may not be sufficient – T cells clones may survive central selectional mechanisms, some antigens may not be able to access the thymus, or some antigens may be expressed only later in life. According to the present hypothesis, DCs from peripheral tissues deliver antigens continuously to lymphoid tissues and induce T-cell tolerance in steady state by anergy, direct depletion of T cells, or by generation of regulatory/suppressor T cells. This has been experimentally proved by loading various

antigens including dying cells remnants[71] to immature DCs which lead to deletion of T lymphocytes *in vivo*[33]. The switch between tolerance or toxicity is regulated in every single DC and depends on their maturational state: antigen-loaded immature tolerogenic DCs[72] activated by CD40-antibody resulted in the up-regulation of a large number of co-stimulatory molecules on DCs and a potent immune response[73].

The mechanism how immature DCs influence T cells to mediate peripheral tolerance is largely unknown. It has been suggested, that the "default" pathway of T cell development *in vivo* leads to the direction of tolerance in every case by entering apoptosis, whenever T cells are stimulated to grow in the absence of further stimuli from maturing DCs, and this may happen similarly to apoptosis of thymocytes during the induction of central tolerance[74]. DCs may also be involved in the deletion of antigen-reactive T cells directly by production of tryptophan metabolites[75]. It has also been suggested, that the induction of tolerance would be related to the generation of NK-T cells[76] or special T cells with suppressor functions. The existence of these T cells (called originally suppressor cells - Tsup) was initially proposed in the early 1970s by Gershon and Kondo[77], but became target of intensive study only in the last decade[78]. Different subsets of T lymphocytes with regulatory/suppressor activities have been identified including regulatory T cells (both $CD25^+$ naturally occurring and CD25-inducible CD25- Treg) and non-antigen-specific CD8+CD62- suppressor lymphocytes[59, 79]. The regulation of both Treg subsets seems to be controlled by DCs[80, 81].

The presence of peripheral tolerogenic DCs may have significance in preventing autoimmunity by tracing self-reactive T cells that have either escaped from the process of central tolerance or have been developed in tissues, but also may play role in the mechanisms how tumors escape from the immune system.

1.2.3. Immuno- and immuno-gene therapies for cancer

Unfortunately, many cancers develop mechanisms to subvert the immune system, including the loss of expression of TAAs or MHC molecules, the production of immune-suppressing cytokines such as TGF-β and IL-10, and/or the induction of regulatory/suppressor cells[22, 25, 34]. To overcome these events, numerous experimental strategies have been developed. These include non-specific activation of the immune system with microbial components or cytokines, antigen-specific adoptive immunotherapy with antibodies and/or T cells, and antigen-specific active

immunotherapy: vaccination. The main goal is to augment immune responses against tumors with the potential to treat even distant, undetectable metastases.

The first reported use of (non-specific) immunotherapy dates back to 1867 when William Coley, an orthopedic surgeon from New York reported to successfully treat a proportion of sarcomas by intratumoral injection of attenuated Streptococci derived from erysipelas[82]. Coley's toxin is the classic example of immune adjuvant therapies. To achieve better results and to avoid dangerous systemic side effects, specific immunotherapies have been developed. Autologous or allogeneic tumor cells have been used as vaccines[61, 83]. The observed results were variable; however, in many cases, a tumor-specific immune response has been induced, and tumor regressions, albeit limited, have occurred. These approaches rely on random encounter of the vaccine with host DCs. *Ex vivo*-generated, antigen-loaded DCs have also been used as vaccines to improve immunity in several trials[61, 83]. The technique in general has the advantage, that theoretically many antigens can be specifically selected *in vitro* when targeting DCs, however, the results of these approaches are not satisfactory, either. As a consequence, the efficacy of this approach has recently been questioned in general[84], because of the undeniably limited rate of objective tumor regressions that has been observed in clinical studies so far. Moreover, the manipulation of dendritic cell progenitors and DCs *ex vivo* is an expensive, time-consuming process and has the potential of infections, which may limit large-scale use of the technique[85].

Because of the limited success achieved by traditional immunotherapies, immuno-gene-therapy has recently been applied. Technically, there are three ways of immuno-gene therapy. *(A)* Autologous or allogeneic tumor cells may be modified *ex vivo* to produce immunostimulatory cytokines or highly immunogenic proteins, and the mixture of the killed cells may be used as a vaccine. This approach provided very promising results (measurable immune response, tumor size regression or stabilization in a proportion of patients, increased median survival) even in some human studies against otherwise very aggressive and therapy-resistant types of malignancies such as non-small-cell lung cancer[86], or pancreatic cancer[87]. *(B)* It is also possible to directly alter the immune cells of the host *in vivo* or *in vitro* to sensitize them to cancer by adding an antigen or stimulatory gene; dendritic cells are the main targets of these therapies[88]. *(C)* The third potential way of immuno-gene therapy is direct transfection of tumors with genetic

material coding for products that would unmask the tumor from immune invasion. Unlike the first two aforementioned cell based immunotherapies, which are currently limited by toxicity, low therapeutic efficacy and by the logistics involved in large scale culture and production of patient specific cells, this approach may be cheap and easy to perform. Several immunostimulatory cytokine-coding vectors have been tested in animal and human models by direct intratumoral delivery. Most of them, like GM-CSF, CD40-L, IL-15, IL-2, IL-12, IL-24, TNF-α, and macrophage inflammatory protein-1a[24, 25, 89, 90] have been proved to be effective in animal models leading to tumor regression and many times induction of long-term immunity. In humans, unfortunately, these cytokines were rather ineffective. Partial objective response was reported only in a few studies – i.e. after gene-delivery of IL-24 into melanomas (regression rate: 2/28)[91], and intratumoral application of TNFerade in a variety of tumors (adenovirus vector coding for TNF-α under control of a radiation-inducible promoter[25, 92, 93].

The identification of Fms-like tyrosine kinase 3 – receptor and its ligand that effects specifically DC development made recently possible to produce large numbers of DCs *in vivo*. The use of Flt3-ligand may be an alternative to the *ex vivo* manipulation of DCs, and become a potential novel target for cytokine-based immuno-gene therapies.

1.3. The Fms-like tyrosine kinase 3 - receptor and ligand

1.3.1. Expression and physiology

Receptor tyrosine kinases bind a large number of ligands that regulate haematopoietic growth[94, 95]. Three of these and their ligands play a central role in hematopoiesis: the receptors for M-CSF, SCF and Flt3[94]. Among these, the Fms-like tyrosine kinase 3 - receptor and ligand (Flt3 – FL) system plays important role in the development of both lymphoid and myeloid cell lineages.

Flt3 is a member of the type III RTK family, which also includes c-kit, c-fms and PDGF-A and -B receptors[94, 96]. Flt3 is highly expressed on common lymphoid and myeloid progenitors as well as steady-state (but not mature) DCs[97]. B cells, T cells, macrophages and monocytes express the receptor in the spleen, peripheral blood and thymus[96]. Among the lymphocyte population, protein expression is restricted to pre-pro-B and pro-B cells and the most immature of (mouse) thymocytes. Week protein expression can be found in non-haematopoietic tissues such as placenta, liver, brain, gonads, kidney, or pancreas, but the mRNA of Flt3 can be detected more widely[94].

Lyman et al.[98] cloned the cDNA of FL for the first time and demonstrated that both membrane-bound and soluble isoforms of the peptide exists; the latter is product of alternative splicing[99]. The membrane bound FL is a 30kD type I transmembrane non-disulphide linked homodimer glycoprotein. Its soluble form (the extracellular 160 amino acid) is also biologically active. The expression of FL is ubiquitous[94]; however, the highest level of expression can be measured on peripheral blood mononuclear cells. It is very rare that the receptor and the ligand are expressed together on the same cell at the same time[94]. Mice that are deficient for FL exhibit a significant reduction in the numbers of progenitor cells in the bone marrow. In addition, these mice have reduced numbers of myeloid cells, B cells, NK cells and dendritic cells[100].

FL alone has weak proliferative effect on defined subpopulations of stem and progenitor cells, but increases their response to other growth factors *in vitro*[96]. Many experiments showed, that FL affects the growth of pluripotent haematologic stem and progenitor cells, as well as a number of lineages in the lymphoid and myeloid pathways [94, 96, 100] *in vivo*. When administered *in vivo*, FL increases (1) the absolute number of immature and mature NK cells in various tissues[101-103], (2) the number of pre-B cells, (3) promotes maturation of NK- B- and T-cells[104], and (4) extremely increases the number of DCs in all lymphoid and parenchymal organs and blood[96, 104, 105].

The capacity of FL to induce DCs is significantly higher than that of other commonly used haematopoietic growth factors or cytokines *in vivo*. Although GM-CSF (and IL-4) is necessary for DC generation from precursors *in vitro*, its *in vivo* effect on DC expansion is not comparable to that of FLs. Overexpression of GM-CSF in GM-CSF transgenic mice does not increase the number of DC in lymphoid tissue, which also suggests that other growth factors are important for DC generation *in vivo*[106]. In contrast, when FL is administered *in vivo*, large portion of the induced DCs belong to the lymphoid subtype[96]. The effect of FL on DC development is dramatic: their absolute number was found to increase 17-fold in the spleen, 4-fold in lymph nodes and 6-fold in peripheral blood after 10 days of daily FL injections[105] in mice. Based on surface marker expression, five distinct spleen populations were identified in mice[105] - importantly, significant amount (three populations) of DCs were found to be mature. Levels of DCs returned to normal level following cessation of FL treatment. The effect of systemically administered FL seems to be attributed mainly to the ability of

expanding the stem and progenitor cell pool, but FL also leads to DC differentiation from the circulating progenitor pool[105, 107]. Local administration of FL or its cDNA could "recruit" DCs to the injection site, which also proves that FL has effect on circulating DC precursors[107-112].

1.3.2. The antitumoral effect of systemic FL application

The unique property of FL to expand the mass of DCs *in vivo* has led to the assumption that it could act as a potential antitumor agent. Antitumoral effect of FL was first demonstrated by Lynch et al[102] in a B10.2 murine fibrosarcoma model: systemic application of recombinant FL induced complete tumor rejection in a subset of animals with adoptive immune memory mediated by $CD8^+$ T cells. In this setting, FL presumably acted by enhancing the antigen-presenting capacity of tumor-bearing mice through the large-scale expansion of DCs. Effective dose of FL was 10 µl/day for ten days. Lynch et al[102] already hypothesized, later Shaw et al[103] and others[53,101,103,113-115] proved that FL has effect on NK cell activity as well - FL promotes NK cell proliferation and suppresses tumor growth even in SCID (T- and B-cell deficient) mice.

The effect of systemic FL administration on tumor growth has been tested in several other murine models including C3[115], MC38 colon carcinoma[113, 115], B16 melanoma[113,116,117], CL-8 melanoma, EL4-lymphoma[116], breast cancer[118], Lewis carcinoma[110], human ovarian tumor[53], TRAMP-C1P3 prostate carcinoma[54, 118-120,] rat colon adenocarcinoma[121], and different sarcoma[113, 114] models. Significant differences in immune changes among different models were reported and the contribution of any of the afore-mentioned cell subsets seemed to be largely dependent on the tumor model itself[113]. Partial response to treatment could be achieved in most, but not all of these experiments by administration of recombinant FL - usually through 8-10 consecutive days. FL administration before exposing animals to tumors could protect from some, but not from all tumors[111].

It was possible to expand significant number of DCs with high doses of recombinant FL in human studies as well [122-126], moreover, a new antigen specific IgG was described in a case[127] - but significant clinical response to therapy couldn't be reached. In a study performed in patients with unresectable metastatic colon cancer, FL injections were mediating DC expansion in peripheral blood, without clinical response[126]. In another study, 20 µg of FL was administered daily for two weeks as an adjuvant to an MHC I

class peptide vaccine. DCs were markedly increased in the peripheral blood of subjects, but augmentation of antigen-presenting cells within the site of vaccination was not observed and significant peptide-specific T-cell responses were not detected.

There are observations that suggest limited applicability of systemic FL therapy in a clinical setting, especially when using large amounts of cytokine. Although high-dose FL therapy even in a subcutaneous 1.25 μg/kg for 28 days regimen was well tolerated in some clinical studies[123, 126], autoimmune hypothyroidism was reported in subjects after low dose FL therapy[125]. Although a rare finding, the number of patients enrolled in FL studies is also low, so the risk of autoimmunity as a side effect can not be excluded. It has been shown, that application of FL protein *in vivo* can lead to significant side effects in animal models as well. Besides organomegaly of lymphoid tissues and liver[96], severe autoimmune phenomena: splenic fibrosis and subsequent death was reported in a murine model after long term FL exposure[128].

Antitumor immunity elicited by administration of FL protein in multiple doses has minimal if any efficacy in humans with cancer. Its efficacy is also limited and not predictable in animal models to protect host from tumor cell challenge and can inhibit tumor growth only in animals with relatively small tumor burdens[94]. Treated animals usually develop tumors after termination of treatment[129, 130]. The half-life of FL in blood stream is short, it binds non-specifically to various receptors and its accumulation in tumors is very low. It has been suggested, that FL gene therapy may induce more potent antitumor immune response than immunotherapy with the soluble protein[108, 110, 131].

1.3.3. FL gene-delivery and cancer

The rationale for using local rFL gene delivery is its unique ability to increase the number of dendritic cells, partly from circulating precursors[107, 105]. Tumor cells engineered *in vitro* to express FL grew slower in murine breast cancer[110], colon carcinoma[111] and hepatocellular carcinoma [112] models. Growth of second, untransduced tumors in vaccinated mice were inhibited in some studies[109, 110], and adoptive transfer of immunity with splenic T-cells from tumor free animals was demonstrated[108]. As shown by these experiments, modification of tumor cells *in vitro* to express FL can generate immunogenic cells, but the antitumoral response may be partial – similarly to systemic administration. Current data is still insufficient in supporting that these modified tumor cells could serve as therapeutic vaccines against established large tumors[85, 130].

The amount of information about immunotherapeutic approaches based on *in vivo* FL-gene delivery is still very limited. In these studies the immunostimulatory effect after local administration of adenoviral or plasmid vectors was found to be accompanied by less dramatic systemic changes compared to FL protein injection. It was shown, that intradermal gene gun delivery of a recombinant chimera of FL linked to the papillomavirus-16 E7 enhanced the effectivity of DNA-vaccination and increased the frequency of Ag-specific $CD8^+$ T-cells[133]. Intramuscular delivery of an rFL-coding plasmid recruited DCs to the injection sites and lymph nodes with potent vaccine adjuvant activity[85]. Similarly, electroporation amplified intramuscular FL-plasmid delivery recruited dendritic cells to the injection site[133] and led to elevation of DC number in spleen[133-134], again showing the potential of local FL gene-delivery for immunotherapeutic use.

In therapeutic setting, when rFL-coding adenoviruses were injected into pre-established tumors, growth inhibitory effect could not be achieved in a MH134 hepatocellular carcinoma model[112] at all; partial growth inhibitory effect was described later in a Hepal-6 tumor model[135], and very recently total tumor regression in the same tumor model from the same authors[130]. This latter study, together with a report of intracranial Ad-rFL application[107] - both published after finishing our experiments - showed, that intratumoral injection of rFL-coding adenovirus can lead to complete tumor regression, and may elicit better response to therapy than FL protein injection or systemic Ad-rFL transfection[130].

1.4. Status of immunotherapy for pancreatic cancer

The recent identification of various tumor-associated antigens expressed by pancreatic cancer, some of them mutated (see also 1.5), may have the capacity to serve as targets for specific immunotherapies[10, 136-140]. Although pancreatic cancer is considered to be weekly immunogen, the promising results of some clinical phase I-II immunological studies including those with non-specific immunotherapies (see also 1.1) drives further experiments in the field.

Unfortunately, specific passive immunotherapy of patients with antibodies against anti-EGFR, VEGF, mesothelin or MUC1 antibodies, or adoptive T-cell transfer has not yet resulted in significant therapeutical benefit[140]. In contrast, DC-based vaccines based on tumor antigens such as mucin-1, CEA or mutated k-ras elicited well measurable

immune response in patients with moderate clinical benefit in clinical immunotherapeutic trials[10, 97, 98, 141]. Therapy with DCs pulsed with synthetic mutant K-ras peptide[141] had some beneficial effect on survival in therapy responders (61 days survival vs. 148) and using the mRNA of CEA may also prolong the survival in some extent, as having been reported[55]. In contrast, DCs loaded with MUC-1 cDNA[142] didn't elicit any clinical response, in spite of detection of antigen reactive T-cells.

Preliminary data about vaccination of patients with allogeneic pancreatic cancer cells engineered to produce GM-CSF combined with surgery has shown impressive phase II results with 76% survival at 2 years compared to the historic average of <50% at 2 years[87]. Similarly preliminary data from a multicentre phase II/III Pancreatic Cancer Clinical Trial with intratumoral TNFerade[93] suggests significant improvement in median survival after treatment. There seems to be hope that immunotherapies could serve as effective adjuvants in the treatment of pancreatic cancer.

1.5. Molecular markers of pancreatic cancer

The identification of mutant or overexpressed proteins have special importance in pancreatic cancer: it is a cancer type very hard to diagnose and nearly impossible to treat. The distinction between well differentiated pancreatic adenocarcinoma and chronic fibrotizing pancreatitis is one of the greatest challenges of the pathologist[143].

A recent report applying microrray technology revealed an average of 63 genetic alterations in pancreatic cancers and defined at least 12 cellular signaling pathways that were altered in 67 to 100% of the tumors[144]. The most important is perhaps K-ras mutation, which is thought to be the initial step in cancer formation and is also characteristic for pancreatic intraepithelial neoplasia[140, 145, 146]. Interestingly, it can also be detected in patients with the rare known risk factors of pancreatic cancer: in smokers and in chronic pancreatitis[144]. Among other frequent tumor-associated mutations (Table 1) p53, p16INK[147] and SMAD4[148, 149] are thought to be milestones of carcinogenesis[150]. These, together with K-ras have already been evaluated as diagnostic factors. However, they are characterized by limited specificity and/or sensitivity and can be used only in the combination with conventional diagnostic tools[148]. To date, molecular pathological methods have shown no advantage in the differential diagnosis of pancreatic cancer [143].

Table 1. Common genetic aberrations in pancreatic cancer[140, 151]

Protein	Frequency %	Protein	Frequency%	Protein	Frequency%
pRb	6	HGF receptor	61–87	CaSm (LSM1)	87
BRCA-2	7–10	IGF-1R	64	Bcl-xL	90
Akt2	20	Notch3	69–74	MUC1	90
Bcl-2	23	Shh	70	Mesothelin	90–100
Gastrin	23–91	K-ras	75–90	Telomerase	92–95
FAK	48–75	BIRC5 (survivin)	77–94	CCK-B receptor	95
p53	50–75	p16INK4A (MTS1)	85	RAAG12	100
Smad4	55				

Many structurally normal proteins are known to be overexpressed in pancreatic cancers; some can be found in the serum. The sialylated Lewis blood group antigen CA19-9 is the most important clinically, being overexpressed in most pancreatic cancers (~70% sensitivity). It represents the major clinical tool to monitor responses to therapy today, but is insufficient for diagnosis: 5-10% of the population do not express the protein and 40% of patients with chronic pancreatitis show elevation[151]. Other serum markers such as mesothelin or specific mucins are under investigation for clinical applicability but still did not produce much better sensitivity and specificity[151]. Other locally overexpressed proteins in pancreatic cancer include EGF and EGFR, TGF-α and β, VEGF, COX-2 and various matrix metalloproteinases – numerous ongoing experiments aim to target cancer cells through these molecules[150].

1.6. The transferrin receptor

Iron is an essential element for cell metabolism and proliferation. Higher proliferation rate is usually accompanied by increased iron requirement - this increased need in dividing cells is probably attributable to the role of iron as a cofactor of ribonucleotide reductase[152]. Free Fe^{3+} ions are toxic due to their oxidative capacity; so they are transported in the plasma by specific protein transporters, mainly by transferrin. The initial step in cellular iron uptake is binding of transferrin-iron complexes to the transferrin receptor-1 (TFRC-1), a homodimer transmembrane protein. Transferrin receptor-2 is expressed mainly by hepatocytes and erythroid progenitors in a cell-cylce dependent manner – its role is secondary in iron uptake and is not yet understood[152, 153]. After binding, the complex may be internalized by endocytosis. The expression of TFRC and the iron storage protein ferritin is regulated primarily at the transcriptional level by iron regulatory proteins (IRPs). These proteins may stabilize the mRNA of

TFRC or transferrin in response to low iron levels or hypoxia after binding to iron responsive elements (IRE) of the mRNAs. As a consequence, iron-deficient cells increase iron uptake via the TFRC-transferrin system and block iron storage by ferritin[152].

TFRC is ubiquitously expressed on normal cells at low levels with only few exceptions (erythroid cells and some erythroid progenitors lacking DNA synthesis and mitochondria). Because of its pivotal role in iron uptake, TFRC is expressed in a larger amount on proliferating e.g. malignant cells than in quiescent cells[154]. Transferrin receptor is also expressed by most carcinomas, sarcomas, and some lymphomas and leukemias[155-158], and its expression was shown to correlate with tumor grade and prognosis in many cases[152]. Due to its increased expression by many malignant cells, transferrin receptor has been suggested as a promising therapeutical target in anticancer therapy[159]. Although the expression of TFRC has been described in many malignant tumors, there is lacking evidence about its presence and role in pancreatic tumors except a single report of two cases of pancreatic cancer that expressed TFRC[154].

1.7. Malignant gliomas - current concept of treatment

Although relatively uncommon, malignant gliomas are associated with high morbidity and mortality: the median survival is only 12-15 months for patients with glioblastomas and 2- 5 years for patients with anaplastic gliomas - despite of treatment. Histologically, glioblastomas account for 60-70%, anaplastic astrocytomas for 10-15%, anaplastic oligodendrogliomas and oligoastrocytomas for 10%, while anaplastic ependymomas and anaplastic gangliogliomas for 5-10% of malignant gliomas. Malignant gliomas are 40% more common in men than in women, and are twice as common in whites as in blacks. Their only established risk factor is exposure to ionizing radiation[160].

Besides standard supportive medical therapy the standard care for newly diagnosed malignant gliomas is surgical resection when feasible; however, malignant gliomas are infiltrative in nature and cannot be completely excised. The addition of radiotherapy to surgery increases survival among patients with glioblastomas from a range of 3-4 months to a range of 7-12 months and is the gold standard of adjuvant and palliative treatment. The use of chemotherapy is still controversial; recently radiotherapy with concomitant temozolomide (oral alkylating agent with good blood-brain barrier penetration) was established as a useful combination for newly diagnosed

glioblastomas[161]. The therapy of anaplastic oligodendrogliomas and anaplastic oligoastrocytomas may be different based on a genetic abnormality (unbalanced translocation of 19p to 1q) making them sensitive to a procarbazine – lomustine – vincristine combinational regimen by an unknown mechanism[162].

Despite of optimal treatment, nearly all malignant gliomas eventually recur. Newer chemotherapeutic agents, targeted molecular agents and antiangiogenic agents may enhance the effectiveness of surgery and radiotherapy. Inhibitors that target receptor tyrosine kinases involved in glioma formation such as EGFR, PDGFR, and VEGFR, as well as signal-transduction inhibitors targeting mTOR, farnesyltransferase, and PI3K may have relevance; but – except the VEGF monoclonal antibody bevacizumab - have shown only very modest activity, with no prolongation of 6-month progression-free survival as single agents[160]. There is urgent need for new therapeutical agents and regimens to augment the effect of current surgical and radiotherapeutic treatments.

1.8. Gene-directed enzyme pro-drug therapies

In gene-directed enzyme pro-drug therapy (GDEPT), the aim is to transduce tumor cells with specific "suicide" genes, which would activate systemically administered non- or mildly toxic pro-drugs to cytotoxic drugs[163]. Acting locally, this may improve the specificity of chemotherapies. The approach is also known in the literature as suicide gene therapy (SGT), virus directed enzyme pro-drug therapy (VDEPT), or gene pro-drug activation therapy (GPAT)[164]. Since the efficiency of *in vivo* transfection is limited in every currently known gene-therapeutical way, the expression of any foreign enzyme can only be increased in a minority of the cells of a targeted tumor. However, there is evidence, that tumor cell killing is not restricted to the transfected cells, but it can also be observed in cells surrounding the ones expressing the suicide gene. This process is called bystander effect or neighboring cell killing effect[163], a phenomenon already being described in traditional radiotherapies before GDEPT. Bystander killing is especially characteristic for GDEPT approaches; in some preclinical studies tumors composed of as few as 10% of pro-drug expressing cells could be fully eradicated[165, 166]. The mechanism of bystander killing is partly based on local drug release from the transduced cells[167, 163], but other mechanisms such as gap-junctional intercellular communication[167, 163] and release/uptake of apoptotic vesicles[168] are also involved. In addition, GDEPT may induce an immunological response by the remnants of the dying

cells mediated by NK and T-cells. This therapeutically beneficial effect is known as the distant bystander effect [163, 164]. Because of the aforementioned low penetration capability of the vectors, induction of a strong bystander effect seems to be crucial in current gene therapies.

To ensure tumor cell-specific expression of the pro-drug activating enzymes, sophisticated methods have been employed including intratumoral delivery, the use of tissue- or tumorspecific promoters or replication-competent tumor specific viral systems[164]. Similarly, a large number of enzyme pro-drug combinational systems have been developed. The most frequently used enzymes are of non-mammalian origin (Table 2) and are different from any circulating endogenous enzymes making the therapy potentially very selective. An important difference or disadvantage compared to the enzymes of mammalian origin is that they are likely to be immunogenic[164].

By January 2006, more than 1100 gene therapy clinical trials have been initiated - around two third of these aimed to cure cancer[163]. Despite of this large number most of them did not get beyond phase I and only few have reached phase III[169]. Most anticancer trials apply some form of GDEPT which have been the most successful so far. The most frequently used GDEPT protocol is the Herpes simplex- derived thymidine kinase (HSV-TK)/ganciclovir system. After phosphorylation of ganciclovir by HSV-TK, an enzyme 1000-fold more efficient than the human TK, the drug is further phosphorylated by cellular kinases to the toxic triphosphate form, which exerts its cytotoxic effects by inhibiting cell DNA polymerases and by competing with dGTP for incorporation into DNA during the S phase of the cell cycle[164].

Table 2. The most frequently used GDEPT pro-drug/enzyme pairs and products. →: the toxic end-product is a result of additional enzymatic change mediated by human enzymes.

Pro-drug	Enzyme	Product
ganciclovir	*HSV* thymidine kinase (HSV-TK)	ganciclovir-monophosphate(→-triphosph.)
5-fluorocytosine	*bacterial/yeast* cytosine deaminase	5-fluorouracil(→fluoro-uridine monophosph.)
5-fluorouracil	*E.coli* uracyl phosphoribosyltransf.	fluoro-uridine monophosphate
ifosfamide	*human* P450 isoenzymes	hydroxy – ifosfamide
cyclophosphamide	*human* P450 isoenzymes	hydroxy – cyclophosphamide

1.9. Status of GDEPT and other gene therapies for malignant gliomas

Similarly to immunotherapies, GDEPT approaches may not necessarily require targeting of the whole tumor cell population due to the bystander effect; this is thought to be the key of their relative success compared to other methods. GDEPT for gliomas

has reached the clinical phase in some settings. In single patients, disappearance of tumor tissue was detected on autopsy after HSV-TK/GCV therapy for recurrent glioblastoma[170]. The largest anticancer gene therapy trial to date, a phase III randomized multicenter study was conducted against glioblastoma multiforme in GDEPT setting[171]. In this trial, neither disease progression nor overall survival was significantly different between the treatment arms (surgery + radiation with or without infiltration of the tumor site with the virus vector, n=124 per group, 1-year follow-up). Partial responses (including increased survival) were reported only from small cohorts of this study. In this, and many other trials[170] packaging cell lines were implanted into the tumor cavity releasing replication incompetent retroviruses. These retroviruses could only infect actively dividing cells – practically only tumor cells in the brain. The transduction rate after such treatment was reported to be very low: less than 0.1%[172]. In contrast to this trial with retroviruses, the use of an adenovirus vector has recently been reported to be impressively effective in the same adjuvant setting: local gene HSV-TK-gene delivery and ganciclovir treatment has led to improved median survival in treated patients (303 enrolled patients, 37.7 vs. 62.4 weeks)[173]. Serious adverse effects were not reported from any of the trials. The inflammatory responses found after therapy at the treatment site was not accompanied by adverse effects[170].

Various other gene therapies are under development for malignant gliomas[170]. The recent use of DNA microarray technology helps to discover more and more genetic changes specific for gliomas supporting specific gene therapies. However, such treatments (replacement/inhibition of a particular gene) would require transfection of whole tumors, and thus are very much dependent on effective vector systems. The replacement of tumor suppressor genes such as p16, retinoblastoma, VHL, DCC, MMCA-1/PTEN[170] or the introduction of pro-apoptotic genes (Fas-L, FADD, caspase-8)[174] is still in preclinical phase and the similar is true for approaches inhibiting genes (i.e. mutated EGFR, PDGF). Only the use of a p53-expressing adenovirus vector progressed to clinical phase I for glioblastomas but did not show clinically measurable benefit[3]. The main problem, the inability to effectively transduce whole solid tumor cell populations is still unsolved. Replication competent or selectively replication competent oncolytic viruses such as the “classical” E1B deleted “ONYX-15” adenovirus that can replicate only in p53 deficient cells may improve results. In terms of gliomas, two

selectively replication competent herpes-simplex vectors has been tried in clinical setting. The "G207"[175] and "NV1020"[3] are mutated so that they have attenuated neurovirulence and cannot replicate in nondividing cells[175]. Their lytic portion of the life cycle directly kills cells and the thymidine kinase that is expressed from the viral genes (an insertion in NV1020) sensitizes cells to ganciclovir. Thus, they provide a combination of oncolytic and GDEPT systems. Clinical trials using these vectors include a phase I trial of G207 and NV1020 for treatment of malignant glioma[3, 175]. Gliomas are characterized by strong immunosuppressive intratumoral milieu, partly attributable to the production of TGF-β-2 by the tumors. To revert this property of gliomas, immuno-gene therapy has also recently been tried with TGF-β-2 antisense oligonucleotides in phase I-II trials[170]. Vaccination with autologous tumor cells transfected with cytokines also reached clinical phase I in some cases (IL-4, IFN-γ)[170].

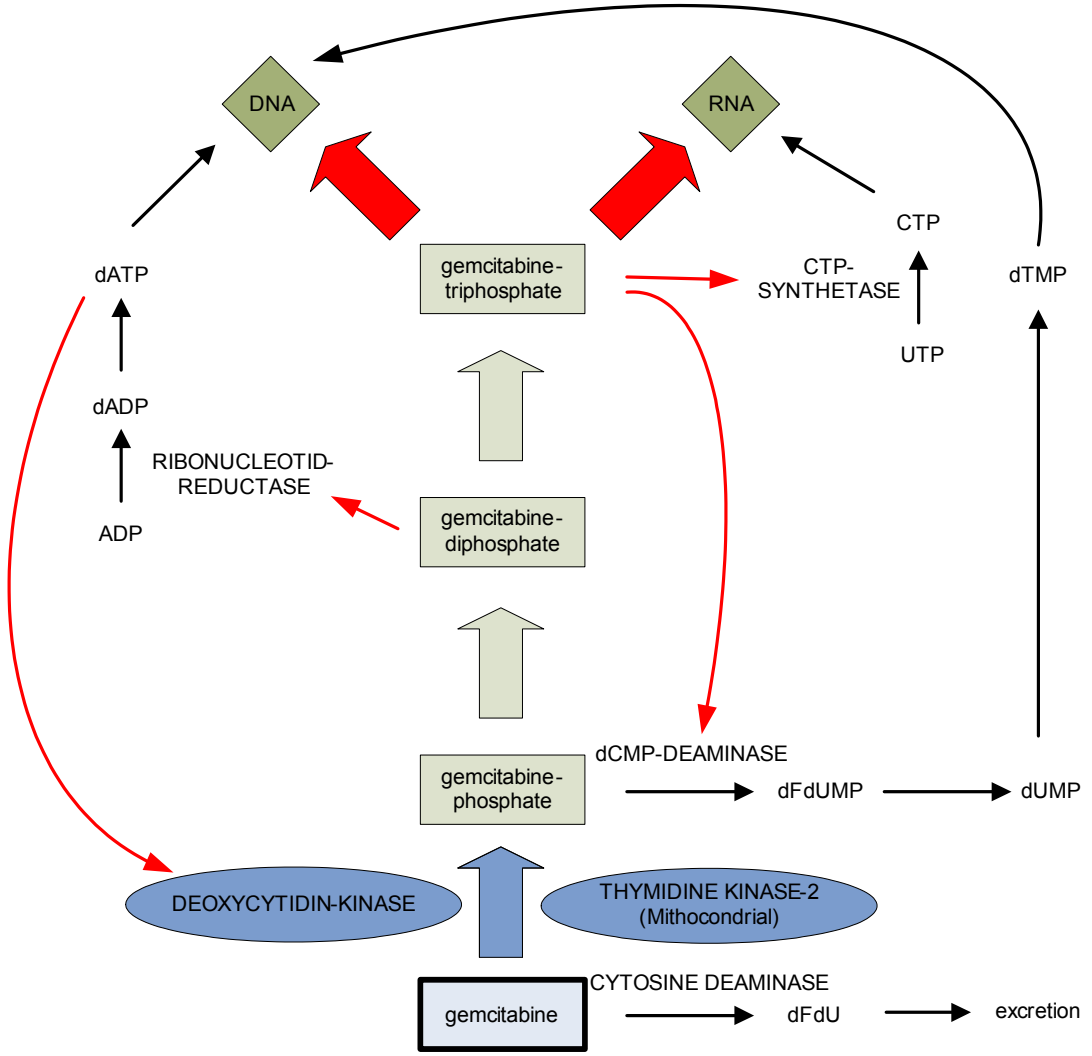
1.10. Gemcitabine – biochemistry and clinical utility

Gemcitabine (2',2'-difluoro-2'-deoxycytidine) is a pyrimidine analogue of deoxycytidine. The molecule was initially synthesized as a potential antiviral drug, later it became a very potent anticancer chemotherapeutic agent[176].

In cells, gemcitabine is mainly phosphorylated by deoxycytidine kinase (dCK) to yield the active metabolite gemcitabine monophosphate and then by other aspecific cellular kinases to give gemcitabine diphosphate (dFdCDP) and gemcitabine triphosphate (dFdCTP)[176]. The first phosphorylation reaction constitutes the rate-limiting step in the activation. dFdCDP reduces the deoxynucleotide pool and thus interferes with DNA synthesis by inhibiting ribonucleotide reductase. The decrease in the deoxynucleotide pool contributes to the more rapid phosphorylation of gemcitabine and decreases the metabolic clearance of gemcitabine nucleotides by deoxycytidine monophosphate deaminase[177]. The triphosphate form (dFdCTP) becomes incorporated into DNA and halts DNA synthesis, or interferes with RNA synthesis and promotes apoptosis[178]. The active metabolites can also inhibit dCMP deaminase and CTP synthetase (Figure 3). Thymidine kinase-2 (TK2) is a mitochondrial enzyme with key role in mitochondrial DNA anabolism[179]. TK2 can also phosphorylate gemcitabine – the phosphorylated forms can not pass the mitochondrial membrane and may cause errors in the mitochondrial DNA. The role of TK2 in the cytotoxicity of gemcitabine is not completely understood. It has been suggested, that in certain cell lines cell death after

gemcitabine treatment would rather be a result of mitochondria-related apoptosis caused by multiple errors in mitochondrial DNA than the disturbance of genomic DNA metabolism[180]. If not phosphorylated, gemcitabine may be deaminated by cytosine deaminase and the resulting dFdU may be excreted from the cell.

Figure 3. Interactions of gemcitabine with nucleotide metabolism. Red arrows: inhibiting effect.



Gemcitabine has a wide range of antitumoral activity against solid tumors[181] and became target of many anticancer chemotherapy protocols (Table 3) and clinical trials targeting bladder cancer, pancreatic cancer, non-small cell lung cancer, breast cancer, head-and-neck cancers and glioblastomas[182-184]. In addition, gemcitabine has been shown *in vitro*[185-190], in animal models[191-193], and also in clinical studies[194] to be a potent radiation sensitizer. Importantly, gemcitabine was proved to be effective against many

tumors that are treated traditionally (partly) by radiotherapy (pancreatic cancer, head and neck cancer, lung carcinomas), placing their combination in the middle of interest. As such another recent example, gemcitabine has been proposed in combination with radiotherapy for the treatment of malignant gliomas and various brain metastases in clinical trials[184, 196-199]. The drug has been shown to pass the blood-brain barrier in GBM patients[184] in effective dose to achieve radiosensitization and is currently progressing through phase I-II clinical testing.

Table 3. Current indications of gemcitabine therapy (www.gemzar.com)

Non-small cell lung cancer	In combination with cisplatin for the first-line treatment of locally advanced (IIIA or IIIB) or metastatic cancer for whom surgery is not possible
Pancreatic cancer	Single agent in the first-line treatment of locally advanced or metastatic cancer (with or without irrradiation). Option to those previously being treated with 5-FU.
Breast cancer	In combination with paclitaxel for metastatic breast cancer after anthracycline therapy for those unable to continue anthracycline.
Ovarian cancer	In combination with carboplatin for recurrent cancer after platinum-based therapy.

1.11. Radiosensitizers

The efficacy of radiotherapy as a single modality treatment in patients with locally or regionally advanced cancers is limited by a number of factors: (I) tumor cells may possess radioresistance associated with hypoxia within the tumor mass or (II) efficient DNA repair mechanisms, or (III) be able to recover from injury in the period between radiation fractions; and (IV) occult tumor cells may exist outside the irradiated field and thus lead to distant recurrence[192]. To overcome these obstacles and/or to increase the effectivity of radiotherapy, radiosensitizing therapies have been developed[200, 201]. There is no universal mechanism that defines the interaction of drugs with radiation leading to sensitization. The classical and emerging agents frequently sensitize through diverse mechanisms involving modification of DNA damage, interference with DNA repair processes, cytokinetic cooperation caused by cell-cycle redistribution, inhibition of proliferation, and enhancement of apoptosis. Manipulation of some basic physical properties of the tissues such as hyperthermia may also enhance radiosensitivity[202]. Radiosensitizing drugs include classic chemotherapeutic agents (5-FU, cisplatin, gemcitabine, bleomycin) and promising newer molecular agents that primarily function via inhibition of angiogenesis, modification of hypoxia (pentoxifylline, nimorazole), interference with signal transduction pathways (EGFR inhibitors) or cell-cycle control mechanisms (checkpoint kinase 1 inhibitors)[195, 203].

Gene therapy has also been applied (mainly in preclinical setting) in order to modulate/enhance tumor radiosensitivity[163]. GDEPT strategies producing radiosensitizing drugs (for example 5-FU) may not surprisingly lead to radiosensitization[193]; but an acyclovir-HSV-TK GDEPT system alone has also been reported to synergize with radiation[204]. Similar results were obtained from double-suicide GDEPT systems, where transfecting viral vectors coded for two prodrug-converting enzymes at the same time[193]. Synergism with radiation may also accompany immunotherapies with cytokine-encoding vectors, or cytokine-secreting cancer cell vaccines[190, 193]. Although not classic radiosensitizers, the use of expression vectors regulated by radiation-induced promoters such as EGR1 may also enhance the effect of radiotherapies (for references see also 1.2.3).

Despite of preclinical results, there is generally limited information about the clinical effectiveness of radiosensitizers. The lack of data is partly attributable to the fact that clinical studies aiming the measurement of radiosensitizing effect require special design. The interaction between drugs and radiation is usually dose and time dependent and rarely specific to tumor cells. Platinum analogs like cisplatin/carboplatin (non-small cell lung cancer, head and neck cancer, gynelogic malignancies) or oxaliplatin (rectal cancer) with radiation are current targets of phase II-III clinical studies[203, 205]. 5-FU is already part of the general treatment of pancreatic and colorectal cancers[205]. A nitroimidazole (nimorazole) became the part of the standard treatment schedule against head and neck cancer after a meta-analysis showing the benefit of this chemical oxygen-mimetic radiosensitizer in Denmark[206, 195]. Tirapazamine, a bioreductive drug selectively killing hypoxic cells has also been shown to be benefical in combination with cisplatin and radiation against head and neck cancer[207].

There is special interest toward radiosensitizers with strong intrinsic antitumoral effect. Gemcitabine, a relatively new nucleoside analog is a drug with such properties.

1.12. Irradiation and gemcitabine

1.12.1. The radiosensitizing properties of gemcitabine

The interference with DNA synthesis, inhibition of ribonucleotide reductase and the reduction of intracellular dATP pools caused by gemcitabine suggested long ago that the drug would have radiosensitizing effect[189, 190]. Later studies validated *in vitro* that gemcitabine enhanced the radiation response up to a dose modifying factor (DMF) of

1.2-2.4 depending on cell line, proliferation status, drug concentration, incubation time and administration schedule[185-190, 195]. Gemcitabine was able to sensitize cells to radiation below its toxic concentration (even in a single dose) both *in vitro* and *in vivo*[193]. The radioenhancement rate was found to be independent of intrinsic cellular radiosensitivity *in vitro*[188], and increased with gemcitabine concentration[188, 210].

There is evidence, that for the maximal radiosensitizing effect, gemcitabine should be given 24h before radiation therapy[192, 208], but the effect may last 48-72 hours[205, 209]. *In vivo* studies confirmed that the administration schedule is critical: DMFs ranged from 1.1 to 3.3 in various studies[195]. Studies on normal tissues showed significant acute drug toxicity on early reacting tissues, while late toxicity was not observed[211-213]. The phosphorylated intracellular metabolites of gemcitabine may remain stabile in the cells for days – a property different from other nucleoside analogs such as 5-FU – making the administration of the drug easier[205]. The majority of trials could not measure significant survival benefit from fixed-dose-rate infusions[214]. In contrary, in a very recent mouse experiment the fixed-dose-rate schedule was found to be superior in terms of radiosensitizing versus bolus administration; presumably due to the production of more intracellular metabolites[215]. For clinical use, a weekly or twice weekly gemcitabine regimen has been proposed before radiation to achieve the best therapeutic index[195]. Unfortunately, gemcitabine chemo-irradiation in the clinic is often accompanied by unacceptable side effects (fatigue, anorexia, vomiting), and the therapeutic dose of gemcitabine often can not be given together with radiation (although radiosensitization begins at lower concentrations). The severity of side effects is very much dependent on the site of tumor (lower doses were tolerated with radiation in head and neck tumors than in pancreatic cancer[193]), the way of irradiation (3-demensional conformal radiotherapy is beneficial[216]), and on individual factors. The combination already seems to be the best treatment in many cases[195, 217], but in the absence of large phase III trials the best schedule for the concomitant use of gemcitabine and irradiation could not yet been identified. It would be beneficial to be able to enhance the toxic/radiosensitizing effect of gemcitabine - preferably specifically in cancers.

1.12.2. Mechanism of action

Although not completely understood, several mechanisms are thought to underlie the radiosensitizing effect of gemcitabine. It is accepted, that dATP depletion and

redistribution of the cells into the early S phase are the main events[216]. These actions are also involved in the intrinsic toxicity of gemcitabine. The dependence on dATP depletion is based on observations that cells transduced with the active subunit of ribonucleotide reductase become relatively resistant to gemcitabine-mediated radiosensitization[205]. Furthermore, radiosensitization did not necessarily correlate with intracellular concentrations of dFdCTP[218], suggesting that dATP pool depletion and not (only) incorporation of dFdCMP into DNA underlies radiosensitization. Gemcitabine can redistribute treated cells to early S phase. Although the S phase is not reported to be the most radiosensitive phase of the cell cycle, this action was found to be in direct correlation with the drugs radiosensitizing potential[219]. However, for strongest radiosensitizing effect, dATP pool depletion and S phase redistribution is needed simultaneously, as was shown after rapid treatment of cells with high concentration of gemcitabine leading to near total depletion of dATP[220].

The mechanism and exact role of S phase redistribution is largely unknown. One hypothesis was the interference with the Chk1/2-Cdc25A (checkpoint kinase, cell division cycle 25 homolog A) pathway. It has been described, that gemcitabine treatment results in the accumulation of the phosphorylated forms of the cell cycle regulators Chk1 and Chk2 and subsequent degradation of Cdc25A[221, 216]. Chk1 inhibition abrogated the G2-M checkpoint, and permitted gemcitabine-treated cells with arrested DNA synthesis to enter mitosis with premature DNA content. Thus, it has been hypothesized, that gemcitabine mediated Chk1 upregulation functions by preventing cells with stalled replication from prematurely entering mitosis[221]. Others suggested, that sensitization to gemcitabine by Chk1 inhibition is mediated by inhibition of the DNA damage response[216]. Chk1 also seems to play a role in the radiosensitization by gemcitabine: a Chk1 inhibitor could enhance radiation sensitivity *in vitro*[216].

Based on the inhibition of deoxynucleotide triphosphate synthesis by gemcitabine, it seemed likely that gemcitabine would have an effect on the repair of radiation-induced DNA damage, which may contribute in part to its radiosensitizing activity. Surprisingly, initial work showed that gemcitabine affects neither radiation induced bulk DNA damage nor repair under radiosensitizing conditions[205, 216, 222]. It is known, that DNA damage induced by ionizing radiation is primarily repaired by the nonhomologous end joining pathway (NHEJ) and, to a lesser extent, through base excision repair and

homologous recombination repair (HRR)[223]. Latter studies showed, that although the NHEJ pathway does not seem to play role in gemcitabine-mediated radiosensitization[224], HRR may be required: the radiation sensitivity of cells deficient in HRR was relatively unaffected by gemcitabine, while cells that were HRR competent, but unable to carry out base excision repair, became radiosensitized[225]. The fact, that HRR may act as the major repair mechanism for cells in S and G2[205] underlies the importance of S phase arrest by gemcitabine. The mismatch repair pathway may also affect gemcitabine mediated radiosensitization: mismatch repair–deficient cells display enhanced radiosensitization after exposure to gemcitabine[218], [226]. It is hypothesized, that mismatch repair may antagonize the radiosensitizing effects of gemcitabine by facilitating the repair of gemcitabine-induced errors in DNA caused by nucleotide pool imbalance.

It has been hypothesized, that the increase in residual DNA damage after radiation in gemcitabine-treated cells leads to radiation-induced apoptosis - although toxicity caused by radiation response itself may not necessarily be a consequence of apoptotic cell death[227]. Data suggest, that the extent of apoptosis produced by the combination of gemcitabine and radiation may correlate with radiosensitization in some cases[193], but its role in overall is cell type and cell cycle dependent. P53 level and/or function did not seem to have a direct effect on gemcitabine radiosensitization[228].

Taken together, it is probable that gemcitabine may radiosensitize cells that progress into S phase by depleting dATP pools, leading to misincorporation and misrepair of incorrect bases after radiation. These lesions produce both apoptotic and non-apoptotic cell death.

1.12.3. Experience in modifying gemcitabine-induced radiosensitization

Attempts to enhance the radiosensitizing effect of gemcitabine by combination with other radiosensitizing agents such as cisplatin, oxaliplatin, 5-FU, irinotecan or capecitabine were clinically not effective (1.1). Other potential targets include cell cycle regulators (Chk1 blockers)[216], and modifiers of the EGFR-signaling pathway. EGFR is indirectly involved in DNA repair[229]; its phosphorylation is enhanced by gemcitabine, acting against radiosensitization[230]. Both approaches are still under development[216, 230].

A theoretically very promising but practically not yet studied approach would be the modulation of the intrinsic (intratumoral) dCK activity to enhance intracellular (intratumoral) activation of gemcitabine.

1.13. Deoxycytidine kinase

The continuous supply of deoxynucleotides for DNA synthesis and repair is provided by *de novo* synthesis or by the deoxynucleotide salvage pathway *in vivo*. Deoxycytidine kinase catalyzes the phosphorylation of 2'-deoxycytidine to its monophosphate form, which is the rate-limiting reaction of the salvage pathway. The enzyme is located both in the nucleus (mainly for DNA synthesis) and in the cytosol in the vicinity of cellular membranes (salvage pathway for phospholipid synthesis)[231]. Transfection of cells with dCK coding vectors leads to disproportionate accumulation of nuclear dCK compared to the cytoplasmic copmpartment by an unknown retention mechanism[231]. dCK has wide substrate specificity: it can also phosphorylate purine nucleosides[231, 232]. The amount of dCK is constant throughout the cell cycle; its basal activity is cell type dependent[231-233] – highest expression is measured in lymphocytes that lack significant *de novo* DNA synthesis. The enzyme was found to be upregulated in several malignant tumors[233].

dCK is responsible for the activation of a number of clinically important anticancer and antiviral drugs, such as 2-chloro-2'-deoxyadenosine, 1-β-D-arabinofuranosylcytosine (araC) and gemcitabine. Impaired dCK expression or activity in cells usually leads to resistance to these drugs[231, 234]. Close correlation was observed between dCK activity and gemcitabine sensitivity in various cancer cell lines and xenografts[235].

The role of dCK activity in the radiosensitizing effect of gemcitabine has not been investigated in detail. Since the radiosensitizing effect of gemcitabine was found to be in relation with the drugs concentration[210, 236], it seems to be very likely that the activity of the rate limiting dCK enzyme itself is an important factor. Some experimental data support this assumption: a gemcitabine resistant cell line lacking dCK was reported to require pretreatment by extremely high concentrations of gemcitabine to become radiosensitized[237]. The suggested good correlation between basal dCK activity and gemcitabine radiosensitization was confirmed in mouse and human cell lines[211].

In our experiment, we chose to apply a dCK gene-directed enzyme pro-drug therapy approach aiming to modify the radiosensitizing effect of gemcitabine treatment. Before this study, dCK-gemcitabine GDEPT has only been reported once in the literature[238]. The use of radiosensitizing drugs in GDEPT such as gemcitabine would possibly help to overcome tumor radioresistance, which may have implication in the treatment of tumors responding weakly to conventional radiotherapies.

1.14. Aims of this study

Up to now, dendritic cell based immunotherapy of pancreatic cancer has been poorly investigated. The immunotherapeutic effect of DCs expanded with FL has not been tested in pancreatic cancer models at all. Very little is known about the effect of non-systemic FL gene-delivery. We aimed

1. to reveal the unknown FL cDNA sequence in rat and create an FL producing non-viral therapeutic vector,
2. to establish a liposome - based gene delivery system for local transfection of DSL6A pancreatic tumors in rats, and
3. to investigate the effect of intratumoral application of rFL cDNA in DSL6A pancreatic cancer, to monitor systemic/local immunological changes, tumor proliferation rate and tumor vasculature.

The expression of transferrin receptor on pancreatic cancers has not yet been investigated in detail. Overexpressed proteins may have relevance in tumor diagnosis or targeting. We aimed

4. to investigate the expression of transferrin receptor on human pancreatic cancer cell cultures and
5. on various human pancreatic tumor samples.

Tumor radioresistance is a severe impediment in the success of radiotherapy. Malignant gliomas are among tumors considered relatively radioresistant[239, 240]. To overcome tumor radioresistance, gene-directed enzyme pro-drug therapies with radiosensitizing chemotherapeutic agents may be helpful. The aim of our next study was

6. to describe the relation between basal dCK activity and gemcitabine sensitivity in human (U373) and rat glioma (C3) cell line,
7. to measure the capability of a dCK-coding adenovirus-vector to elevate the intracellular dCK activity of the selected tumor cell lines,
8. to describe the effect of chemo-irradiation on the transduced cells *in vitro*, and
9. to investigate the effect of increased dCK expression on chemo-irradiation *in vivo.*

2. Materials and methods

2.1. Materials

Table 4. Reagents

3-(N-morpholino) propanesulphonic acid (MOPS)	Carl Roth GmbH (Karlsruhe, Germany)
Agarose "Electrophoresis Grade"	Gibco BRL (Gaithersburg MD, USA)
Ammonium chloride (NH_4Cl)	Sigma-Aldrich (St. Louis, MO, USA)
Ammonium hydroxide (NH_4OH)	Sigma-Aldrich (St. Louis, MO, USA)
Ammonium formiate	Sigma-Aldrich (St. Louis, MO, USA)
Ampicillin 100 mg/ml	Sigma-Aldrich (St. Louis, MO, USA)
Bacto-Agar	BD Diagnostic Systems (Sparks, MD, USA)
Bind Silan	Amersham Pharm Biotech(Piscataway NJ,USA)
Bovine Serum Albumin	Sigma-Aldrich (St. Louis, MO, USA)
Copper sulfate pentahydrate ($CuSO_4 \times 5H_2O$)	Sigma-Aldrich (St. Louis, MO, USA)
DDT (1,1,1-Trichloro-2,2-bis(4-chlorophenyl) ethane	Sigma-Aldrich (St. Louis, MO, USA)
DEAE (diethylaminoethanol) discs	Whatman (Dassel, Germany)
Dextrose	Sigma-Aldrich (St. Louis, MO, USA)
Deoxy[5-3H]cytidine	Amersham Biosciences (Little Chalfont, UK)
Diethylpyrocarbonat (DEPC)	Sigma-Aldrich (St. Louis, MO, USA)
Dimethylsulfoxid (DMSO)	Merck (Whitehouse Station NJ, USA)
EDTA-free protein inhibitor cocktail	Roche (Mannheim, Germany)
Ethanol p.a.	Merck (Whitehouse Station NJ, USA)
Ethidium bromide tablets (1 mg/ml solution)	BIORAD (Hercules CA, USA)
EGTA	Sigma-Aldrich (St. Louis MO, USA)
Ethylenediaminetetraacetic acid (EDTA)	Merck (Whitehouse Station NJ, USA)
Fetal Calf Serum (FCS)	CCPro (Neustadt, Germany)
Folin & Ciocalteu's phenol reagent	Sigma-Aldrich (St. Louis, MO, USA)
Gemcitabine (Gemzar)	Lilly (Fegersheim, France)
Glacial Acetic Acid	Serva (Heidelberg, Germany)
Glucose	Merck (Whitehouse Station NJ, USA)
Glutaraldehyde	Serva (Heidelberg, Germany)
Glycerin 86%	Carl Roth GmbH (Karlsruhe, Germany)
Goat Serum	DAKO (Hamburg, Germany)
Heparin-Sodium 25000 IU/5ml	B.Braun Melsungen AG(Melsungen, Germany)
HEPES	Sigma-Aldrich (St. Louis, MO, USA)
Hydrochloric acid (HCl)	Carl Roth GmbH (Karlsruhe, Germany)
Hydromount	National Diagnostics (Atlanta, GA, USA)
Iscove's Modified Dulbecco's Medium (IMDM) (1X)500 ml	Gibco BRL (Gaithersburg MD, USA)
Isopropanol	Serva (Heidelberg, Germany)
Isopropyl-beta-D-1-thiogalactopyranosid (IPTG)	Sigma-Aldrich (St. Louis, MO, USA)
Kanamycin 20 mg/ml	Sigma-Aldrich (St. Louis, MO, USA)
Levamisole	DAKO (Hamburg, Germany)
Magnesium chloride ($MgCl_2$)	Gibco BRL (Gaithersburg MD, USA)
Magnesium sulphate ($MgSO_4$)	Merck (Whitehouse Station NJ, USA)
Mayer's acid hemalum	Fluka (Steinheim, Germany)
N, N, N', N' - Tetramethylethylendiamin (TEMED)	Serva (Heidelberg, Germany)
NP-40	Sigma-Aldrich (St. Louis, MO, USA)
Paraplast Tissue Embedding Medium	Tyco Healthcare (Mansfield, MA , USA)
Pefablock	Roche (Mannheim, Germany)

Penicillin/Streptomycin solution	Life Technologies (Karlsruhe, Germany)
Perchloric acid ($HClO_4$, PCA)	Sigma-Aldrich (St. Louis, MO, USA)
Phosphate Buffered Saline (PBS)	Gibco BRL (Gaithersburg MD, USA)
Folin - Ciocalteau reagent: Phenol reagent - 2 N	Sigma-Aldrich (St. Louis, MO, USA)
Potassium bicarbonate ($KHCO_3$)	Sigma-Aldrich (St. Louis, MO, USA)
Potassium chloride (KCl)	Merck (Whitehouse Station NJ, USA)
Potassium ferricyanide, potassium ferrocyanide	Sigma-Aldrich (St. Louis, MO, USA)
Potassium hydrogene phosphate (KH_2PO_4)	Merck (Whitehouse Station NJ, USA)
Potassium tartatrate	Sigma-Aldrich (St. Louis, MO, USA)
RPMI 1640 Medium	Gibco BRL (Gaithersburg MD, USA)
Saccharose	Merck (Whitehouse Station NJ, USA)
Silane - 2% TESPA in acetone	Sigma-Aldrich (St. Louis, MO, USA)
Sodium acetate (Na+CH3COO-)	Merck (Whitehouse Station NJ, USA)
Sodium carbonate (Na_2CO_3)	Sigma-Aldrich (St. Louis, MO, USA)
Sodium chloride (NaCl)	Merck (Whitehouse Station NJ, USA)
Sodium deoxycholate, Sodium dodecyl sulphate	Sigma-Aldrich (St. Louis, MO, USA)
Sodium hydroxide (NaOH)	Carl Roth GmbH (Karlsruhe, Germany)
Sodium iodide	Sigma-Aldrich (St. Louis, MO, USA)
Sodium phosphate monohydrate ($NaH_2PO_4\,H_2O$)	Merck (Whitehouse Station NJ, USA)
Sodium phosphate dibasic (Na_2HPO_4)	Merck (Whitehouse Station NJ, USA)
Sodiumdodecylsulphate (SDS)	Carl Roth GmbH (Karlsruhe, Germany)
TRIS buffered saline	DAKO (Hamburg, Germany)
TRIS-(hydroximethyl)aminoethan	Carl Roth GmbH (Karlsruhe, Germany)
Trypsin	Sigma-Aldrich (St. Louis, MO, USA)
Trypan Blue	Sigma-Aldrich (St. Louis, MO, USA)
Trypton	BD Diagnostic Systems (Sparks MD, USA)
Urea	Gibco BRL (Gaithersburg MD, USA)
X-Gal, Xylenes, Reagent Grade, Xylencyanol	Sigma-Aldrich (St. Louis, MO, USA)
Yeast Extract	BD Diagnostic Systems (Sparks, MD, USA)

Table 5. Commercial reagents and kits

100 base pair ladder	Pharmacia (New York, NY, USA)
1 kb Ladder	MBI Fermentas (St. Leon-Rot, Germany)
BamHI Restriction enzyme + buffers	Roche (Mannheim, Germany)
Big-Dye terminator DNA-sequencing Kit	Perkin Elmer Life Sci. (Boston, MA, USA)
Capillary electropohoresis consumables (ABI PRISM 310)	Applied Biosystems (Foster City, CA, USA)
DAKO LSAB@2 and New Fuchsin Kit	DAKO (Hamburg, Germany)
Eco RI Rrestriction Enzyme	Roche (Mannheim, Germany)
GlassMAX RNA Microisolation Spin Cartridge System	Life Technologies, Inc. (Karlsruhe, Germany)
Qiaquick Gel Extraction Kit, EndoFree Plasmid Maxi Kit	Qiagen (Hilden, Germany)
In Vivo GeneSHUTTLE Transfection System	Quantum Biotechnologies (Montreal, Canada)
pcDNA3.1 Plasmid	Invitrogen (Karlsbad, CA, USA)
Quantikine M-Mouse Flt-3 Ligand Immunoassay	R&D Systems (Minneapolis, MN, USA)
Quantikine Rat TNF-α and IFN-γ Immunoassay	R&D Systems (Minneapolis, MN, USA)
LacZ Reporter Assay (for *in vitro* use)	InvivoGen (San Diego, CA, USA)
LSAB 2-Kit	Dako (Hamburg, Germany)
Quantum Prep Plasmid Miniprep Kit	Biorad (Hercules, CA, USA)
Superscript II Rnase H-Reverse Transcriptase	Life Technologies, Inc. (Karlsruhe,Germany)
TA Cloning Kit	Invitrogen (Karlsbad, CA, USA)
T4 DNA ligase + buffer	Roche (Mannheim, Germany)
Taq DNA polymerase	Life Technologies, Inc. (Karlsruhe, Germany)
XbaI restriction enzyme + buffers	Roche (Mannheim, Germany)

Table 6. Buffers and solutions

1x TE (500 ml)

5.0 ml 1M Tris-HCl + 2.0 ml 250 mM EDTA ad 500 ml aqua bidest at pH 7.2

2X HEBS (HEPES buffered saline, 500 ml)

8.0g NaCl + 0.37g KCl + 0.099 g Na2HPO4 + 1g Dextrose + 5g HEPES at pH to 7.2

6x DNA loading buffer

0.25% (w/v) Bromophenol blue + 0.25% (w/v) Xylencyanol + 15% (v/v) Ficoll Typ 400

10% Ammonium persulfate

10% (w/v) APC in aqua bidest

10x TBE (1000 ml)

108 g TRIS + 55g boric acid + 40 ml 0.5 M EDTA ad 1000 ml aqua bidest.

50x TAE (1000 ml)

242 g TRIS + 57.1 ml glacial acetic acid + 100 ml 0.5 M EDTAQ ad 1000 ml aqua bidest

20% Acrylamide/bisacrylamide stock solution

100g Acrylamide/Bisacrylamide ad 500 ml aqua bidest.

20x MOPS

83.6 g MOPS + 8.2 g sodium acetate + 7.4 g EDTA ad 1000 ml aqua bidest. at pH to 7.0

Acrylamide/bisacrylamide - working solution (500 ml)

155 ml Acrylamide/Bisacrylamide stock solution + 210 g urea + 60 ml 10xTBE ad 500 ml aqua bidest.

Blocking buffer for immunohistochemistry

2 ml goat serum ad 10 ml Immunowash solution

DEPC water

1 ml DEPC ad 1000 ml aqua bidest.

Dulbecco's Modified Eagle Medium
Iscove's standard cell culture medium

550 ml IMDM +55 ml FCS + 5.5 ml Penicillin (10000 U/ml)/Streptomycin(10 mg/ml)

Erythrocyte lysis buffer (1000 ml)

8.29 g NH_4Cl + 1 g KHCO3 + 0.0371 EDTA ad 1000 ml aqua bidest.

IPTG solution 40 mg/ml

Immunowash solution (for immunohistochemistry)

0.05 M TRIS buffered saline in PBS

LB-Medium (fluid)

1% Trypton + 0.5% yeast erxtract + 1% NaCl in aqua bidest.

LB-Medium (solid)

LB-Medium (fluid) + DIFCO-BACTO Agar in aqua bidest.

LacZ Fixative solution (50 ml, whole mount tissue protocol)

0.4 ml 25% gutaraldehyde + 0.5 ml 0.5 M EDTA + 5ml 1 M $MgCl_2$ ad 50 ml PBS

LacZ Staining buffer (100 ml, whole tissue protocol)

4 ml 25 mg/ml X-gal in DMSO + 0.21 g pot.ferrocyanide + 0.16 g pot.ferricyanide ad 96 ml LacZ Wash buffer

LacZ Wash buffer (500 ml, whole mount tissue protocol)

1 ml 1 M $MgCl_2$ + 5 ml 1% Sodium deoxycholate + 5 ml 2%NP-40 ad 500 ml PBS

Lowry Reagent

'A'100g sodium carbonate (Na_2CO_3) in 1000 ml 0.5 M NaOH

'B'1g copper sulfate pentahydrate ($CuSO_4$ x $5H_2O$) in 100 ml aqua bidest.

'C': 2 g potassium tartatrate in 100 ml aqua bidest.

PBS (1000 ml)

8g NaCl + 0.2 g KCl + 1.44 g Na_2HPO_4 + 0.24 g KH_2PO_4 ad 1000 ml aqua bidest.

PBS/EDTA solution

500 ml PBS + 1g EDTA

RIPA buffer

1% NP-40 + 0.5% sodium deoxycholate + 0.1% SDS + 1mM DDT + 4% EDTA-free protein inhibitor cocktail + 0.5mM Pefablock in PBS

RNA loading buffer (48 ml)

24 ml formamide + 8.56 ml formaldehyde + 2.4 ml 20 x MOPS + 4.8 ml 10% Ficoll 400 + 9.8 mg bromophenol blue + 8.24 ml DEPC water

SOC Medium

2% Tryptone+0.5% yeast extract + 0.4/% glucose + 10mM NaCl + 2.5mM KCl + 5mM $MgCl_2$ + 5mM $MgSO_4$

Trypsin solution

0.115 % Na_2HPO_4 + 0.125% Trypsin + 0.125% EDTA + 0.01% $CaCl_2$ x H_2O + 0.01% $MgSO_4$ + 0.8% NaCl + 0.02% KCl + 0.02% KH_2PO_4

Table 7. Software

Software name	Application	Manufacturer
BLASTN	Sequence analysis	http://www.ncbi.nlm.nih.gov/blast/Blast.cgi
Bio Linx V2.20	plate reader software	Dynatech Laboratories, Inc. (Chantylly, VA, USA)
Expo 32 ADC 1.1B	flow cytometry – data aquisition	Beckman Coulter, Inc. (Miami, FL, USA)
Expo 32 ADC Analysis	analysis of flow cytometric data	Beckman Coulter, Inc. (Miami, FL, USA)
Microsoft Office 2003	word processing	Microsoft Corporation (Redmond, WA, USA)
Photoshop 3.0	digital image analysis	Adobe Systems Incorporated (San Jose, CA, USA)
SPSS (Version 11.5.1.)	statistical analysis	SPSS Inc. (Chicago, IL, USA)

Figure 4. Annealing sites of primers in the rat rFL sequence. The previously unknown 3'-sequence is shown with dots.

1	**A T G A C A G T G C T G G C G C C A G C** c t g g a g c c c a a a t t c c t c c t t g t t g	F
46	c t g t t g c t g c t g c t g c t g a g c c c t t g c c t g c g g g g g a c a c c t g a c	
91	t g t t a c t t c a g c c a c a g t c c c a t c t c c t c c a a c t t c c a c a t g a g g	
136	a t t a g c g a g t t g a c t g a c t a c c t g c t t a a a g a t t a c c c a **G T C A C T**	for2
181	**G T G G C C A T C A A T C T T** c a g g a c g a g a a a c a c t g c a g g g c c t t g t g g	
226	a g c c t c t t c c t g g c c c a t c g c t g g a t a g a g c a a c t g a a g a c t g t g	
271	g c a g g g t c t a a a a t g c a a a a a g c t t c t g g a g g a t g t c a a t a c g g a g	
316	a t a c a t t **T T G T C A C C T C G T G T A C C T T C C** a g c c c c t a c c a g a a t g t	rev
361	c t t c g a t t c g t c **C A G A C C A A C A T C T C C C A C C T** c c t g c a g g a c a c c	for1
406	t g c t c a c a g c t g t t a g c t c t g a a g c c c t g t a t c g g g a a a g c c t g c	
451	c a g a a t t t c t c t c g g t g c c t g g a g g t g c a g t g c c a g c c g g a c t c c	
496	t c c a c c c t g c t t c c c c c a g a g a g t c c t g g a g c c c t a g g a g c c a c g	
541	g a g c t t c c a a a g c	
586	. . . . .	
631	. . . . . **C C C**	R
676	**C T C C C C T C C C A T C C C T A G** - 3'	

Table 8. Antibodies

Host	Antibody (clone)	Catalog Number	Producer
Flow Cytometry - anti-rat antibodies			
Mouse	anti-CD25 (OX39)	559980	BD Pharmingen (Sandiego, CA, USA)
Mouse	anti-CD28 (JJ319)	559982	BD Pharmingen (Sandiego, CA, USA)
Mouse	anti-CD18 (WT.3)	554977	BD Pharmingen (Sandiego, CA, USA)
Mouse	anti-CD11c (WT.1)	554861	BD Pharmingen (Sandiego, CA, USA)
Mouse	anti-CD80 (3H5)	MCA1961	Serotec (Düsseldorf, Germany)
Mouse	anti-CD86 (24F)	MCA1962	Serotec (Düsseldorf, Germany)
Mouse	FITC-conjugated anti-NKR-P1A	555008	BD Pharmingen (Sandiego, CA, USA)
Mouse	FITC-conjugated-anti-CD4 (OX35)	554837	BD Pharmingen (Sandiego, CA, USA)
Mouse	FITC-conjugated-anti-CD8a (OX8)	554856	BD Pharmingen (Sandiego, CA, USA)
Mouse	FITC-conjugated-anti-CD62L (HRL1)	554963	BD Pharmingen (Sandiego, CA, USA)
Mouse	FITC-conjugated-anti-CD40 (HM40-3)	553723	BD Pharmingen (Sandiego, CA, USA)
Goat	PE-conjugated-anti-mouse	550589	BD Pharmingen (Sandiego, CA, USA)
Control	PE-conjugated mouse IgG_1-κ	554680	BD Pharmingen (Sandiego, CA, USA)
Control	FITC-conjugated mouse IgG_1-κ	550616	BD Pharmingen (Sandiego, CA, USA)
Flow Cytometry - anti-human antibody			
Mouse	FITC-labeled anti-TRFC (DF1513)	MCA1148F	Serotec (Düsseldorf, Germany)
Immunhistochemistry - anti-rat antibodies			
Mouse	anti-CD4 (OX35)	22022D	BD Pharmingen (Sandiego, CA, USA)
Mouse	anti-CD8 (OX-8)	22072D	BD Pharmingen (Sandiego, CA, USA)
Mouse	anti-CD161a (10/78)	555006	BD Pharmingen (Sandiego, CA, USA)
Mouse	anti-Reca-1 (HIS52)	MCA970	Serotec (Düsseldorf, Germany)
Mouse	anti-PCNA (PC10)	M0879	Dako (Hamburg, Germany)
Control	mouse IgG_1	X0931	Dako (Hamburg, Germany)
Immunhistochemistry - anti-human antibodies			
Mouse	anti-cytokeratin (clone AE1/AE3)	M3515	Dako (Hamburg, Germany)
Mouse	anti-TFRC (clone clone Ber-T9)	M0734	Dako (Hamburg, Germany)
Rabbit	anti-transferrin, polyclonal	A0061	Dako (Hamburg, Germany)
Control	Rabbit IgG, polyclonal	A0107	Dako (Hamburg, Germany)

Table 9. Oligonucleotides and their application in FL cDNA cloning

Name	Sequence (5'→3')	Application
oligo d(T)	TTTTTTTTTTTTTTTT	3` RACE - RT
adaptor	GACTCGAGTCGACATCG	3` RACE - PCR
Frohman	TTTTTTTTTTTTTTTTTGACTCGAGTCGACATCGA	3` RACE - RT
for1	CAGACCAACATCTCCCACCT	amplification of 3` end
Ufor1	CGACGTTGTAAAACGACGGCCAG	sequencing (pCR2.1)
BamHI-F	*BamHI*-ATGACAGTGCTGGCGCCAGC	cohesive-end cloning
R-XbaI	CCCTCCCCTCCCATCCCTAG-*XbaI*	cohesive-end cloning
BamHI	GGATCC	Restriction site
XbaI	TCTAGA	Restriction site
for2	GTCACTGTGGCCATCAATCTT	screening for insert
rev	TTGTCACCTCGTGTACCTTCC	screening for insert
Ufor2 (T7 promoter)	TAATACGACTCACTATAGGG	sequencing (pcDNA3.1)
Urev2 (BGH rev. pr. site)	CCTCGACTGTGCCTTCTA	sequencing (pcDNA3.1)

Table 10. Equipment

Name	Manufacturer
Molecular biology	
Hitachi U2000 - Spectrophotometer	Hitachi (Tokyo, Japan)
ABI Prism 310 Genetic Analyser	Applied Biosystems (Foster City, CA, USA)
Camera Polaroid MP4+ land camera (+film)	Polaroid (Offenbach, Germany)
Electrophoresis chamber	PerkinElmer Life And Anal. Sci., iIc. (Boston, MA, USA)
OmniGene Thermocycler	Hybaid Ltd (Ashford, United Kingdom)
Photometer UV-Visible Spectrophotometer	Pharmacia (New York, NY, USA)
Pipetboy	Tecnomara (Wallisellen, Switzerland)
Power supply Power Pac 300	Bio-Rad Laboratories (Hercules, CA, USA)
Speed Vac 100 - Vacuum concentrator	Savant (Farmingdale, NA, USA)
Thermomixer 5436	Eppendorf AG (Hamburg, Germany)
Vortex - Genie 2 (G-560-E)	Scientific Industries, Inc. (Bohemia, NY, USA)
Vortex MS1 Minishaker	IKA Works, Inc. (Wilmington, NC, USA)
Water bath	Julabo, Seelbach, Germany
Cell culture	
Binder CB - CO_2 incubator for cell culture	Wolf Laboratories Ltd (Pocklington, UK)
Sterilgard Biological Safety Cabinet (Class II)	The Baker Company (Maine, USA)
Neubauer haemocytometer	Sigma-Aldrich (St. Louis, MO, USA)
Histology	
CF 20/4DX Digital Camera	Kappa GmbH (Gleichen, Germany)
Leica CM 3050 - Cryostate	Leica (Bensheim, Germany)
Leica DMRB - Microscope	Leica (Bensheim, Germany)
Leica Jung RM 2035 - Microscope	Leica (Bensheim, Germany)
Rotary Microtome	Leica (Bensheim, Germany)
TissueTek Embedding Console (Model: 4715)	Sakura FineTek Inc. (Giessen, Germany)
Centrifuges	
Biofuge Fresco - Centrifuge (Rotor: 75003328)	Heraeus Sepatech GmbH (Osterode, Germany)
Kontron Hermle ZK 365 - Centrifuge	HERMLE Labortechnik (Wehingen, Germany)
Megafuge 1.OR (Rotor: 3360)	Heraeus Sepatech GmbH (Osterode, Germany)
Suprafuge 22 (Rotor: 5154)	Heraeus Sepatech GmbH (Osterode, Germany)
Varifuge 3.OR - Centrifuge (Rotor: 5310)	Heraeus Sepatech GmbH (Osterode, Germany)
Other	
50 µl-syringes	Hamilton (Reno, NV, USA)
Coulter Epics XL-MCL - Flow cytometer	Beckman Coulter, Inc. (Miami, FL, USA)
Dynatech MR5000 - Plate reader	Dynatech Lab., Inc. (Chantilly, VA, USA)
Gammatron-3 radiation chamber	Siemens, Erlangen, Germany
LKB-Wallac-1217 liquid scintillation counter	Rackbeta, Turku, Finland
Refrigerator 4 °C, -20 °C	Liebherr (Ochsenhausen, Germany)
Refrigerator –80 °C	Revco (München, Germany)
Sartorius GD603 - Precision weighing balance	Sartorius GmbH (Göttingen, Germany)
Sartorius GE7101 - Precision weighing balance	Sartorius GmbH (Göttingen, Germany)
THX-250 therapeutic X-ray source	Medicor, Budapest, Hungary

2.2. Characterisation and cloning of the rat FL cDNA

2.2.1. RNA isolation from rat spleen

A male Lewis rat was euthanized in CO_2. Total spleen was removed, frozen in liquid nitrogen and cut to 50 µm sections on cryostat. Samples were dissolved in 800 µl of guanidinium-isothiocyanate containing 8% mercaptoethanol. RNA was pelleted (13000g, 4°C, 5 minutes) and resuspended in 450 µl sodium iodide - 3M sodium acetate solution. RNA was isolated using the GLASSMAX RNA MicroIsolation Spin Cartridge System. To assess the quality of the product, RNA was denaturated (65°C for 5 minutes) and agarose gel electrophoresis was performed (1% agarose gel with 1x MOPS buffer and 0.5 µg/ml. ethidium bromide, 80V). RNA concentration was measured by UV spectrophotometry (260/280 nm).

2.2.2. RT and 3'-RACE - PCR

At the time of the study, only a fragment of the rat FL cDNA located in the 5' region was accessible from public databases (BF522463 and CB606325, Figure 4.). To obtain the full length cDNA that could be used for gene therapy, 3'-rapid amplification of cDNA ends (RACE) was performed. RACE-PCR, first described by Frohman[241] in 1988 is an anchor PCR modification of RT-PCR. The rationale is to amplify sequences between a single previously characterized region in the mRNA (cDNA) and an anchor sequence that is coupled to the 3' end. A primer is designed from the known internal sequence and the second primer is selected from the relevant anchor sequence.

Samples containing total RNA were treated with DNase (Table 11). The following steps were performed on ice in a safety cabinet. The whole mRNA pool was transcribed to single strand cDNA by reverse transcription - the 3' polyA-tail was used as a non-specific binding site for an oligo(dT) primer, coupled to a linker sequence to allow more specific binding (oligo(dT)17-primer, Frohman primer, Table 9). Two independent 3' RACE - PCR reactions were performed. PCR was used to amplify the double-stranded product. The assembled sequence of two rat ESTs available in public databases, BF522463 and CB606325, containing the 5' sequence of the rat FL was used to design a forward primer (for1, Figure 4, Table 9) ; the adaptor oligonucleotide was used as a reverse primer. Primers were produced in the German Cancer Research Center, Heidelberg. Agarose gel electrophoresis (1% agarose in 0,5x TAE buffer, 50-100V for

45 minutes) was used to separate the end products. The appropriate DNA sample at 400 bp was excised from the gel and purified with the QIAquick Gel-Extraction Kit.

Figure 5. Double stranded cDNA synthesis using 3' RACE. DNA products at 400 bp were detected in two independent reactions (*), the stronger was selected for sequencing (first lane)

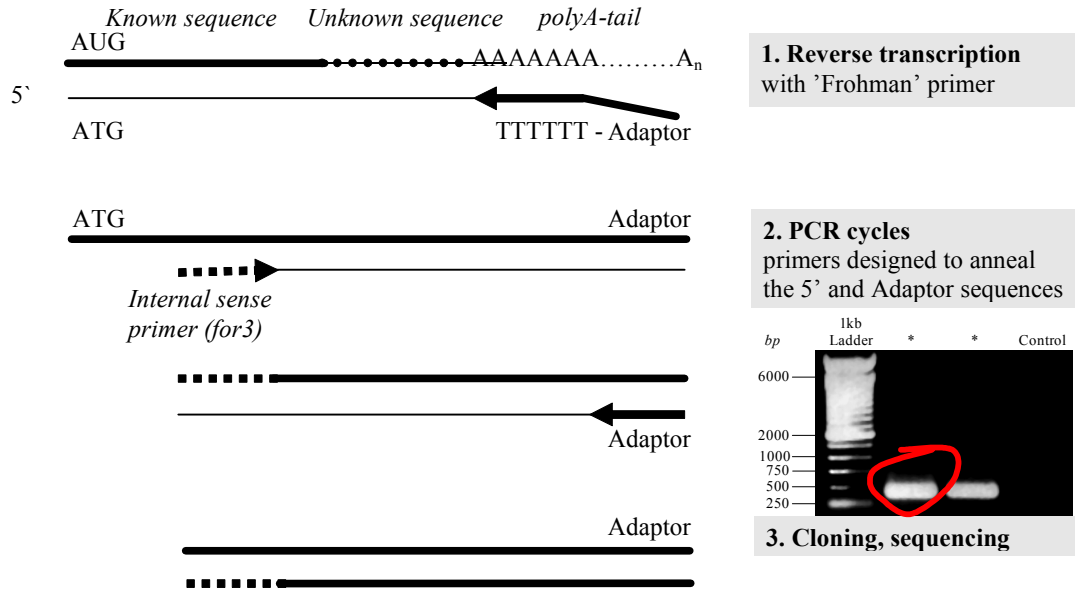


Table 11. DNase treatment

1µg RNA in 8µl water
1µl 10x Dnase – buffer
1µl 1U/µl DNaseI
incubation at room temperature for 15 minutes
1µl 20mM EDTA
incubation at 65°C for 10 minutes
incubation at 65°C for 10 minutes

Table 12. Reverse transcription

4µl 5x RT-buffer
2µl 0,1M DTT
1µl 10mM dNTPs
1µl 3µM Frohman-primer
1µl 200 U/µl MMLV reverse transcriptase
incubation at 42°C for 1 hour
enzyme inactivation at 52°C for 30 minutes

Table 13. Components of PCR

5µl 10x PCR – buffer
5µl 2mM dNTPs
1.5µl 50mM MgCl2
0.5µl 25µM primer1 + 0.5µl 25µM primer 2
0.2µl 5 U/µl Taq DNA-polymerase
5µl cDNA
32.3µl water

Table 14. PCR protocol. One cycle ishighlighted (30 cycles)

denaturation	93°C	1,5 min
denaturation	93°C	30 sec
annealing	59°C	30 sec
elongation	72°C	120 sec
elongation	72°C	6 min
cooling to 4°C		

2.2.3. Multiplication and sequencing of the rat FL cDNA

DNA was ligated into pCR2.1 plasmid with the TA Cloning Kit and transformed into competent bacteria (50 µl competent INValphaF´ E. coli strain (Invitrogen) + 2 µl 0,5M mercaptoethanol + 3 µl ligated DNA incubated on ice for 30 minutes, heat shock at -42°C for 30 sec., incubation on ice for 2 minutes, addition of 450 µl SOC medium and incubation again at 37°C for 45 minutes). Bacteria were plated on LB-agar containing 0.1mM X-gal and 100µg/ml kanamycin. After 12-18 hours of growth seven uncolored clones were transferred into 10 ml tubes containing 3 ml LB-medium with 30 ng/ml kanamycin and cultured at 37°C for 10 hours on shaker (250 rpm). DNA was extracted using the Quantum Prep Plasmid Miniprep Kit. The length of the insert was controlled after digestion by EcoRI (17 µl plasmid DNA + 2 µl 10x Incubation buffer H (Roche) + 1 µl EcoRI enzyme incubated at 37°C for 2 hours) by agarose gel electrophoresis. Samples containing the 400 bp insert were precipitated with sodium acetate (183 µl of DNA + 18 µl 3M sodium acetate + 458 µl 95% ethanol incubated at -80°C for 30 minutes, washing with 70% etanol, drying, resuspending in water) for sequencing.

The unknown 3´ region of the rat rFL cDNA was determined by fluorescent sequencing using the Big-Dye terminator DNA-sequencing Kit and an ABI PRISM 310 Genetic Analyzer (primer: Ufor1 - Table 9, cycle sequencing reaction: denaturation 96 °C - 10 sec, annealing 50 °C - 5 sec, elongation 60 °C - 4 minutes, 25 cycles). Electrophoresis was performed according to the "Standard POP-4 polymer" protocol. Sequence data was collected on computer.

2.2.4. Cloning and purifying large quantities of plasmid for experimental use

The full length cDNA was cloned into the mammalian expression vector pcDNA3.1 by directional cohesive-end cloning (Figure 5). Reverse transcription was performed from splenic RNA extract as described before (2.2.1, 2.2.2). PCR was performed (Table 13, Table 14) with a forward primer linked to the BamHI restriction sequence (BamHI-for) and a reverse primer annealing to the 3' end of the rFL cDNA linked to the XbaI restriction sequence (rev-XbaI). PCR products and pcDNA3.1 plasmid were double digested with BamHI and XbaI restriction endonucleases and ligated (Table 15, Table 16).

Competent bacteria (E. coli INValphaF`) were transformed and plated on LB-plates containing 100 µg/ml ampicillin. 18 clones were selected after 12-18 hours of growth

and cultured in 5 ml liquid LB-medium containing 30 ng/ml ampicillin for 10 hours at 37ºC on shaker (250 rpm). Cultures were stored -70ºC after addition of 20% glycerol. Plasmids were extracted from samples (Quantum Prep Plasmid Miniprep Kit) and screened for insert using primers annealing to the originally known sequence of the rFL cDNA (for2 and rev) in a standard PCR. Presence of the entire sequence was tested by PCR using two universal primers (Ufor2, Urev2) and sequenation ('Ufor2' primer, see 2.2.3).

Figure 6. Schematic figure of cloning of the full length rFL cDNA into pcDNA3.1 plasmid. ATG: start codon. TAA: stop codon. T7: bacterial promoter allowing transcription in the sense direction. Human cytomegalovirus immediate-early promoter (P_{CMV}) allows expression of insert in eukaryotic cells.

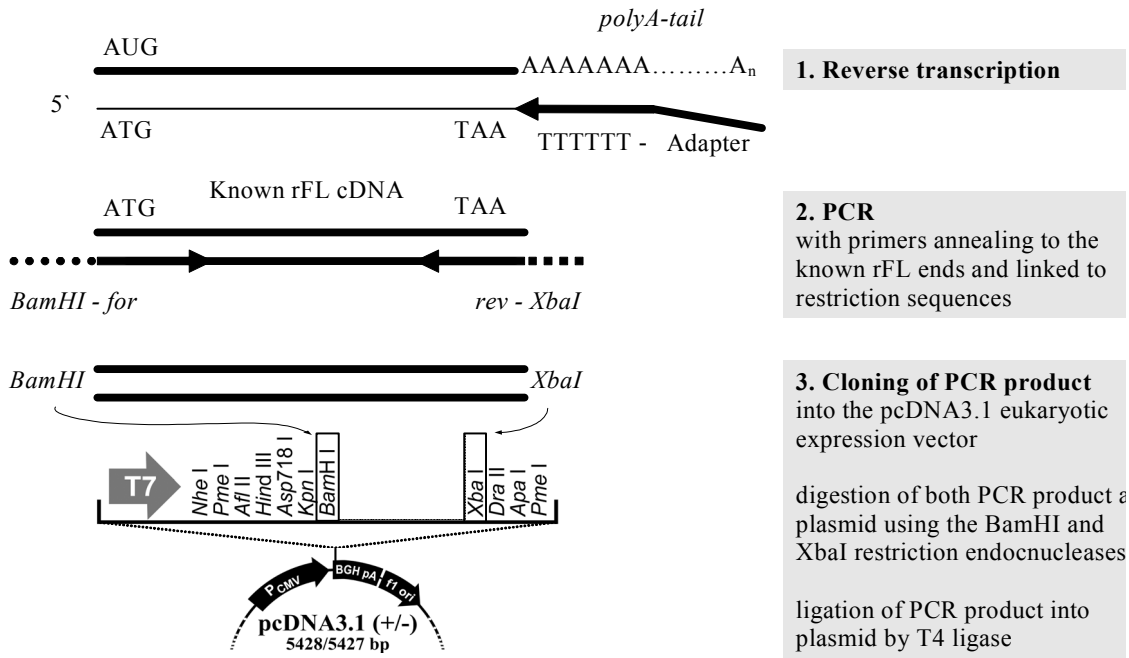


Table 15. Double digestion

1µg DNA, 1µg pcDNA3.1 plasmid
4µl 10x Incubation buffer B (Roche)
1µl BamHI enzyme (10U)
1µl XbaI enzyme (10U)
ad 40µl aqua bidest.
incubation at 37°C for 3 hours,
inacticvation at 65°C for 15 minutes

Table 16. Cohesive end ligation

4µl from restriction reaction
water to 18µl
heat to 45°C for 5 minutes
put on ice
2µl 10x Ligase Buffer (Roche)
1µl T4 DNA Ligase 4U/µl
incubation overnight at 14°C

Large quantities of pcDNA3.1-rFL for experimental use were produced by re-culturing the stock bacteria in LB-liquid media. Samples of the culture were stocked at -70ºC with 20% glycerol. Plasmid purification was performed using the EndoFree Plasmid Maxi Kit.

2.3. The DSL6A tumor model and the liposome-based transfection system

2.3.1. Tumor cell line, cell culture, animal and tumor model

The rat DSL6A duct-like pancreatic carcinoma cell line was originally derived from an azaserine treated Lewis rat[242, 243]. Being syngeneic in Lewis rats, this tumor model allows immunological studies. Histologically, the tumor is a desmoplastic, duct-like differentiated carcinoma which strongly resembles human pancreatic cancer in contrast to the acinar morphology of most other types of rat tumors of the exocrine pancreas[243]. It shows local invasion and develops satellite metastases. If left in the pancreas, the tumor is lethal after 3-4 months of growth[244].

DSL6A cells were maintained under standard conditions. Aliquots of various cell types were stocked in liquid nitrogen in our laboratory and were melted at room temperature before seeding. $5x10^5$ cells were seeded in large cell culture flasks (A=150 cm^2). Cells were grown for 2-3 days prior to investigation in Iskove's standard cell culture medium at 37°C in humidified atmosphere with 5% CO_2.

For *in vivo* experiments, subcutaneously growing DSL6A tumors were induced in syngeneic Lewis rats. Male Lewis rats (Charles River) were kept under pathogen-free conditions in the Central Animal Facility of the University of Heidelberg and received food and water *ad libitum*. *In vitro* growing DSL6A cells were brought to suspension by trypsinization (incubation in 0.25% trypsine for 10 minutes followed by washing twice in PBS). 10^6 cells in 100 µl were injected subcutaneously into the abdominal flank of Lewis rats weighing 200-220 gramms with a fine needle syringe (Hamilton) under short-term CO_2 narcosis. After 10-12 weeks, the diameter of tumors reached 5 mm.

2.3.2. Preparation of DNA - liposome complexes

The pcDNA3.1-rFL plasmid or the pcDNA3.1-LacZ plasmid was formulated in cationic liposomes (*In Vivo* GeneSHUTTLE, N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate [DOTAP]:cholesterol = 1:1) for *in vivo* gene delivery at room temperature according to the suppliers recommendation. The final

concentration of the plasmid was set to 0.5 μg/μl. DNA:liposome complexes were prepared immediately before injection. Optical density based quality control of the complexes was performed in ever case (OD_{400}: 0.7 - 0.9).

2.3.3. Transfection efficacy measurements

The ability of pcDNA3.1 plasmid to propagate into DSL6A cells was tested *in vitro* by transfection with pcDNA3.1-LacZ (cloned in our laboratory before), using a standard Ca-phosphate transfection protocol (500 μl 1x HEBS buffer + 10 μg pcDNA3.1-LacZ + 30 μl 2.5 M CaCl in HEPES to cell culture in 8 ml medium; control: pcDNA3.1). β-galactosidase activity was visualized with the LacZ Reporter Assay.

Transfection efficiency with liposome-coated plasmid-DNA on two subcutaneous DSL6A tumors at a diameter of 5 mm was measured after a single intratumoral injection of 25 μg pcDNA3.1-LacZ formulated in cationic liposomes. Tumors were removed two days post transfection and subjected to whole mount Lac-Z staining (1. fixation: 90 minutes in LacZ Fixative solution, 2. washing: 5x5 minutes with LacZ Washing solution, 3. staining: 24 hours in LacZ Staining buffer on shaker at RT, 4. washing in PBS, 5. fixation in 4% paraformaldehyde). Untreated tumors served as negative controls. After staining, tissue was embedded in paraffin (washing in 4ºC PBS 2x30 minutes, dehydration in 50-70-90-100% ethanol - each for 30 minutes at rT, incubation in 10ml toluene for 2x30 minutes, equilibration by 1:1 toluene:Paraplast solution: 3x 60ºC for 20 minutes, embedding in Paraplast). 5 μm sections were cut on microtome, collected on silane pre-treated slides, allowed to dry at RT and stored at 4ºC. Before staining, paraffin was removed by immersing the sections in toluene for 2x10 minutes at RT, followed by a series of 100-80-70-50-30% ethanol and washing by PBS. Sections were counterstained in Mayer's acid haemalum and mounted Transfection efficacy was assessed by computer analysis as proposed by Lehr et al[245]. Briefly, four 0.74 mm^2 areas of a representative histological section from each tumor were randomly chosen by light microscope, digitalized by a colour video camera (CF 20/4DX). The images were edited in Photoshop 3.0. The selection of specific areas representing LacZ positive or negative cells was accomplished according to the similarity of colour properties of the pixels on the image using the "magic wand", "select similar" and "histogram" functions. Transfection efficacy was calculated according to the size of the stained region compared to the total area in each image.

To confirm the FL production of transfected DSL6A cells, 10^6 cells were transduced by pcDNA3.1-rFL or pcDNA3.1 *in vitro* using the calcium phosphate transfection protocol. Cell culture supernatant was collected 48 hours post transfection. *In vivo,* subcutaneously growing DSL6A tumors (5 mm in diameter) were transfected by a single intratumoral injection of liposome-coated pcDNA3.1-FL or left untreated. Tumors were removed 48 hours post transfection, cut into 50µl sections, put into Eppendorf tubes and lysed on ice for 15 minutes by adding 4°C RIPA buffer. Cell debris was pelleted by centrifugation at 13000rpm. FL level of cell culture supernatants and tumor lysates was assessed by specific ELISA (R&D, Minneapolis, MN, USA); the result was always expressed normalized to the total protein content. Total protein content was measured by the method of Lowry[246]. RIPA buffer has been reported to modify the results of immuno-assays, so we examined the impact of the buffer used for lysis on our ELISA-assay. Various dilutions with RIPA buffer didn't change the linearity of the assays at normal FL concentrations and the method was therefore applicable for protein extraction with ELISA (data not shown).

2.4. Local rFL immuno-gene therapy in the DSL6A pancreatic cancer model

2.4.1. Therapy regimen and follow-up

The experimental protocol was approved by the Animal Welfare Board of the Regierungspräsidium Karlsruhe, Germany (certification 35-9185.81/69-01). After s.c. tumor inoculation animals were randomized into the following groups: (a) no treatment (n=8), (b) pcDNA3.1-treatment (n=8), (c) rFL-pcDNA3.1-treatment (n=8). Four animals died during the narcosis, so the groups were modified as follows: (a) control, no treatment (n=6), (b) pcDNA3.1-treatment (n=6), (c) rFL-pcDNA3.1-treatment (n=8). Therapy of each animal was initiated when the tumors reached 5 mm in diameter (approximately 10-12 weeks after tumor inoculation). Intratumoral injection of 50 µl liposome:DNA complex was performed on days 1, 3, 5, 8, 10, and 12 under short-term CO_2 narcosis, leaving the pin of the needle in an intratumoral position for 30 seconds to prevent outflow of the injected fluid. Tumor growth had been monitored for five weeks. The tumor volume was calculated by the formula: *{0.52 x (shortest diameter)^2 x (longest diameter)*[247] and expressed in percent of the baseline value. Animals that showed obvious reduction of tumor volume (tumor burden <100% for at least two weeks) were defined as responders. At the 6th week animals were euthanized by CO_2. Tumors were

harvested, snap frozen and stored in liquid nitrogen. The size and weight of spleens was measured. The general status and body weight of each animal was assessed at the start and at the end of experiments.

2.4.2. TNF-α and INF-γ ELISA

1 ml blood was drawn into tubes containing 1 U of heparin from rats from the peribulbar plexus on days 1, 5 and 12 in CO_2 narcosis. TNF-α and INF-γ levels were measured by ELISA from the plasma., while the cell fracture (centrifugation at 2500 rpm for 10 minutes) was subjected to flow cytometric analysis. IFN-γ is peptide secreted by NK cells and T lymphocytes, most prominently by CD8 cytotoxic T cells in response to mitogens or to antigens to which they are sensitized. It is a product exclusively of lymphocytes[248]. It plays an important role in the coordinated regulation of expression of the immune response via the stimulation or repression of key genes[249]. TNF-α is a cytokine produced primarily by monocytes and macrophages, or by activated NK and T cells[250]. Both INF- γ and TNF- α are non-specific markers of immune activation[251, 252].

2.4.3. Flow cytometry

FACS of peripheral blood leukocytes (control group: 3 animals, pcDNA3.1 group: 3 animals, rFL-pcDNA3.1 group: 5 animals) was performed at days 1, 5 and 12; splenic leukocytes were examined at the end of experiments (control group: 6 animals, pcDNA3.1 group: 3 animals, rFL-pcDNA3.1 group: 8 animals). Spleens were minced with a scalpel and were gently passed through a sterile nylon mesh with 50 μm pore size to prepare single-cell suspension. Splenocytes and blood cells were incubated 1:10 in erythrocyte lysis buffer on ice for 10 minutes and washed twice in 4ºC PBS.

A concentration of 100 cells/ml was set in PBS before labeling. Direct or indirect fluorescent labeling was performed by incubating the monodisperse cell suspension with appropriate antibodies / combinations (Table 8, Table 17). 100 μl cell suspension was incubated with 2 μl of antibody for 15 minutes on ice followed by washing with PBS (6000 rpm at 5 minutes in a cooled table centrifuge). Labeled or unlabeled mouse isotypic antibody was used as negative control. Samples were protected from light during incubation and transportation. Measurement was performed on an EPICS-XL flow cytometer (10.000 events/sample was acquired and analyzed with the Expo ADC32 software.)

Cell debris, cell population containing lymphocytes and NK cells or the population of large monocytoid cells was gated according to size and granularity using forward and side scatter data. Fluorescent activity was analyzed separately for each population.

Table 17. Flow cytometry protocol. For antibody details see Table 8.

control	Iso-FITC + PE	Washing (PBS)		Washing		Washing
1	anti-NK (FITC)					
2	Anti-CD80		anti-mouse (PE)			
6	Anti-CD86		anti-mouse (PE)		anti-CD40(FITC)	
7	Anti-CD11b/c		anti-mouse (PE)		anti-CD40(FITC)	
3	Anti-CD25		anti-mouse (PE)		anti-CD4(FITC)	
4	Anti-CD28		anti-mouse (PE)		anti-CD8(FITC)	
5	Anti-CD18		anti-mouse (PE)		anti-CD62L(FITC)	

The applied antibodies are commonly used in the literature (Table 8). The anti-NK 10/78 antibody clone reacts with NKR-P1A, a homodimer stimulatory receptor expressed on all NK cells – it is widely used for NK cell detection[253]. The OX-39 antibody reacts with the α-chain of the IL-2 receptor (CD25). CD28 is a co-stimulatory receptor required for T cell activation; its ligands include CD80 and CD86[79]. CD18 (β integrin) is found on the majority of leukocytes; it is involved in cell adhesion. CD62L (L-selectin) is detected on most neutrophils, peripheral lymphocytes and on some splenic lymphocytes; it is required for lymphocyte homing to peripheral lymph nodes and contributes to neutrophil emigration at inflammatory sites[254]. CD62L and CD18 regulate leukocyte adhesive phenotype and are widely used markers of T-cell activation. The OX-42 antibody reacts with the CR3 complement receptor found on dendritic cells. It recognizes a common epitope shared by CD11b and CD11c (integrin αM and αX chains). It is a marker extensively used to mark DCs. All murine DC subsets express CD11b or CD11c[47]. CD40 is a glycoprotein expressed on monocytes and DCs. CD40-CD40-ligand signaling by MHC-restricted, activated CD4+ T cells induces differentiation of DCs; CD40 is up-regulated when DCs migrate from the periphery to draining lymph nodes (DLN) in response to antigen challenge and maturation[255]. CD80 and CD86 are transmembrane proteins expressed primarily on antigen presenting cells; their ligands include CD28 and CTLA4[32]. The binding of their ligands on T cells provide the "second signal" to induce immune response. CD80 and CD86 are commonly used markers of dendritic cell maturation; their expression is increasing during this process[57].

2.4.4. Immunohistochemistry

To analyze the proliferation rate mean vessel density and lymphocyte infiltration of tumor tissue indirect three-step immunohistochemistry was applied using the LSAB-2 kit. Proliferating cell nuclear antigen 1 (PCNA) is an intranuclear strongly expressed during the S-phase; it is considered to be an index of cell proliferation[256]. RECA-1 is an endothelial cells surface antigen used for immunohistological evaluation of microvascular density in solid rat tumors[252]. 5μm-sections were cut on cryostate, air-dried and fixed in acetone for 2 minutes and stored at –20°C. Antibodies were incubated (Table 18) on sections. Counterstaining was made with Mayer's acid hemalum for 4 minutes. Specimens were immersed 10 times into baths containing ammonia water and were placed into distilled water for 2 minutes. Slides were mounted and coversliped by Hydromount. Spleen of a healthy, untreated animal was used as positive control. The extent of lymphocyte infiltration was estimated double-blind using the scale: (+)-low, (++)-moderate and (+++)-high. Quantitative analysis of PCNA and RECA staining was performed by computer-assisted image analysis: four microscopic fields of 0.74 mm^2 were randomly chosen by light microscope and digitalized (CF 20/4DX video camera). The number of blood vessels was counted and expressed per 1 mm^2 of surface. Number of PCNA-positive and PCNA-negative tumor cells was counted and expressed in percent of the total number of tumor cells.

Table 18. Application of primary antibodies for immunohistochemistry in DSL6A tumors.

	CD4	CD8	CD161-a	Reca-1	PCNA	control - IgG_1
Dilution of antibody	1:100	1:100	1:50	1:20	1:100	1:100
Incubation time	10 min	10 min	1 hour	10 min	10 min	1 hour

2.5. Transferrin receptor expression of human pancreatic cancer cell lines

2.5.1. Cell culture

To investigate the expression of transferrin receptor *in vitro* nine human cell lines of ductal pancreatic cancer were grown in culture, namely: AsPC1, Capan1, MiaPaca1, KCl MOH1, Panc1, FAMPAC, Patscl52, PaTu8902 and PaTu8988t. All of these cells were isolated from human pancreatic cancer tissues and are widely used in experimental setting[257] (see also: www.cabri.org). Aliquots of various cell types were stocked in liquid nitrogen in our laboratory and were melted at room temperature before seeding.

$5x10^5$ cells were seeded in large cell culture flasks ($A=150cm^2$). Cells were under standard conditions (2.3.1). Cells were harvested 2-3 days after seeding by a mechanical scraper to protect the cell surface proteins that would have injured by tripsinization; they were non-confluent and proliferating actively at this time. Cells were washed twice in PBS and the number of living cells was assessed by diluting a 100 µl sample with equal volume of Trypan Blue and counting the non-stained cells in a Neubauer haemocytometer.

2.5.2. Flow cytometry for TFRC

Flow cytomtry was performed as described before (2.4.3). Direct fluorescent labeling was performed FITC-labeled anti-TFRC antibodies (100 µl cell suspension + 2 µl of appropriate antibody for 15 minutes). FITC-labeled mouse isotypic antibody served as negative control. Samples were protected from light during incubation and transportation. Measurement was performed on an EPICS-XL flow cytometer. 10.000 events were counted per sample. Data was acquired and analyzed with the Expo ADC32 software.

2.6. Transferrin receptor expression of human pancreatic tumor samples

2.6.1. Patients

Patients admitted to the study examining the TFRC expression underwent surgery for pancreatic tumors at the Department of Surgery of the University of Heidelberg. All patients gave their informed consent to the protocol which was approved by the Ethics Committee at the University of Heidelberg. 51 samples of ductal pancreatic carcinoma (12 metastases and 39 primary tumors) and twelve samples of neuroendocrine tumors (five insulinomas, one gastrinoma, one glucagonoma, one carcinoid, four neuroendocrine carcinomas) were collected. Hystological diagnosis was made by pathologists of the University of Heidelberg. Eight samples of non-malignant pancreas tissues were obtained from the healthy part of the resected pancreas from the operated patients. The samples were obtained 5-10 cm away from the tumor. Each sample was snap frozen and stored in liquid nitrogen till further analysis.

2.6.2. Immunohistochemistry (cytokeratin, transferrin receptor and transferrin)

Immunohistochemistry was done using the LSAB-2 kit as described before (2.4.4). Anti-cytokeratin, anti-TFRC, and polyclonal anti-transferrin antibodies were incubated

for 10 minutes on sections. Counterstaining was made with Mayer's acid hemalum. Positive expression of TFRC was defined if more than 80% of cells were stained. Heterogeneous expression meant positivity at 25-80% of the cells. Staining of less than 5% of cells was defined as negative expression.

2.7. Glioblastoma cell lines

Human U373 (U373-MG) and rat C6 glioblastoma cell lines were purchased from ECACC (Salisbury, UK). The human glioblastoma-astrocytoma cell line U-373-MG was originally derived from a human glioblastoma and shows morphological features typical of its neuroectodermal origin. It has been shown that close biological similarities exist between this experimental glioblastoma and high-grade human astrocytic tumors obtained directly from surgical resections[258, 259]. The cells are widely used as an *in vitro* model of human malignant gliomas.

The cloned C6 glioma line was originally derived from a rat brain tumor induced by methylnitrosourea[260, 261]. Similarly to human neurogen tumors, it expresses S100. Histologically, the C6 cell line has been classified as an astrocytoma. C6 demonstrated the greatest number of genes among many similar cell lines whose expression was similar to that reported for human brain tumors[262]. The only drawback of this tumor model is its immunogenicity: C6 does not have syngeneic host. Despite of this fact the model resembles human gliomas both genetically and morphologically very well and is one of the most frequently used murine glioma models.

All glioma cell lines were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal calf serum and 10 mg/ml Streptomycin under standard conditions.

2.8. Overexpression of deoxycytidine kinase in gliomas

2.8.1. The human deoxycytidine kinase encoding adenovirus vector (Ad-hudCK)

The construction of Ad-hudCK was accomplished before these studies in our laboratory; we hereby only briefly summarize the method. A bacterial plasmid expression vector encoding the human deoxycytidine kinase (dCK) gene was the generous gift of Dr. Talianidis. In the absence of appropriate restriction enzyme recognition sites the dCK insert was amplified by polymerase chain reaction using the following primers: 5'-TTGAATTCCACCATGGCCACCCCGCCCAAG-3'; and 5'AAGGATCCTCACAAA

GTACTCAAAAACTC-3'. The primers carried an EcoRI and a BamHI restriction enzyme recognition site at their 5' ends, respectively. The PCR product was digested with EcoRI-BamHI and subcloned into the pUC18 plasmid vector. The nucleotide sequence of the dCK insert was checked using the "*fmol* DNA Sequencing System" (Promega, Madison, WI, USA). Ad-HudCK was generated using the AdMax™ Adenovirus Creation Kit (Microbix Biosystems Inc. Toronto, Canada) as suggested by the supplier. Briefly, first the EcoRI–BamHI dCK fragment was cloned into the pDC516 shuttle plasmid. Then, human embryonic kidney 293 (HEK293) cells (ECACC) were co-transfected with the dCK containing shuttle plasmid and pBHGfrtΔE1,3FLP genomic plasmid using the MBS Mammalian Transfection Kit (Stratagene, La Jolla, CA, USA). Cells were maintained in 0.33% agarose containing DMEM until lytic plaques appeared (8-10 days). Individual plaques were isolated, and tested for human dCK expression. Large-scale adenovirus vector isolation was performed using HEK293 suspension cell cultures as described[263]. The viral solution was stored at -80°C - titers of viral stocks were determined by plaque assay.

2.8.2. In vitro transduction with adenoviral vectors

To determine transduction efficiency of the adenoviral vector on the experimental cell lines, 10^4 U373 and C6 cells were seeded in 12-well dishes dishes and infected 24h later with the adenoviral vector AdexCAlacZ encoding the lacZ gene. After removal of the medium, the virus was added to the dishes in 200 µl serum-free medium at different multiplicities of infection (MOI) and incubated for 1 hour. The medium was then refreshed and lacZ activity visualized by the LacZ Reporter Assay.

2.8.3. Preparation of cellular extracts and measurement of dCK activities

To be able to analyze the relationship between dCK activity and gemcitabine sensitivity, the basal, specific and post-transfectional dCK activity of C6 and U373 cells was determined in four independent experiments.

Cellular extracts were prepared from wild-type or Ad-HudCK transduced cells as described[264]. Briefly, cells were harvested, pelleted, washed in PBS. Cells were deep frozen at -70ºC and melted three times, and then lysed in 1 ml Soluene:H_2O=8:2 solution. Protein content was measured. Deoxycytidine kinase activity was determined by an enzymatic reaction, where the reaction mixture contained 50 mM Tris-HCl (pH: 7.6), 10mM NaF, 5 mM ATP, 10µM CdR, 0.1 mg/ml BSA, 2 mM DTT, 5 mM $MgCl_2$,

and 0.5 μCi (22 pmol) deoxy[5-^{3}H]cytidine. Reaction was carried out in a total volume of 50 μl and was initiated by the addition of 5 μl cellular extract to 45 μl of reaction mixture, followed by incubation at 37°C for 30 minutes. To stop the reaction 30 μl of the reaction mixtures were dropped on DEAE filter disks and washed 2x10 min in 1.5 mM ammonium-formiate. The filters were incubated for 2 min in 200 μl of 0.5 M PCA and their radioactivity was measured in a liquid scintillation counter (LKB-Wallac-1217). Since phosphorylation of deoxycytidine can be performed by the thymidine kinase 2 (TK2) enzyme as well, the specific dCK activity was also determined. The TK2 enzyme, which has a much better affinity to thymidine than to deoxycytidine was saturated by the addition of 1.2 mM thymidine to the reaction mixture.

Deoxycytidine kinase activity was expressed in nmol/hour/mg protein and was calculated as follows: dCK activity = c_1/c_2 x dC x 2/prot; where: c_1 represents $cpm_{sample}cpm_{blank}$; c_2 represents $cpm_{maximum}$-cpm_{blank}; dC represents total amount of labeled and unlabelled deoxycytidine in the reaction mixture applied on the filter paper (expressed in nmol/ml); 2 represents a constant to correct enzyme activity for 60 min; prot represents total protein amount present in the reaction mixture applied on the filter paper (expressed in mg/ml).

2.9. Sensitivity of dCK overexpressing glioma cell lines to chemoradiation

The growth curve of C6 and U373 cells is described in the literature[259-261]. To determine the sensitivity of the C6 and U373 cell lines to gemcitabine, cells were seeded in 25 cm^2 tissue culture dishes in duplicates at day one using the following cell densities: 12x10^3, and 6x10^3 cells/cm^2 for U373 and C6 cells, respectively. On the next day, the exponentially growing cells were treated with increasing concentrations of gemcitabine (5-500 nM). The number of surviving cells was determined three days later. Cells were harvested by trypsinization (2.3.1); the number of trypan-blue non-staining viable cells was counted by microscope. Surviving fraction was determined by considering the control, untreated cells 100%, and calculating the percent of cells in the various treatment groups relative to the control group. Every experiment was performed in duplicates. Three independent experiments were performed.

To determine sensitivity to Ad-HudCK, cells were seeded as mentioned above, and the next day cells were transduced with Ad-HudCK at increasing MOIs (0, 20, 100, 300). Transduction was performed by removing the culture medium from the cells and adding the viral stocks at appropriate concentrations to the flasks in 2 ml of serum-free DMEM

for 1 hour; cell culture medium was than refilled. Cell survival and surviving fraction were determined as described above three days after the viral transduction in three independent experiments.

To determine the combined effects of Ad-HudCK transduction, gemcitabine treatment and irradiation, cells were seeded in duplicates on day one in 25 cm^2 tissue culture dishes at the following cell densities: $6x10^3$ and $2x10^3$ cells/cm^2 for U373 and C6 cells, respectively. Using these cell densities, confluence was not reached even after 7 days in culture. On day two, cells were transduced with Ad-HudCK. Transduction was done with 100 MOI for U373 and 300 MOI for C6. At these rates, transduction efficiencies of the cell lines were very similar (between 65 and 75%) and dCK enzyme activities were 8.165, and 8.313 nmol/hour/mg protein for U373 and C6 cells, respectively (2.8.3). On day three, cells were treated with gemcitabine. Gemcitabine was added to the cells in their exponential growth phase. The following gemcitabine concentrations were used: 25 nM for C6 and 250 nM for U373. Cell survival was between 60 and 75% for both cell lines. Irradiation was performed on day four, 24 hours after gemcitabine treatment with 4 Gy ^{60}Co-γ radiation (Gammatron-3, dose rate: 0.1 Gy/min). On day seven, cell survival was determined by harvesting the adherent cells by trypsinization and counting the trypan-blue non-staining viable cells by microscope. Surviving fraction was calculated as described above. The enhancement rate of the combination therapy was determined as follows: surviving $fraction_{c1}$ x surviving $fraction_{c2}$/100/surviving $fraction_{combination}$, where c1 and c2 represent the two components of the combination therapy. For the triple combination, c1 represents surviving fraction after the combined treatment with gemcitabine and irradiation and c2 represents surviving fraction after Ad-HudCK transduction.

2.10. Animal studies with the C6 rat glioma model

Animal studies were carried out in accordance with Hungarian regulations under the permission of the Institutional and National Body of Animal Care and Testing. All treatment groups consisted of 4-5 animals and experiments were repeated three times. Male Wistar rats (Charles River) were kept in the Animal Facility of the "Frédéric Joliot-Curie" National Research Institute for Radiobiology and Radiohygiene, and received food and water *ad libitum*.

The C6 rat glioma models were used. For intracranial tumor implantation of C6 rat glioma cells, 5-6 week old male Wistar rats were anesthetized by intramuscular injection of 150mg/kg ketamin and 10mg/kg xylazin solution and placed in a stereotactic apparatus. The scalp was opened and a burr hole was made at the level of Bregma and 2 mm to the right of the cranial midline suture. Injection was done with a 50 µl Hamilton syringe; the needle was inserted to a depth of 5.0 mm below the dura, into the striatum. Tumor inoculation was performed either with wild-type or with Ad-HudCK transduced C6 cells. 5 µl cell suspensions, containing $1.5x10^5$ C6 cells in Hank's balanced salt solution were injected over a period of 5 minutes. Viral transduction of C6 cells was done *in vitro* at 300 MOI 24 hours before tumor implantation. Three days after tumor implantation rats were treated with intraperitoneal injection of 60 mg/kg body weight gemcitabine, and 24 hours later the head of the anesthetized rats was irradiated with 4 Gy X rays (THX-250 therapeutic X-ray source, dose rate: 1,003 Gy/min). A lead tube shielded the other parts of the body to protect from radiation.

Animals were killed when they were moribund or 100 days after tumor cell inoculation and were carefully autopsied.

2.11. Statistical analysis

Statistical analysis was performed using the SPSS software. All data are given as mean ± SD.

Differences of DSL6A tumor volume, cytokine level and vessel density were compared with Mann-Whitney *U* test. Comparison of number of responders to rFL therapy, the extent of leukocyte infiltration, and transferrin receptor expression in primary and metastatic tumors was compared with the chi-square (χ^2) test between the groups. *P* values <0.05 were considered to be statistically significant.

Analysis of survival data of glioma-bearing animals was performed using the Kaplan-Meier method. Comparisons of survival curves were done with the Kaplan-Meier log-rank test. Statistical significance of data was tested by Student's t-test.

3. Results

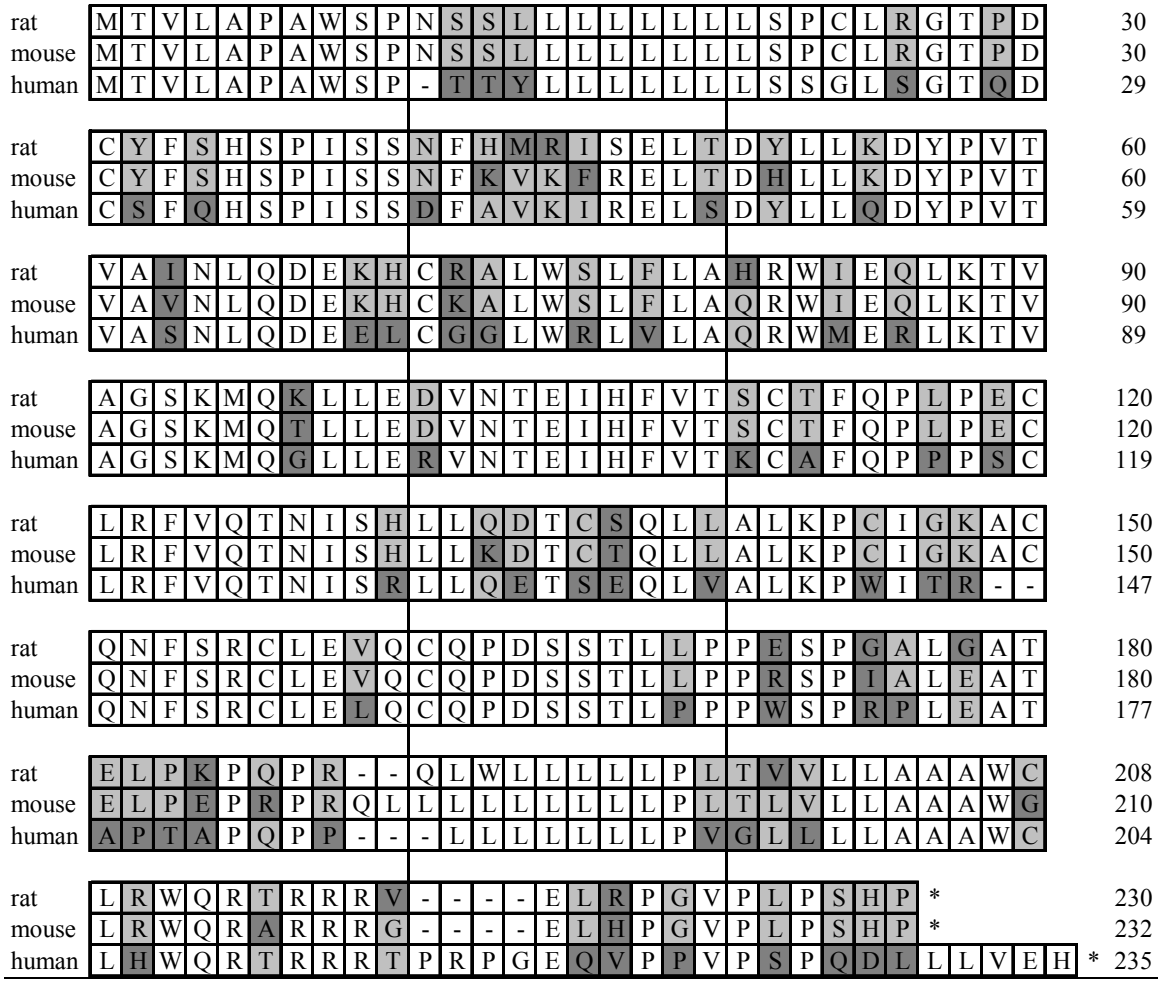
3.1. The rat rFL sequence

The previously unknown full-length rat FL cDNA was amplified, sequenced (Figure 7) and the translational product was compared to its mouse and human equivalents according to public databases (Figure 8). The rat rFL cDNA consists of 693 bases. It's translational product is estimated to be 230 amino acids long. Comparison of the translated rat FL amino acid sequence to human and mouse FL proteins showed 72.9% and 89.7% identity, respectively.

Figure 7. The rFL cDNA sequence. Amino acid sequence was calculated from nucleotide sequence. Newly sequenced bases are written in bold upper case. Base numbering is shown on the left. *: stop codon.

```
  1 a t g a c a g t g c t g g c g c c a g c c t g g a g c c c a a a t t c c t c c t t g t t g
    M   T   V   L   A   P   A   W   S   P   N   S   S   L   L
 46 c t g t t g c t g c t g c t g c t g a g c c c t t g c c t g c g g g g g a c a c c t g a c
    L   L   L   L   L   L   S   P   C   L   R   G   T   P   D
 91 t g t t a c t t c a g c c a c a g t c c c a t c t c c t c c a a c t t c c a c a t g a g g
    C   Y   F   S   H   S   P   I   S   S   N   F   H   M   R
136 a t t a g c g a g t t g a c t g a c t a c c t g c t t a a a g a t t a c c c a g t c a c t
    I   S   E   L   T   D   Y   L   L   K   D   Y   P   V   T
181 g t g g c c a t c a a t c t t c a g g a c g a g a a a c a c t g c a g g g c c t t g t g g
    V   A   I   N   L   Q   D   E   K   H   C   K   A   L   W
226 a g c c t c t t c c t g g c c c a t c g c t g g a t a g a g c a a c t g a a g a c t g t g
    S   L   F   L   A   H   R   W   I   E   Q   L   K   T   V
271 g c a g g g t c t a a a a t g c a a a a g c t t c t g g a g g a t g t c a a t a c g g a g
    A   G   S   K   M   Q   K   L   L   E   D   V   N   T   E
316 a t a c a t t t t g t c a c c t c g t g t a c c t t c c a g c c c c t a c c a g a a t g t
    I   H   F   V   T   S   C   T   F   Q   P   L   P   E   C
361 c t t c g a t t c g t c c a g a c c a a c a t c t c c c a c c t c c t g c a g g a c a c c
    L   R   F   V   Q   T   N   I   S   H   L   L   Q   D   T
406 t g c t c a c a g c t g t t a g c t c t g a a g c c c t g t a t c g g g a a a g c c t g c
    C   S   Q   L   L   A   L   K   P   C   I   G   K   A   C
451 c a g a a t t t c t c t c g g t g c c t g g a g g t g c a g t g c c a g c c g g a c t c c
    Q   N   F   S   R   C   L   E   V   Q   C   Q   P   D   S
496 t c c a c c c t g c t t c c c c c a g a g a g t c c t g g a g c c c t a g g a g c c a c g
    S   T   L   L   P   P   E   S   P   G   A   L   G   A   T
541 g a g c t t c c a a a g c C T C A G C C C A G G C A G C T G T G G C T C C T G C T G C T A
    E   L   P   K   P   Q   P   R   Q   L   W   L   L   L   L
586 C T G C C T C T C A C A G T G G T G C T G C T G G C A G C C G C C T G G T G C C T T C G C
    L   P   L   T   V   V   L   L   A   A   A   W   C   L   R
631 T G G C A A A G G A C A A G A A G G A G G G T G G A G C T C C G C C C T G G G G T G C C C
    W   Q   R   T   R   R   R   V   E   L   R   P   G   V   P
676 C T C C C C T C C C A T C C C T A G  693
    L   P   S   H   P   *
```

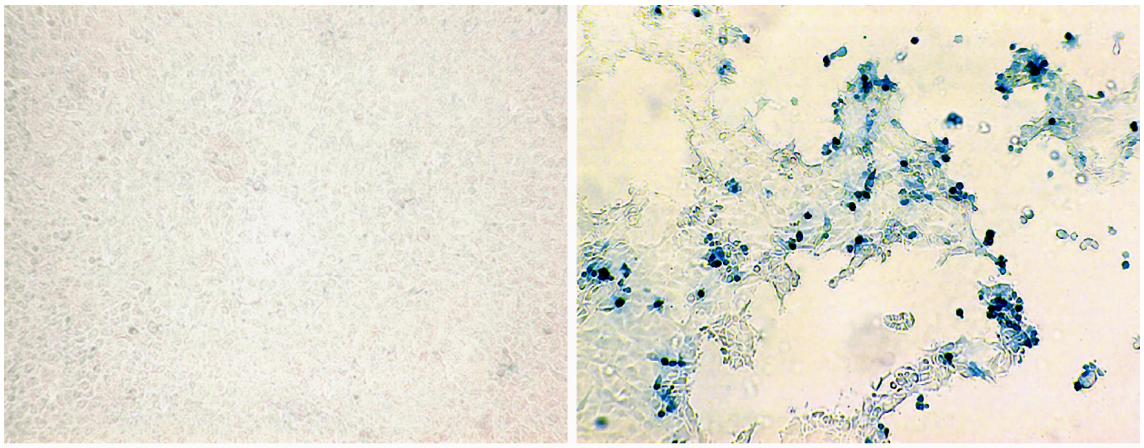
Figure 8. Peptid sequence comparison of human/mouse/rat FL. The rat amino acid sequence was calculated from the nucleotide sequence. Differences are highlighted with grey. Right side: amino acid numbering. Sequence identity: human-mouse 73.55%, human-rat 72.92%, mouse-rat 89.65%.



3.2. Transfection of DSL6A tumors with pcDNA3.1 constructs

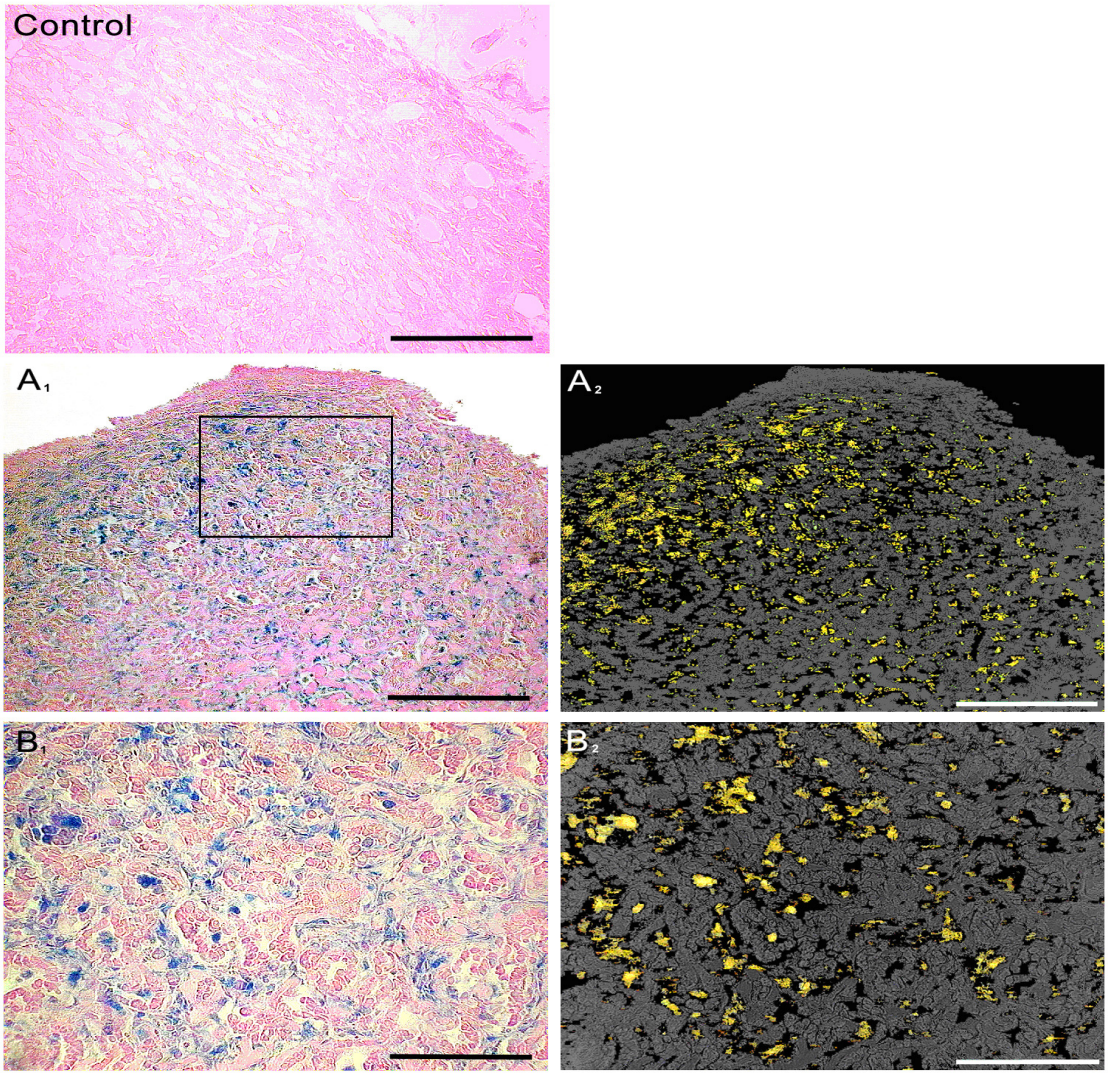
To confirm the ability of the expression vector pcDNA3.1 to transfect DSL6A cells, DSL6A cells were transfected with pcDNA3.1-LacZ. LacZ expression could be visualized in transfected but not in control cells (Figure 9).

Figure 9. Transfection of DSL6A cells with LacZ-pcDNA3.1 plasmids *in vitro*. Left: untransfected DSL6A cells. Right: DSL6A cells expressing β-galactosidase after LacZ-pcDNA3.1 transfection (B-gal staining).



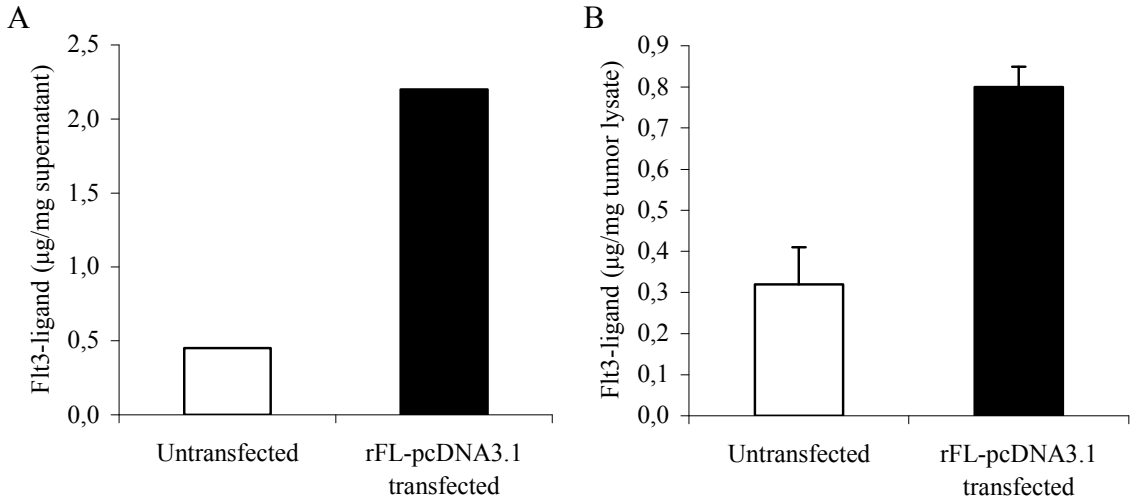
In vivo, single transfection of two subcutaneously grown DSL6A tumors with pcDNA3.1-LacZ coated in cationic liposomes resulted in high expression of LacZ in both of the tumors compared to total absence of LacZ activity in controls as demonstrated by whole-mount tissue β-gal staining (Figure 10). Both ductal and stromal cells expressed LacZ. Light microscopy didn't reveal noticeable alteration in expression activity throughout different sections of the same tumor. A representative section made at the highest diameter of each tumor was selected for quantitative computer analysis. This showed slight differences throughout images being central staining somewhat weaker than peripheral. Peritumoral staining was not seen. Transfection efficiency was 10.3% in average (12.2%, 8.4%).

Figure 10. Transfection of s.c. DSL6A tumors with pcDNA3.1-LacZ formulated in cationic lyposomes. Untransfected (Control) or transfected (A, B: same section) tumors were stained following a whole-mount tissue β-Gal protocol. A2, B2: Chromogen separation by computer analysis. Left: original images. Right: images are divided to three layers. Yellow: LacZ positive areas, grey: LacZ negative areas, black: background. Bars: 250 μm (Control, A); 100 μm (B).



The ability of rfL-transfected DSL6A cells to produce FL was than tested *in vitro* and *in vivo*. The soluble form of FL can be expressed by DSL6A cells as proved by ELISA, which showed elevated level of FL transfected DSL6A cell culture supernatant compared to control. FL quantity normalized to total protein content and was 0.45 µg/mg and 2.2 µg/mg in control and treated samples, respectively. FL expression of two liposome coated rFL-pcDNA3.1 transfected subcutaneous DSL6A tumors was measured from whole tumor lysates by the same ELISA. Normalized FL content of tumor lysates was 2.5 fold higher in rFL-pcDNA3.1 treated tumors than in controls after two days of a single injection (0.8 µg/mg±0.09 and 0.32±0.05 µg/mg, respectively).

Figure 11. Flt3-ligand production of rFL-pcDNA3.1 transfected DSL6A tumor cells. (A) *In vitro* growing tumor cells were transfected and the soluble FL was measured from the cell culture supernatant. (B) Two subcutaneously growing DSL6A tumors were transfected by a single injection of liposome-coated rFL-pcDNA3.1 and FL amount was quantified from whole tumor lysate. FL-specific ELISA was used for measurements.

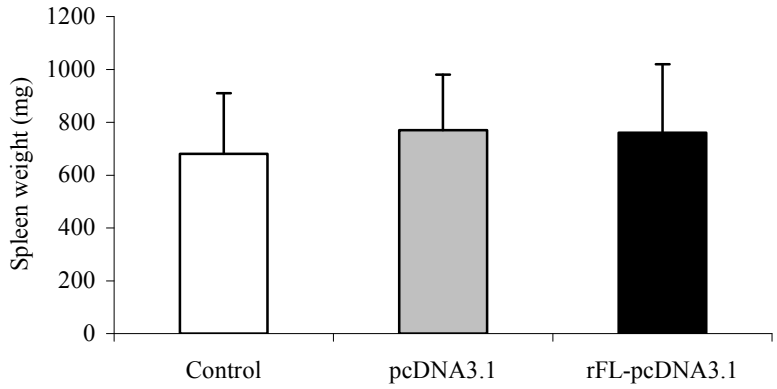


3.3. Animal experiment with rat DSL6A pancreatic cancer

3.3.1. Tumor growth

DSL6A tumors grew 10-12 weeks after inoculation to reach 4-6 mm in diameter, when we started the therapy. At the sixth week of follow-up several tumors developed transcutaneous ulceration which affected the size and further growth of the tumor. Therefore, the measurement of tumor volume at the sixth week was excluded from the final analysis. Animals didn't have macroscopic metastases at this time. Splenomegaly was not observed - significant difference in the mean weight of spleens was not found (Table 19).

Table 19. Weight of spleens of DSL6A bearing animals at the sixth week. Data represent mean + SD.



All tumors in the untreated and pcDNA3.1treated group showed continuous growth. Statistically significant difference was not found in the growth of untreated and pcDNA3.1-treated tumors at any time point of the follow-up (Figure 12). In contrast, some tumors showed tumor volume reduction in the rFL-pcDNA3.1 treated group. There was significant reduction of tumor growth during the first 3 weeks in the rFL-pcDNA3.1 group compared to both other groups (Figure 12, Mann-Whitney U test, $p<0.05$). Four out of eight tumors responded to rFL-pcDNA3.1 therapy (tumor burden lower than 100% for at least two weeks, Table 20, Figure 13). Response to therapy was not seen in other groups. Tumor growth suppression may have been attributable to therapy (χ^2 test, $p=0.04$).

Figure 12. Growth curve of DSL6A tumors. Significant tumor growth reduction was found during the first three weeks in the rFL-pcDNA3.1 group compared to other groups (*, Mann-Whitney U test, $p<0.05$). Statistically significant difference was not found between the control and pcDNA3.1 groups at any time of the follow-up. Bar: SD.

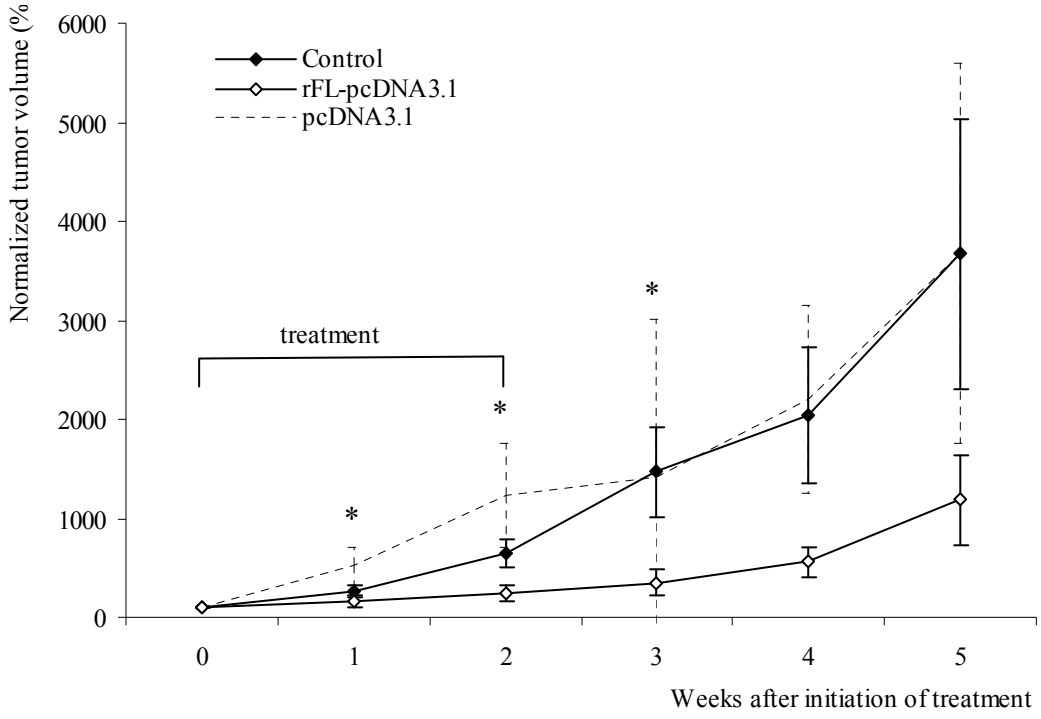
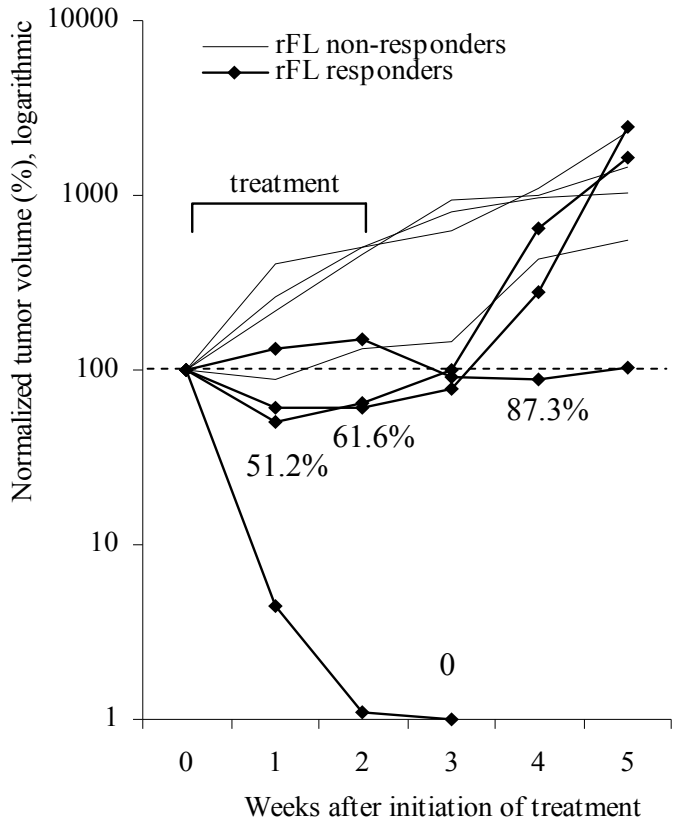


Table 20. Number of animals responding to therapy (response: tumor burden below the initial size for at least 2 weeks).

	Responders	Non-responders
Control	0	6
pcDNA3.1	0	6
rFL-pcDNA3.1	4	4

Among responders, total regression of tumor was observed in one case, regression and stabilization of tumor volume in another case following minimal initial growth. Size of the other two responder tumors decreased during the twelve-days-long therapy regimen and remained smaller at the third week than it was initially, but started to expand and reached the mean size of non-responding tumors between the fourth and fifth week. A fifth tumor, which is considered to be non-responder showed minimal size reduction at the first week but started to grow rapidly thereafter.

Figure 13. Growth curve of the eight rFL-pcDNA3.1-treated DSL6A tumors. Only 4/8 tumors responded to therapy (response: tumor shrinkage for at least 2 weeks). We observed total regression in one case and stabilization of tumor burden in a second case. The lowest tumor volume of responders is indicated. Tumor volume reduction was not seen in any other groups.



3.3.2. Flow cytometry

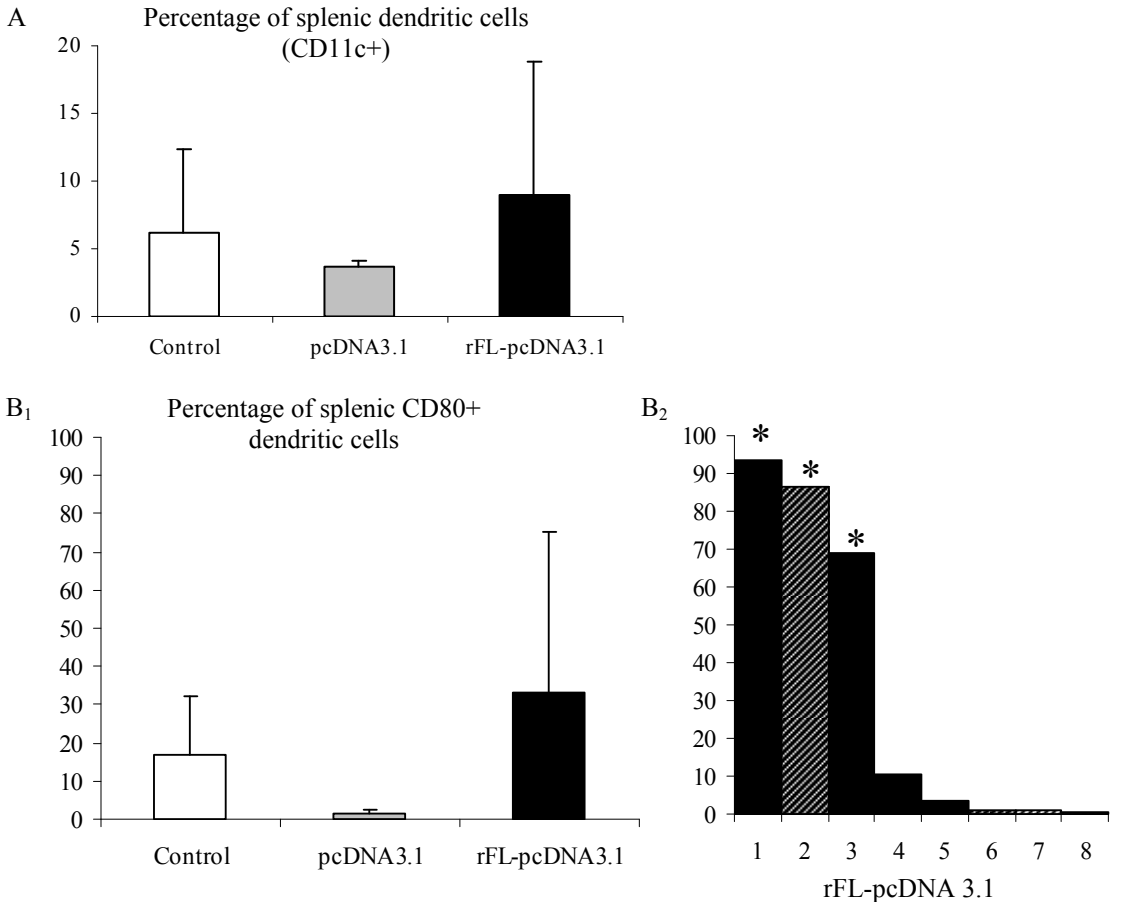
The co-expressional pattern of the following leukocyte antigens was examined from blood samples and spleens to control the systemic effect of immunotherapy: NKR-P1A, CD4-CD25, CD8-CD28, CD18-CD62L, CD11b/c-CD40, CD80, and CD86-CD40.

Analysis of blood leukocytes on days 1, 5, and 12 didn't show significant difference in expression of any of these antigens (or their combinations) in any of the subpopulation of leukocytes (gated by size and granularity) among animal groups. There was no time dependent change in these markers on blood leukocytes during therapy.

Screening of splenocytes after the sixth week with the same setting of antibodies showed obvious elevation (>70%) in the number of CD80 (B7.1) expressing large monocytoid cells (Figure 14B) in 38% of rFL treated animals (two responders and one non-responder), but not in animals from other groups. The same population of

splenocytes showed CD11b/c expression and was considered to be the population of DCs. The alteration is not statistically significant. In spite of the elevated level of the maturation marker CD80 in some rFL-pcDNA3.1 treated animals, the total number of splenic DCs was not increased in any of the animals as revealed by unaltered CD11b/c expression (Figure 14A).

Figure 14. Percentage of splenic dendritic cells at the 6th week following initiation of treatment of DSL6A tumors (A, mean+SD). The difference in the mean is not significant. Percentage of splenic CD80 expressing dendritic cells (B1, mean+SD): uniquely increased expression was found in three rFL treated animals (B2, marked with *), but not in animals from other groups. Streaked columns: response was not seen to therapy.



Flow cytometry revealed significantly elevated NK-cell number in spleens of rFL-pcDNA3.1 treated animals responding to therapy compared to non-responders and controls ($p<0.05$, Figure 15). Other meaningful differences were not found during the analysis of leukocyte markers with the above mentioned set of antibodies (Figure 16-18).

Figure 15. Percentage of NK cells among splenic (A) and peripheral blood (B) lymphocytes. Peripheral blood leukocytes were examined at days 1, 5 and 12 during therapy (control group: 3 animals, pcDNA3.1 group: 3 animals, rFL-pcDNA3.1 group: 5 animals). Splenic leukocytes were examined at the 6th week following initiation of treatment (control group: 6 animals, pcDNA3.1 group: 3 animals, rFL-pcDNA3.1 group: 8 animals). Significantly elevated number (*) of NK cells was found in spleens of animals responding to therapy in the rFL treated group compared to non-responders and controls (A, $p<0.05$). NK cell number was unaltered in the peripheral blood during therapy (B). Bars: mean+SD.

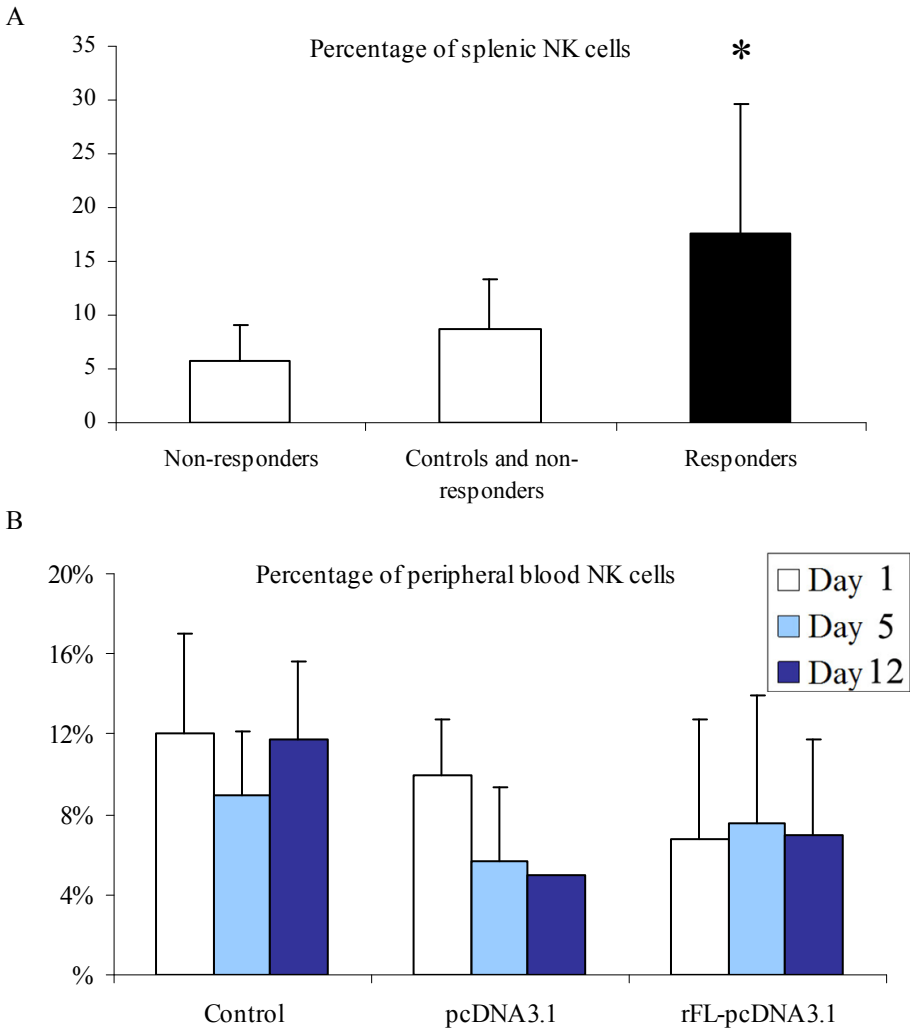


Figure 16. Percentage of splenic and peripheral blood CD4+CD25+ and CD8+CD28+ lymphocytes. Peripheral blood leukocytes were examined at days 1, 5 and 12 during therapy (control group: 3 animals, pcDNA3.1 group: 3 animals, rFL-pcDNA3.1 group: 5 animals). Splenic leukocytes were examined at the 6th week following initiation of treatment (control group: 6 animals, pcDNA3.1 group: 3 animals, rFL-pcDNA3.1 group: 8 animals). The number of CD4+CD25+ and CD8+CD28+ cells remained unaltered during and after therapy. Bars: mean+SD.

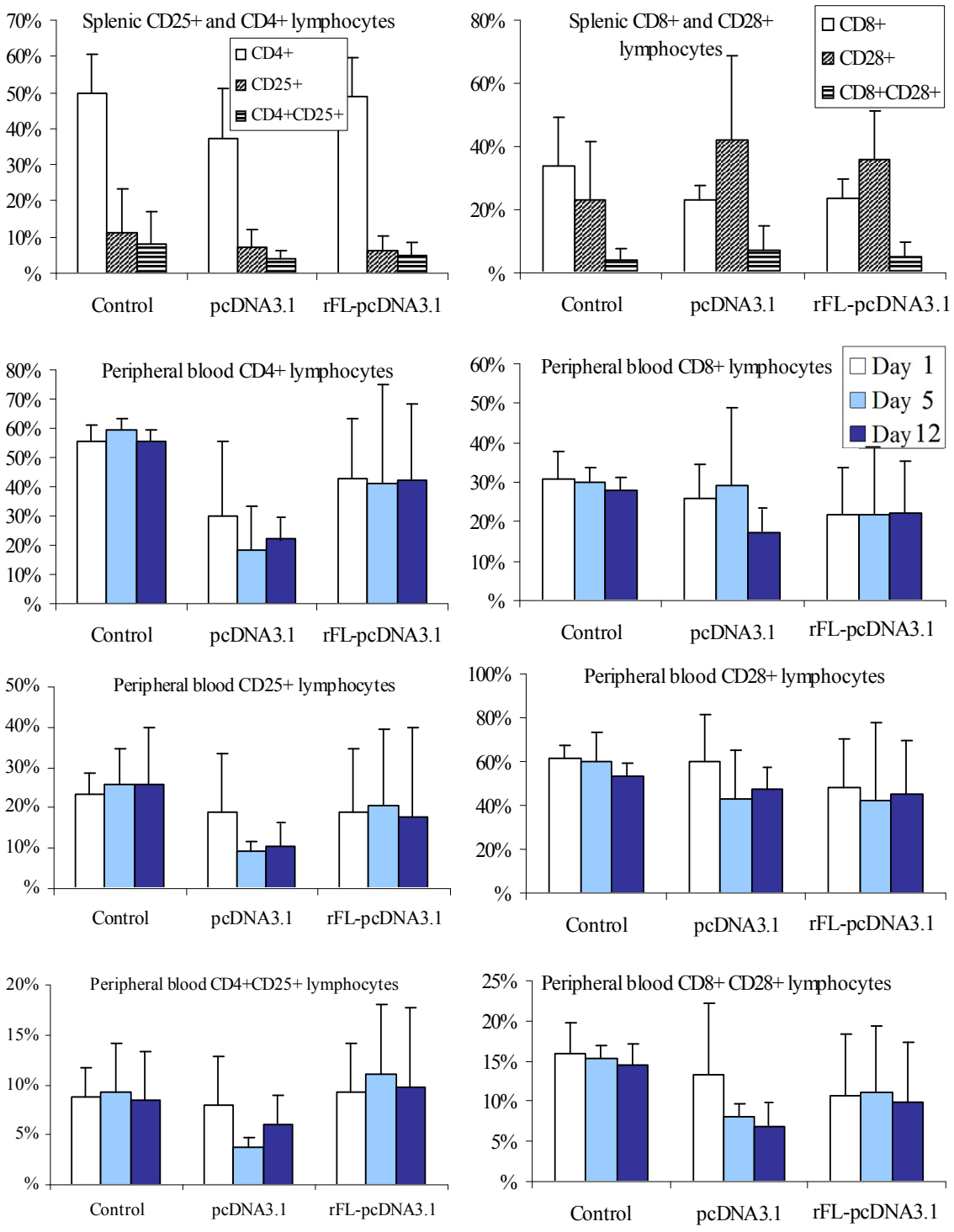


Figure 17. Percentage of splenic and peripheral blood CD62L+CD18+ lymphocytes and large monocytoid cells. Peripheral blood leukocytes were examined at days 1, 5 and 12 during therapy (control group: 3 animals, pcDNA3.1 group: 3 animals, rFL-pcDNA3.1 group: 5 animals). Splenic leukocytes were examined at the 6th week following initiation of treatment (control group: 6 animals, pcDNA3.1 group: 3 animals, rFL-pcDNA3.1 group: 8 animals). The number of CD62L+CD18+ lymphocytes and large monocytoid cells remained unaltered during and after therapy. Bars: mean+SD.

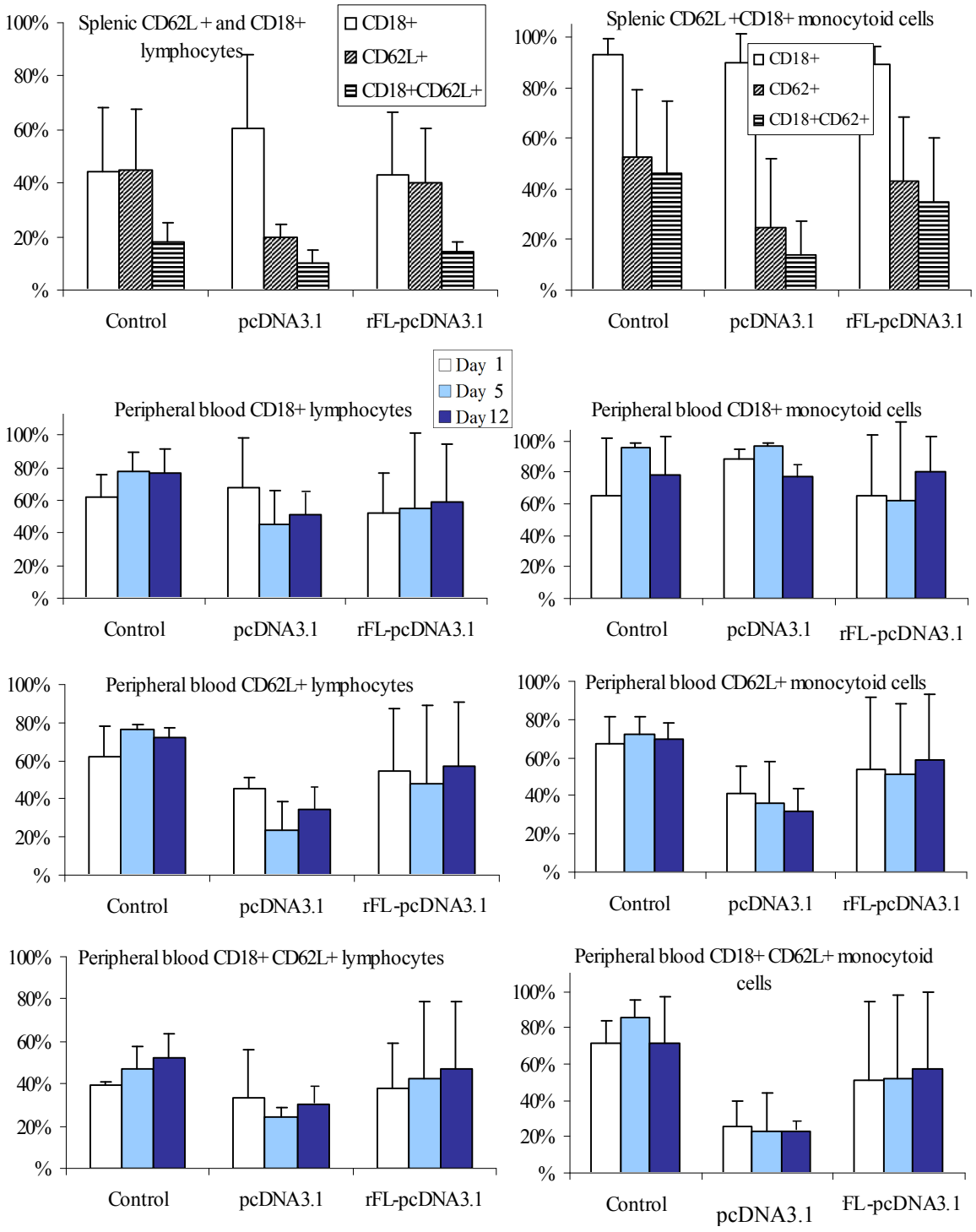
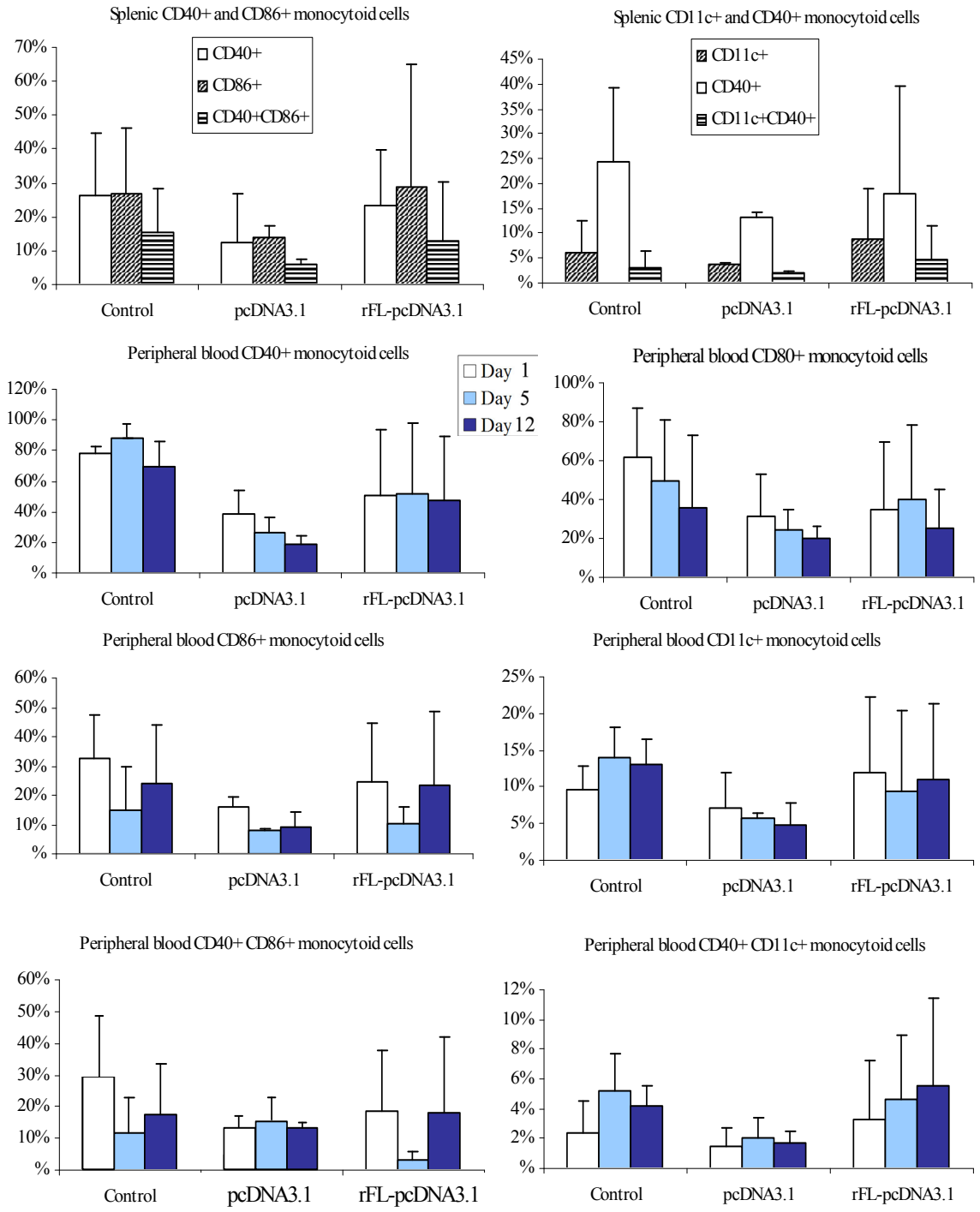


Figure 18. Percentage of splenic CD40+CD86+ and CD11c+CD40+ large monocytoid cells; and percentage of peripheral blood CD40+CD86+, CD11c+CD40+ and CD80+ large monocytoid cells. Peripheral blood leukocytes were examined at days 1, 5 and 12 during therapy (control group: 3 animals, pcDNA3.1 group: 3 animals, rFL-pcDNA3.1 group: 5 animals). Splenic leukocytes were examined at the 6th week following initiation of treatment (control group: 6 animals, pcDNA3.1 group: 3 animals, rFL-pcDNA3.1 group: 8 animals). The number of leukocytes expressing the investigated markers remained unaltered during and after therapy. Bars: mean+SD.



3.3.3. Cytokine measurements and immunohistochemistry

The plasma level of INF-γ was below the detectable level in all measurements by ELISA. Significant difference was not observed in plasma levels of TNF-α during the first two weeks of therapy among the animal groups. Time dependent change could also not be observed.

Infiltration of DSL6A tumors by CD4+, CD8+ and NK-cells was analyzed by immunohistochemistry. The presence of intratumoral CD4+, CD8+ and NK-cells was very generally very low. CD4+ and CD8+ cells were absent in most tumors. Significantly differences were not detected among the groups (Table 21).

No alteration of mean vessel density or tumor cell proliferation rate was observed in treated animals compared to controls by immunohistochemistry (RECA and PCNA staining, Figure 19, Figure 20).

Table 21. Presence of DSL6A tumor infiltrating CD4, CD8 and NK cells after rFL-pcDNA3.1 therapy. The extent of positivity was estimated double-blind using the scale: (+) low, (++) moderate or (+++) high infiltration based on immunohistochemistry.

		Control (6)	pcDNA3.1 (6)	rFL-pcDNA3.1 (7)
CD4	no	2	3	5
	+	4	3	2
	++	0	0	0
	+++	0	0	0
CD8	no	4	4	6
	+	2	2	1
	++	0	0	0
	+++	0	0	0
NK	no	1	0	0
	+	5	3	7
	++	0	3	0
	+++	0	0	0

Figure 19. Mean vessel density (RECA staining, n/mm^2) and tumor proliferation rate (PCNA staining, percentage) of DSL6A tumors. Data represent mean of 6 tumors in each group. Error bars: SD. Significant differences were not found.

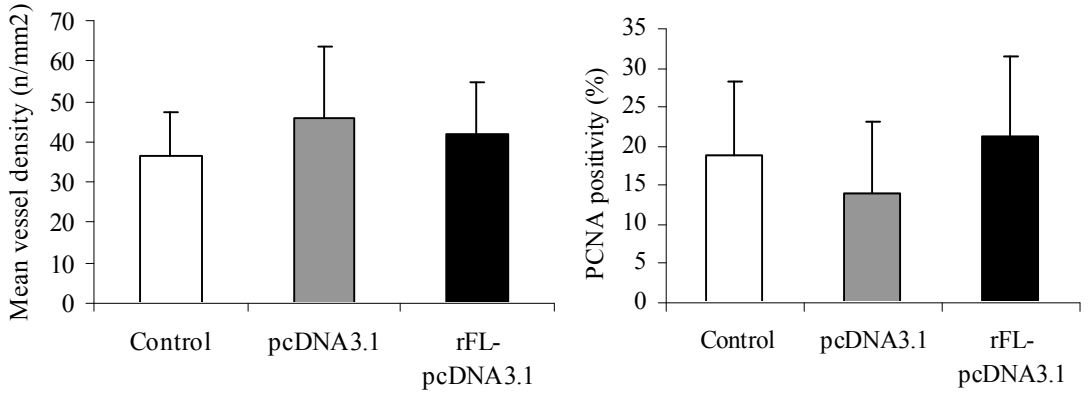
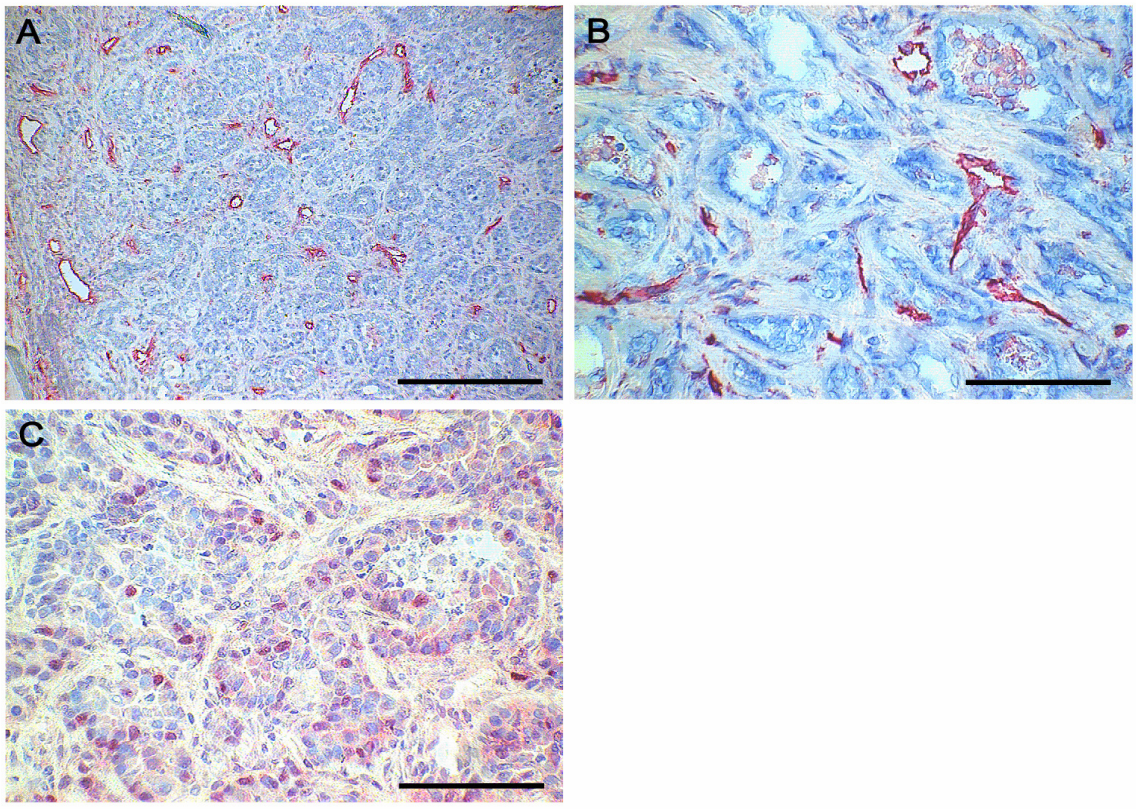


Figure 20. Representative sections of RECA (A, B) and PCNA (C) staining of untransfected DSL6A tumors. Bars: 250 µm (A), 100 µm (B, C).



3.4. Transferrin receptor expression of human pancreatic tumors

Human pancreatic tumors and normal pancreatic tissue samples were analyzed for transferrin receptor expression by immunohistochemistry. Normal pancreatic tissue, normal stromal and endothelial cells were not stained by anti-TRFC antibodies on any of the sections. In contrast, 93% of pancreatic adenocarcinomas showed positive (82%) or heterogeneous (11%) expression (Table 22, Figure 21). TFRC was strongly expressed by malignant epithelial cells; these cells were identical to cells positively stained by anti-cytokeratin antibodies (Figure 21). Primary adenocarcinomas and metastases showed similar extent of expression ($p>0.05$, Chi-square test). Three primary tumors and two metastatses demonstrated negative TFRC expression. The distribution of transferrin was diffuse and homogeneous (Figure 21).

Figure 21. Immunohistochemical staining of transferrin (A), transferrin receptor (B) and cytokeratin (C) in ductal pancreatic cancer. Transferrin shows diffuse staining. Malignant epithelial cells are exclusively stained by anti-TFRC and anti-cytokeratin antibodies. A-C: same tumor. Bar: 250 µm.

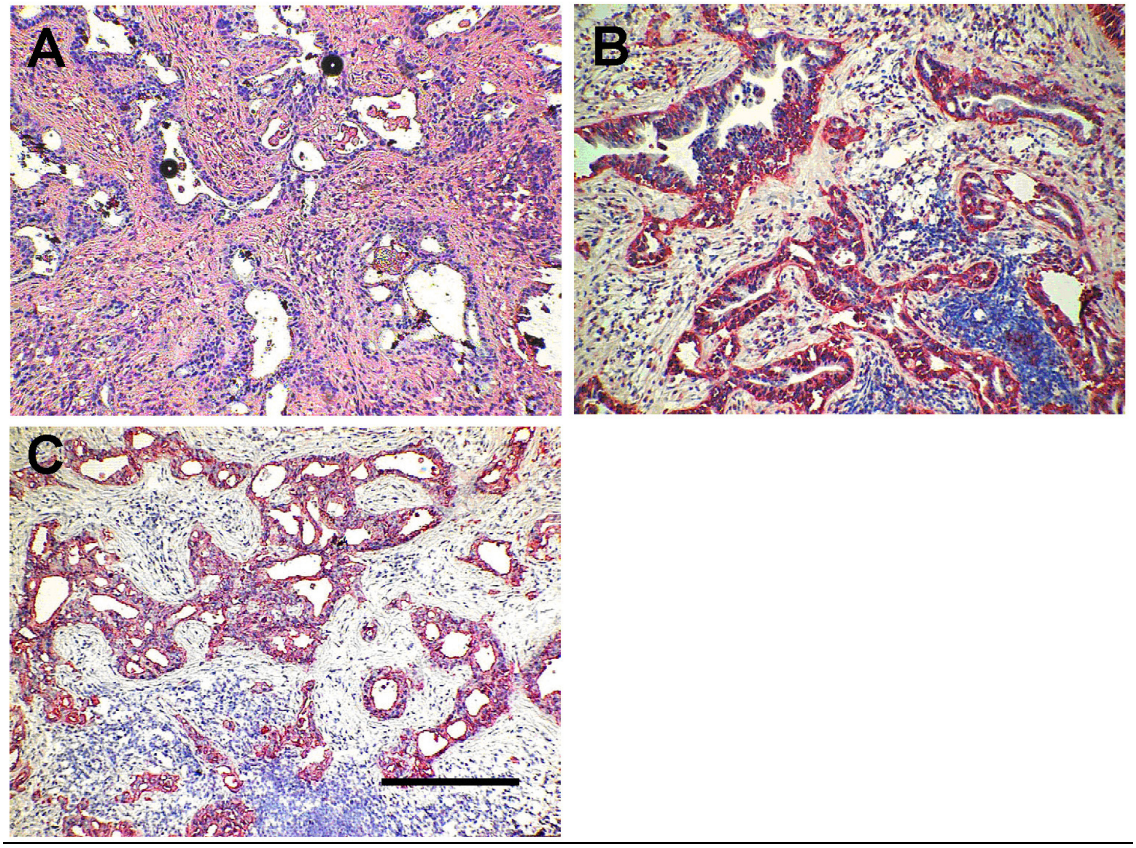


Table 22. Transferrin receptor staining of human pancreatic adenocarcinomas. Primary adenocarcinomas and metastases showed statistically similar extent of expression. Positive: >80% of cells stained; heterogeneus: 25-80% staining; negative: <25% staining.

TFRC expression	Primary tumors (39)	Metastases (12)	All tumors (51)
positive (>80% of cells stained)	32 (82%)	9 (75%)	41 (80%)
heterogeneus (25-80% staining)	4 (11%)	1 (8%)	5 (10%)
negative (<25% staining)	3 (7%)	2 (17%)	5 (10%)

TFRC expression was absent in most neuroendocrine tumors (all benign tumors: insulinomas, gastrinoma, glucagonoma and one neuroendocrine carcinoma, Table 23). Three of four neuroendocrine carcinomas were characterized by strong expression of TFRC, which was related to malignancy (Table 23).

Figure 22. Immunohistochemical staining of transferrin receptor in normal pancreas (A), ductal pancreatic cancer (B), insulinoma (C) and neuroendocrine carcinoma (D). Only malignant cells in ductal pancreatic cancer and neuroendocrine carcinoma were stained. Bar: 250 µm.

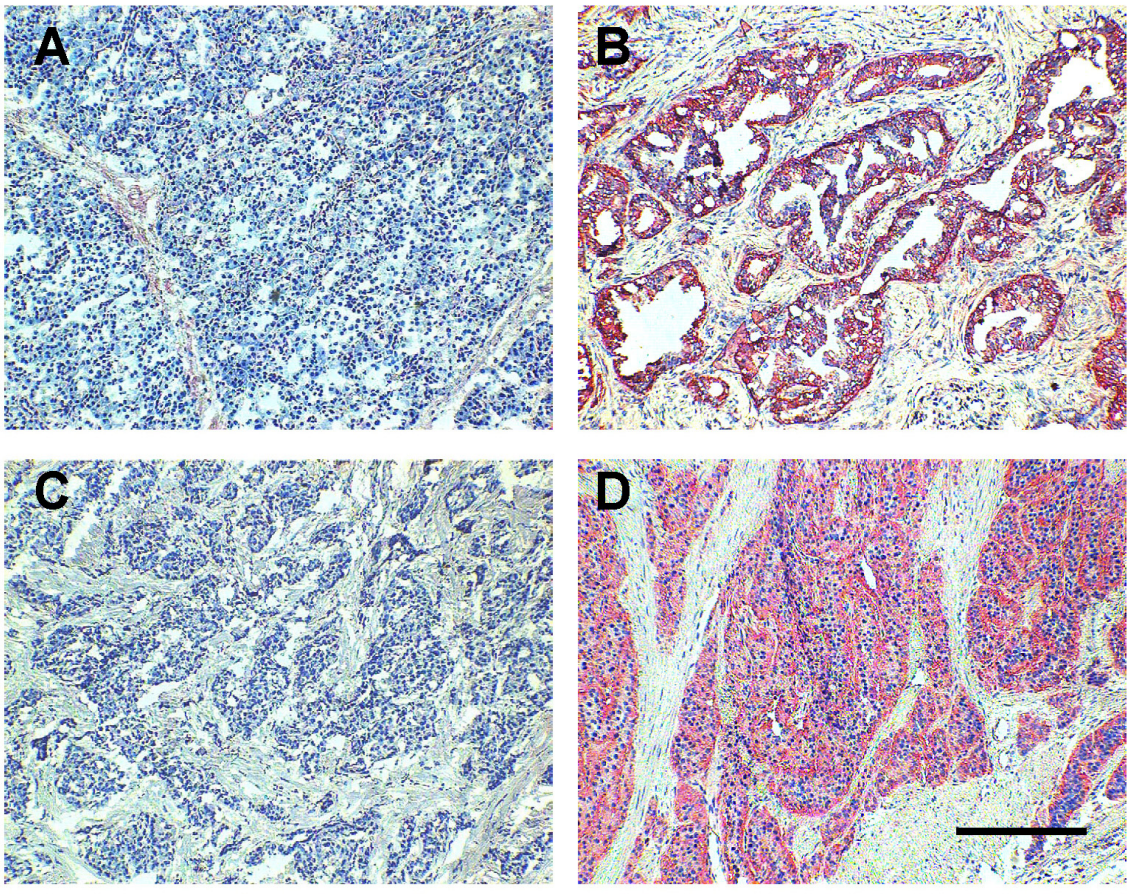


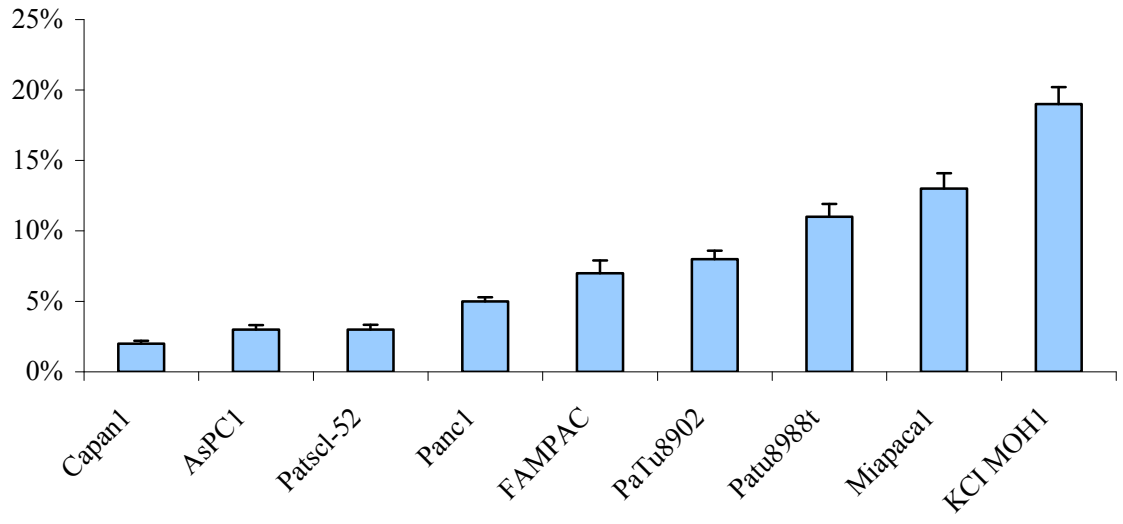
Table 23. Expression of transferrin receptor by tumor cells in neuroendocrine tumors of the pancreas.

TFRC expression	Neuroendocrine carcinoma	Insulinoma	Gastrinoma	Glucagonoma	Carcinoid
positive	3	0	0	0	0
negative	1	5	1	1	1

3.5. Transferrin receptor expression of human pancreatic cancer cell lines

TFRC expression of nine *in vitro* cultured and exponentially growing human pancreatic cancer cell lines was examined by flow cytometry. Only the minority of the pancreatic cancer cells expressed TFRC: the rate of expression was generally low. (Mean±SD = 8.9±5.6%. Capan1: 2%; AsPC1: 3%; Patscl-52: 3%; Panc1: 5%; FAMPAC: 7%; PaTu8902: 8%; PaTu8988t: 11%, MiaPaca1: 13%; KCI MOH1: 19%).

Table 24. Transferrin receptor expression of human pancreatic cancer cell lines in their exponential phase of growth *in vitro* by flow cytometry. Columns represent the mean of 3 independent measurements. Error bars: SD.



3.6. Basal dCK activity and gemcitabine sensitivity of glioma cell lines

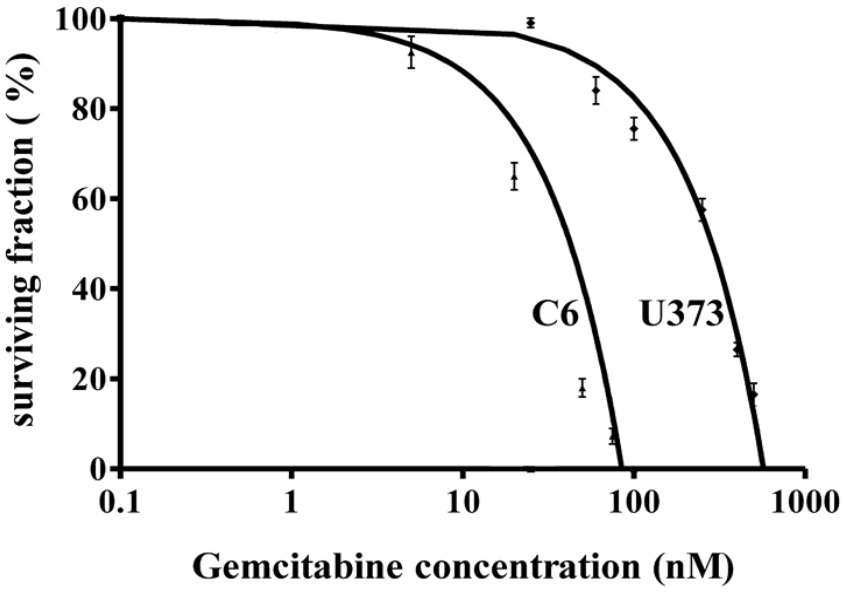
The relationship between basal dCK activity and gemcitabine sensitivity was investigated in two well characterized and frequently used glioma cell lines (human U373 and rat C6). Both the total and specific dCK activities were determined and strong differences found between the cell lines. Bigger difference was observed in C6 cells between the specific and total dCK activity than in U373 cells (Table 25).

Table 25. dCK activities of wild-type and Ad-HudCK-transduced glioma cell lines (mean values±SD). Specific activity of the dCK enzyme was determined after saturation of TK2 by an excess of thymidine. Enzyme activities are expressed in nmol/h per mg protein. All reactions were performed independently four times.

Cell line	Total activity	Specific activity
U373	0.549 ± 0.084	0.308 ±0.048
C6	0.177 ± 0.015	0.058 ±0.003

The *in vitro* gemcitabine sensitivity of the cell lines was compared: U373 cells were more resistant to the drug (Figure 23). The IC50 values were 39 and 282 nM for C6 and U373 cells, respectively.

Figure 23. Gemcitabine sensitivity of human U373 and rat C6 glioma cell lines. Surviving fraction was determined 3 days after treatment. Data points represent the mean±SD of three independent experiments.



3.7. Deoxycytidine kinase activity of Ad-HudCK transduced cells

Transduction efficiency of the investigated glioma cell lines with adenoviral vectors was calculated. U373 and C6 cells were infected with AdexCAlacZ at different MOI and LacZ activity measured (Table 26). Transduction efficiency in U373 cells was 75% at 100 MOI. Lower transduction efficiency was found in C6 cells, where 40% and 65% of the cells were infected at 100 and 300 MOI. The AdexCAlacZ construct was non-toxic for the tested cell lines even at 300 MOI.

Table 26. Transductional efficiency of C6 and U373 cell lines with LacZ expressing adenoviruses. Stained fraction of cells was compared to total cell number. Data represent the mean±SD of three independent experiments. MOI: multiplicity of infection.

MOI	C6	U373
20	n.a.	50.1 ± 6 %
100	40.2 ± 5 %	75.2 ± 8 %
200	45.9 ± 4 %	89.2 ± 5 %
300	65.3 ± 7 %	93.3 ± 5 %

Next, cells were transduced with Ad-HudCK at increasing MOI and dCK activities determined. Enzyme activities increased with the applied MOI in each cell line (Table 25). In U373 cells, enzyme levels were saturated at around 100 MOI (8.16 nmol/hour/mg protein), probably because adenoviral transduction efficiency was close to 100%. High relative increase in dCK level was detected in C6 cells, where enzyme activity at 200-300 MOI was about 140-fold higher than the basal dCK activity and reached a plateau at around 8.3 nmol/hour/mg protein.

Table 27. Total dCK activities of Ad-HudCK-transduced U373 and C6 glioma cell lines (mean values±SD). Enzyme activities are expressed in nmol/h per mg protein. All reactions were performed independently four times. MOI: multiplicity of infection.

Cell line	Ad-HudCK transduction rates (MOI)			
	20	100	200	300
U373	6.01 ±0.148	8.165±0.508	8.284±0.479	8.501 ±0.442
C6	0.839±0.113	5.312±0.734	8.066±0.148	8.313 ±0.241

During the transduction experiments with Ad-HudCK we noted that high dCK levels might be toxic for the cells. Therefore, the potential toxic effects were investigated. Cells were infected with Ad-HudCK at increasing MOI and survival followed. A strong cytotoxic effect of dCK overexpression following Ad-HudCK transduction was detected in U373 cells. The C6 rat glioma cell line tolerated high dCK levels well, since only mild cytotoxicity was detected even at 300 MOI (Table 28).

Table 28. Cytotoxicity of Ad-HudCK in U737 and C6 gliomas *in vitro*. Cells were transduced with Ad-HudCK at increasing MOI; cell survival (surviving fraction) was determined by cell counting. Data represent the mean values of three independent experiments±SD.

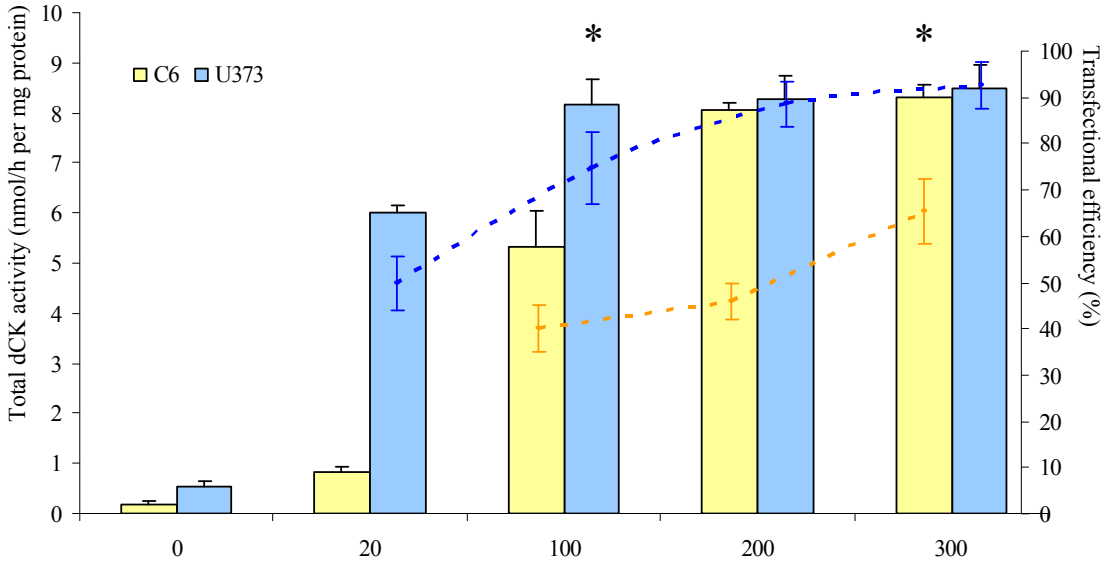
Cell line	Ad-HudCK transduction rates (MOI)			
	0	20	100	300
U373	100%	63 ± 4.16%	52% ± 5.86%	28 ± 2.08%
C6	100%	99 ± 9.61%	95% ± 17.52%	82 ± 13.89%

3.8. The effect of deoxycytidine kinase overexpression on chemoradiotherapy

3.8.1. Gemcitabine and radiation in vitro

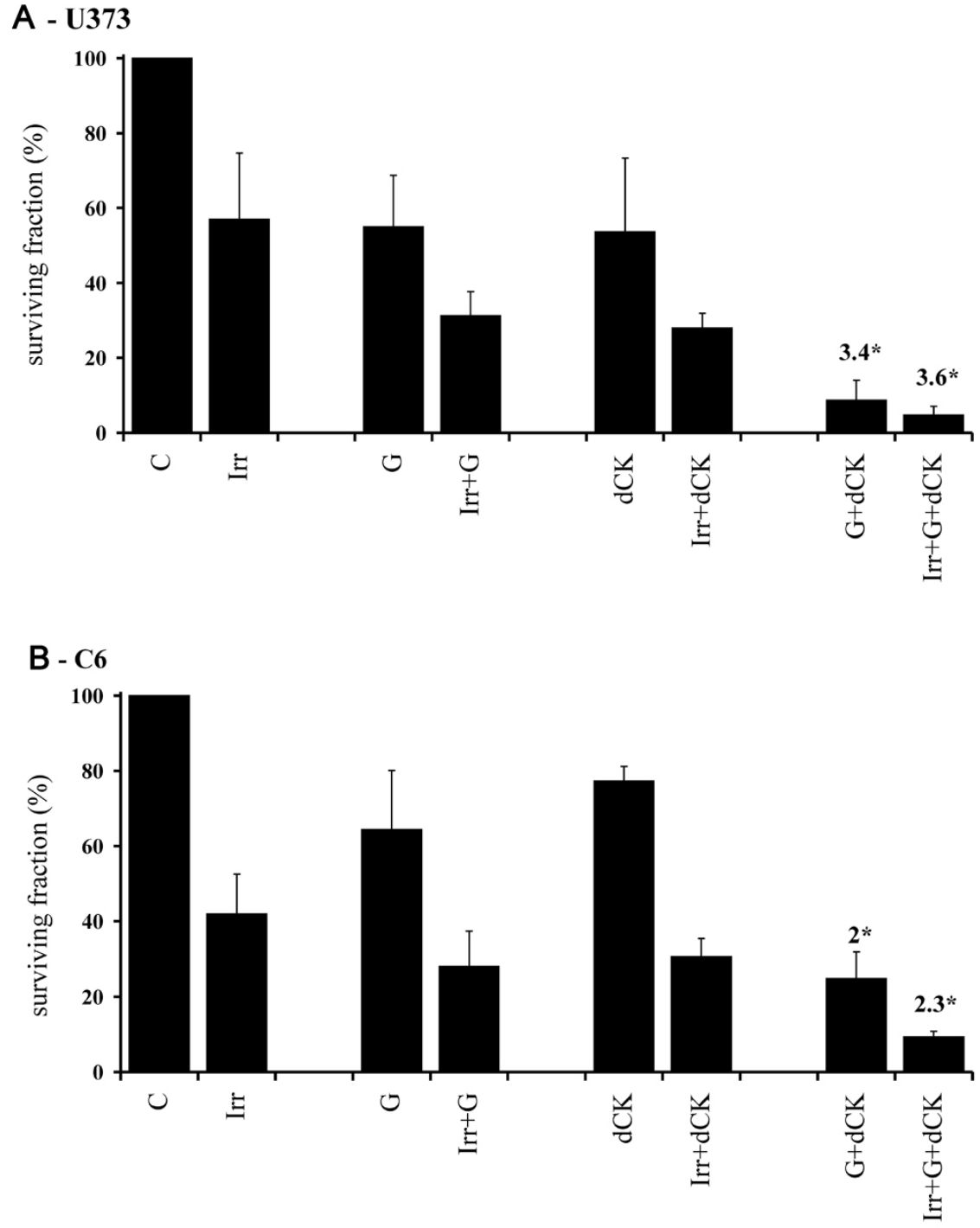
We investigated whether the introduction of the dCK gene into C6 and U373 glioma cells could increase the direct cytotoxic and radiosensitizing effect of gemcitabine. In these experiments, the different sensitivity of the cell lines toward gemcitabine treatment and adenoviral transduction was taken into consideration. Cells were treated with gemcitabine concentrations, which had very similar cytotoxic effects on their own, and were only moderately toxic to the non-transduced cells (60-80% survival; Figure 23). It was important to use drug concentrations that were only moderately toxic, otherwise the enhancement in the cytotoxic or radiosensitizing effect would have been difficult to evaluate. Thus, the following gemcitabine concentrations were used: 25 nM for the C6 cells and 250 nM for the U373 cells (estimated survival rate for U373 cells: 62%, C6 cells: 69%). When choosing the suitable viral transduction rates, two conditions should be fulfilled: to get similar transduction efficiencies and dCK activities in all three cell lines. Thus, the following transduction rates were used: 100 MOI for the U373 cells and 300 MOI for the C6 cells. These adenoviral transduction rates (MOI) gave nearly identical transduction efficiencies. The dCK activities in C6 and U373 cells were identical (around 8 nmol/hour/mg protein).

Figure 24. Increase in dCK activity after Ad-HudCK transduction and transfectional efficiency of the adenovirus vector. Transfectional efficiency of C6 and U373 cell lines at different MOI was measured after AdexCAlacZ transfection (dotted lines, %). Total dCK activity was measured after transfection of cells at the same MOI with Ad-HudCK (columns, nmol/h/mg protein). Identical dCK activities were reached at 300 MOI in C6 and at 100 MOI in U373 cells at comparable transfectional efficiencies (*). Data points represent the mean±SD of three (AdexCAlacZ) or four (AdHudCK) independent experiments.



In the experiments combined with irradiation, cells were treated with 4 Gy 60Co-γ-radiation (Figure 25, 35-60% survival in non-transduced cells). Deoxycytidine kinase overexpression had different toxic effects: about 60% and 80% survival in U373 and C6 cells, respectively. Increased dCK activity had no radiosensitizing effect in the studied cell lines under *in vitro* circumstances. In the absence of dCK overexpression, the combination of gemcitabine treatment and irradiation had only additive effects in U373 and C6 cells. Deoxycytidine overexpression enhanced gemcitabine toxicity in both investigated cell lines (2 and 3.4-fold enhancement in C6 and U373 cells, respectively). Deoxycytidine kinase overexpression also improved the radiosensitizing effect of gemcitabine, by a degree very similar to this in the studied cell lines: the effect was strong both in C6 and U373 cells (2.3 and 3.6-fold, respectively).

Figure 25. Radiosensitivity of glioma cell lines after transduction with Ad-HudCK and treatment with gemcitabine and irradiation. Cells were transduced with Ad-HudCK at 100 MOI (U373) or 300 MOI (C6) 1 day after plating. The next day cells were treated with 250 nM (U373) or 25 nM (C6) gemcitabine. Twenty-four hours later, cells were irradiated with 4 Gy. Cell survival and enhancement rates were determined as described in Methods on day 7. C:untreated; Irr: irradiated; G: treated with gemcitabine; dCK: transduced with the Ad-HudCK vector. Data represent the mean of three independent experiments. Error bars: SD. The values above the columns represent the enhancement rates of the different combinations. Values marked with * show significant differences to the calculated additive effects of the corresponding combinations ($P<0.05$).



3.8.2. Gemcitabine and radiation in vivo

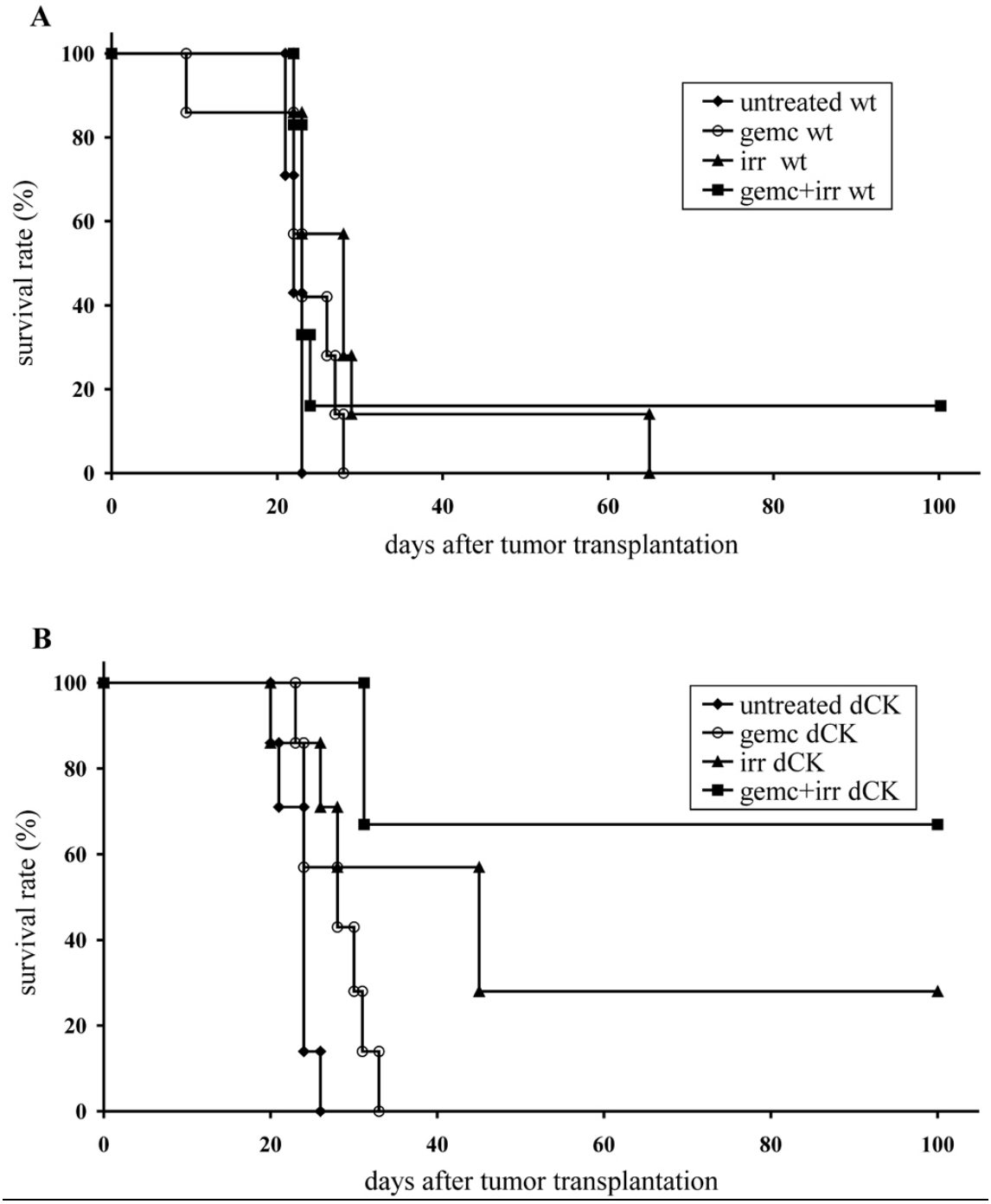
The rat C6 intracranial tumor model was used to investigate the *in vivo* effects of dCK overexpression. Intracranial tumors were established by implanting either wild-type or dCK overexpressing tumor cells. To create dCK overexpressing cells, *in vitro* growing C6 cells were transduced with Ad-HudCK (300 MOI) one day before tumor transplantation. This MOI was selected for transduction as the one that increases intracellular dCK levels substantially (Table 28). Animals were treated with gemcitabine and local tumor irradiation.

Overexpression of the dCK gene had no effect on *in vivo* tumor growth. Gemcitabine treatment alone had minor effect on tumor growth, and dCK overexpression could not improve this effect. In contrast, dCK overexpression increased the anti-tumor effect of local tumor irradiation: 28% survival rate was found among irradiated animals that were implanted with pre-transfected C6 cells (Figure 26). In the rat C6 model, the combined treatment with gemcitabine and tumor irradiation in the absence of dCK overexpression resulted in 16% survival and practically no increase in the animal life span (Table 29). Significantly improved ($P<0.0162$) survival rate (nearly 70%) and animal life span (about 41%) was detected when gemcitabine treatment and irradiation was combined with dCK overexpression (Figure 26B, Table 29). dCK overexpression, apart of its own minor *in vivo* radiosensitizing effect could improve the combined effect of gemcitabine treatment and local tumor irradiation.

Table 29. Changes in the life span of animals treated with gemcitabine + irradiation or Ad-HudCK + gemcitabine + irradiation. „Cured" (=survived longer than 100 days) animals were excluded from calculation of the mean survival times. Increase in life span was calculated as follows: MSTtreated animals/MSTcontrol animals–1) x 100; MST: mean survival time

Treatment group	Cure rate	Mean survival time (MST, days)	Increase in life span
Untreated	0 %	22.14 (±0.9)	-
Gemc.+irradiation	16 %	23 (±0.7)	4 %
Gemc.+irrad.+ Ad-HudCK	67 %	31.3 (±0.58)	41 %

Figure 26. Survival of C6 glioma bearing rats after gemcitabine treatment and local irradiation. Gliomas were induced in rats by intracranial transplantation of either wild-type (wt) C6 cells (A) or C6 cells transduced with the Ad-HudCK (dCK) vector (B). Rats were treated with gemcitabine (gemc) and local tumor irradiation (irr). Survival curves represent the pooled data of three independent experiments. Each group consisted of 4-5 animals. P<0.0162 for the group treated with Ad-HudCK, gemcitabine and local irradiation versus the group treated with gemcitabine and irradiation.



4. Discussion

4.1. The used tumor models

The used pancreatic cancer and glioma cell lines represent well established systems for *in vitro* experiments (see also 2.3.1 and 2.7). Experimental murine cancer can also be induced by administration of carcinogens *in vivo*[265], but the long time of incubation, the impossibility to predict the exact time of tumor development, and the poor organ specificity makes it impossible to use such a system for (immuno-) gene therapeutical approaches of specific tumor types. There are two possibilities regarding the site of tumor cell implantation: orthotopic or non-orthotopic (e.g. subcutaneous) implantation. We used a subcutaneous model in the immunological study. There are two major advantages of this model. First, it allows easy and controllable manipulation of the tumors including intratumoral injection of various substances during the therapy. Second, consecutive tumor growth could be relatively accurately documented. Orthotopic growth of tumors resembles the clinical situation better; some environmental factors such as lymphatic and blood vessel connections are obviously different at every other implantational site. For radiosensitizing GDEPT, we used an orthotopic glioma model by implanting pre-transfected tumor cells by intracranial stereotaxic injection, which is a well reproducible method. This model system resembles the human situation of anti-glioma chemo-radiotherapies very well. We did not need to measure the size of brain tumors - survival analysis was selected to assess the efficacy of the treatment.

4.2. The rat Flt3-ligand

The amino acid sequence homology between the mouse and human FL was reported to be 72%[266,267]. Recently, the canine and the feline FL has also been cloned[268]; the canine FL amino acid sequence showed 71% and 52.8% homology to human and mouse, respectively. This number was 70.2% and 54.1% in the feline-human and feline-mouse comparison. In this work we described the rat FL cDNA sequence for the first time. The used method of sequencing and cloning has been described before in detail[238, 269]. Not surprisingly, analysis of the cDNA sequence and its translational product *in silico* revealed higher identity between mouse and rat (90% in amino acid sequence), than between human and rat (73%) peptides. However, difference between mouse and rat

sequences still exist, and this information may have important implication on further studies with FL in rats.

Former murine studies about FL-based immunotherapies were accomplished mainly in mice models or hamsters, very rarely in rats. It is varying if human or mouse protein or cDNA was applied. However, in spite of the high homology between mouse (mFL) and human FL (hFL) and the fact that they were reported to be able to cross-react[94], they were found to act differently in murine models[111]. In this latter study mFL was not only proved to be less potent in expanding DCs, but the mobilized DCs had different phenotype and failed to protect against tumors, where hFL was protective. Differences in activity of murine versus human FL have been already reported before, but the authors did not emphasize its importance[110]. This observation may have considerable implication on how we interpret previous data, since hFL was used in most of murine studies. Our goal in this experiment was not to compare the therapeutic effect of all the known FLs from different species, but to study the species-specific gene product: a situation that is undoubtedly the most easily imaginable in humans.

4.3. Liposomal transfection of DLS6A tumors *in vivo*

4.3.1. Possibilities of gene transfer – general considerations

Many gene delivery systems have been developed in the last decades to improve the effectivity of transfections compared to naked-DNA injection - a method which was described as one with very poor efficiency[270] – including viral and non-viral carriers. Naked DNA-injection is usually not efficient in a therapeutical setting and did not became the standard of gene therapeutical approaches. The half life of DNA in the blood stream is short and it is rapidly cleared when injected locally[271]. Interestingly, recent data questioned some of these statements[272] as liver and muscle cells were be able to efficiently take up DNA[273] even when injected systemically. Thus, naked DNA has been recently used for local gene delivery into muscles – together with other techniques facilitating DNA uptake such as electroporation[272]. Still, despite of some promising results[70, 167, 274-276], the applicability of the technique in tumor gene therapy is restricted.

An effective alternative is the use of viral vectors. Since viral systems generally ensure the highest transfection rates currently available, they stay in the middle of interest in vector engineering. Among them adenoviruses emerged as the leading candidate for

gene delivery - they can be produced in high titers, do not integrate into the host chromosome, and have a wide tropism. Adenoviral vectors infect both dividing and non-dividing cells, have high stability *in vivo*, and have a relatively high capacity for gene transfer[277]. Although viral vectors are still superior in their transfection ability compared to any other gene delivery system[278], there are some considerations that may limit their use. Application of viruses has raised a number of safety concerns[279, 280], including possible mutagenesis and immunological responses, apart from limitations of their large-scale production. The use of non-viral gene delivery systems in sensitive immunological studies may be especially important taken into consideration that viral particles themselves interact with the immune system[281].

4.3.2. Lipoplexes

As non-viral gene delivery systems, lipoplexes offer a highly effective and extensively used alternative both for free DNA and viral vectors and stay in the first line of gene therapeutical experiments in human trials[270]. We chose to use lipoplexes in our immunological experiment since they are (1) capable for effective local transfection, (2) they are less immunogenic than viral vectors and (3) it is very easy to obtain them.

Lipoplexes are complexes of DNA with cationic lipids. In aqueous media, cationic lipids are assembled into a bilayer vesicular-like structure, liposomes. Since Felgner and colleagues exploited the first cationic lipid N-[l-(2,3-dioleyoxi)propyl]-N,N,N-trimethyl-ammonium chloride (DOTMA)[270] as vehicle for the transfer of DNA into eukaryotic cells in 1994, many new cationic lipids have been synthesized. The principle of action is the same in all of these systems. Due to charge interaction, cationic lipids spontaneously associate with nucleic acids, resulting in the formation of lipoplexes. Nucleic acids are condensed on the interior of invaginated liposomes between two lipid bilayers[282]. These membranous structures are taken up by cells by endocytosis after non-specific binding to cellular receptors; this is followed by the release of the DNA into the cytoplasm. The mechanism of nuclear import of the DNA is largely unknown, but is most likely a protein-mediated process[270].

Cationic liposomes as carriers for genetic material are usually very efficient *in vitro*[270]. In contrary, their *in vivo* efficiency is worse, usually not higher than 4% when applied intratumoraly[283]. Many physical factors influence the stability, complex formation and transfection efficiency of lipoplexes such as particle size, DNA/liposomes ratio and

ionic strength of the used medium[284, 270]. Correlation between *in vitro* and *in vivo* experiments can usually not be obtained[284]. It has been stated that only empirical findings would remain the main source of data about structure/activity relationship for cationic lipids[284,285].

Cationic lipids have been successfully used for direct intratumoral gene delivery. An obvious advantage of liposomes is their ability to remain in the tumor, while naked DNA is cleared from tissues within hours[167]: direct injection of DNA in a lipoplex was reported to be significantly more efficient than naked DNA in induction of interferon-α2 expression in human basal cell carcinoma[286] and UM449 tumor model[287]. Many groups have demonstrated preclinical efficacy using intratumoral cationic lipid-mediated gene transfer of cytokines[288, 289], alloantigens[290, 291], and prodrug-converting enzymes[292]. It has been reported, that intratumoraly injected cationic lipids can inhibit transfection of cells in some tumors but not in others[287]; the mechanism is still unknown. These findings indicate that the measurement of transfection efficiency is crucial before experiments with cationic lipids.

We used here a novel DOTAP - cholesterol liposome formulation, a construct that is very easy to preparate and has already been evaluated in human gene therapy[293]. Because of their poor stability (i.e. continuous aggregation), the lipoplexes had always be administered directly after their formation[284]. In our study, high *in vivo* transfection rates at about 10% could be reached with this carrier system. This means that we reached high transfection rates superior to the ones reported before with this lipoplex, or even with adenoviruses in some cases. The β-gal staining technique is a standard method widely accepted for the quantitative analysis of transfectional efficiency. However, most of the data from the literature examining transfection efficacy of lipolexes is based on flow-cytometric analysis of whole tumor lysates which allows the measurement of the entire tumor tissue and not only ‘representative’ sections that was done in our case by light microscopy. Although the sections analyzed here were picked from different parts of the tumors, and the sections seemed to be stained homogenously, it can not be excluded that the error of our method is larger than that of the flow-cytometric measurement. The computer assisted method for quantifying the transfection rate is an accepted method used by pathologists[245] and should theoretically have given the same result to the manual method of cell counting, supposing that the size of

transfected and non-transfected cells does not differ significantly. Since every kind of cell in the tumor was expressing LacZ, the technique was believed to be feasible[245]. We didn't examine liposome:DNA ratios in these experiments other than the one offered by the product vendor. It would be interesting to further investigate the limits of DOTAP:cholesterol based liposomal transfection in this model.

4.3.3. FL expression of transfected DSL6A cells

FL specific ELISA after transfection of DSL6A cells both *in vitro* and *in vivo* showed that transfected tumor cells express FL (Figure 11). It is known, that both soluble and membrane-bound forms of FL exists[99], with similar activity and effect *in vivo*[110, 294]. Transfected DSL6A cells efficiently produced soluble FL, the spliced variant of the membrane bound form *in vitro*, as proved by ELISA from cell culture supernatants.

Elevated FL expression was detected after *in vivo* transfection of s.c. implanted DSL6A tumors as well; this measurement detected both the membrane-bound and soluble forms of the protein. Others also proved successful transfection by FL-expressing vectors: elevated level of mRNA (70-270 fold increase 1 or 3 weeks after injection of plasmid vectors into skeletal muscle)[107], or FL protein production (350-fold increase from brain lysate 1-5 weeks after striatal injection of adenoviruses)[130] was reported following local transfection. Single intratumoral injection of liposome-coated plasmid-rFL resulted only in moderate FL elevation in our case. This is perhaps attributable to the used liposomal system itself; adenoviruses used in the aforementioned study[130] are known to be superior in transfection efficiency and protein production. The used expression unit in our experiments did not contain a replication origin for continuous expression, proteins production was temporally limited. In the first referred study[107] the plasmids coding for FL were injected repeatedly, which also may have contributed to the high elevation in mRNA levels; although it is hard to speculate the increase of consecutive protein expression, which was not directly measured.

We measured FL levels 48h post transfection; this time was selected based on literature data. In a previous study describing the kinetics of local cytokine (IL-2) production following intratumoral transfection with cationic liposomes the peak of expression was detected 48h after transfection, but the level of local IL-2 dropped rapidly by day five[287].

4.4. Impact of rFL therapy on DSL6A tumor growth, response to therapy

Efficacy of therapy was assessed by weekly measurement of the size of the tumors in the DSL6A pancreatic cancer model. The size of subcutaneously growing tumors could be safely measured after they reached 5 mm in diameter. After analyzing the growth curves, we termed animals as “responders” or “non-responders” to therapy for statistical analysis.

“Therapeutical response” can be defined according to various parameters including longer survival, reduction of tumor burden or inhibition of tumor growth (stabile disease), or according to “softer” criteria such as changes in immune parameters or improvement in the general condition of the animals. Appropriate definition of “response to therapy” in tumor-immunological studies is not always easy, since the effects of immunotherapeutic treatments are often mild. Theoretically, being able to recognize any alteration of the immune system could already be interpreted as "response" of the host, even without antitumoral effect. We defined “response to therapy” according to the growth curve of the tumors; it is perhaps the clinically most relevant feature and is the most important factor in regard of transferability to the human situation. “Response” was defined as a relatively long-lasting and obvious decrease in the tumor burden that can never be seen in untreated DSL6A tumors: a reduction of the tumor volume for at least 2 weeks. After DSL6A tumors reach the 5 mm in diameter, they grow continuously and exponentially for several weeks[295]; this was reproduced in this study (control group). We chose the time limit of at least two weeks (3 measurements) to exclude the potential error of a measurement at the early phase of growth. The decrease in the tumor burden is also important: untreated DSL6A tumors do not show reduction of their volume until they reach a diameter of several centimeters and develop central necrosis. Since most tumors recovered from the initial tumor growth suppression, a survival analysis did not seem to be reasonable in the pancreatic cancer study.

The size of tumors represents the basis of response to therapy in many human studies as well. However, there is often confusion as one aims to compare these data[84], because some authors use softer criteria than others when interpreting results. Especially by patients with large tumor burdens or disseminated disease or where the prognosis is dismal has it been proposed that even "stabile disease" or "no progression" could be

evaluated as positive response[28, 84]. However, such phenomena can occur in the natural course of human tumor growth[84]. Standard oncologic criteria for evaluating and reporting objective clinical responses to treatment are well established in oncology, and adherence to these guidelines is essential in comparing the results of treatment protocols in humans[84, 142]. A set of criteria proposed recently is the Response Evaluation Criteria in Solid Tumors (RECIST): a 30% reduction in the sum of the maximum diameters of lesions to indicate a response, along with the appearance of no new or progressive lesions. The most commonly used definition of objective clinical response, however, is at least a 50% reduction in the sum of the products of the perpendicular diameters of all lesions without the 25% growth of any lesion or the appearance of new lesions. These guidelines say that objective loss of tumor burden must be reached, which is still a rare finding in immunotherapeutic approaches against cancer, but not in many conventional chemotherapeutical approaches.

As seen here, the used guidelines for humans offer more strict criteria than the ones used in this and other[113, 120] animal studies, mainly because the growth of human tumors may not be so regular than that of the animal models and the control groups are not so homogenous. It is easier to show the effects of therapy in animal models of cancer, since the tumors are genetically similar in every animal along with other factors such as site of implantation or absence of concomitant diseases. The growth of these tumors should not differ too much. This was the case in our model, where expansion of DSL6A tumors was similarly fast in non-treated animals after forming a small initial tumor nodule felt by palpation under the skin. The only difference between the tumors was the time they needed to reach the initial 5 mm in diameter (10-12 weeks) after implantation, but this is probably attributable to the differences in the implanted tumor cell number and is a usual phenomenon. Dramatic alteration from these growth curves or even tumor regression was only found in rFL treated animals, so we believe that the designated "response" to therapy as a clearly visible decrease of tumor burden which is never seen in untreated animals reflects the effect of rFL expression by the treated tumors.

It has been shown in former studies that intratumoral therapy with liposomes alone can cause tumor growth inhibition or can influence the metastatic capability of tumors [296]. Untreated and liposome treated tumors grew similarly without significant difference at

any time point of follow up in our experiments, showing no impact of needle punctures or presence of liposomes on tumor growth.

The present study demonstrated that in a model of subcutaneously implanted rat pancreatic cancer, repeated intratumoral transfection with plasmid-rFL augmented by lipoplexes may lead to tumor growth retardation (responders in the rFL group: 50%) or total tumor regression (12.5% in the rFL group). However, tumor growth could not be influenced in every case, and most of the tumors (87.5%) began to expand similarly to controls shortly after therapy. This observation is consistent with data from the majority of studies applying rFL systemically[118, 121] or locally[135], where the authors described unaltered tumor growth after the cessation of treatment. However, two recent papers – both published after the completion of our experiments - described total tumor regression in every treated animal after local transfection with adenoviral vectors [107, 130].

From the analysis of the growth curves we conclude that intratumoral injection of FL coding plasmids (1) may lead to total or partial tumor regression in the DSL6A pancreatic cancer model, and (2) this effect is attributable to FL and not other factors of therapy. However, it seems that most of even those tumors that were partially inhibited in growth overcame the immune attack after a short period of time.

4.5. Immune changes after rFL–gene therapy, mechanism of action

Increasing the number of antigen presenting cells in the host may break tolerance in case of some but not all mice models of cancer, as shown by numerous experiments where rFL was administered systemically[53-56, 102, 103, 297, 110, 114-118, 120, 121, 298]. It is very probable, that the increase in the number of DCs leads to amplified uptake of tumor cell antigens that results in an amplified anti-tumoral immune response. However, similarly to our data, growth inhibition of tumors was many times only a temporary finding [54, 56, 102, 118, 120, 121, 135, 297, 298]. Several mechanisms can account for a limited antitumoral immune response against tumors; all of which might have been responsible for the failure of our therapy as well. (1) Tumors may not express (enough) antigens that are recognized by the immune system. (2) The number of the effector and/or antigen presenting cells may be insufficient in the host, where they must traffic to and infiltrate the tumor stroma. (3) APCs and effectors may remain immature or not activated in the host or inside the tumors and thus may not mediate antitumoral response, or may even

mediate tolerance to the acquired antigens. (4) Tumors themselves may inhibit or directly delete the effectors (e.g. T cells) of the immune system[22].

We neither have found alteration in the examined leukocyte numbers in peripheral blood during therapy, nor experienced lymphadenomegaly or splenomegaly showing rather limited changes of the immune system. Splenic cell compartments were also found to be roughly unaltered in terms of major leukocyte compartments and markers of activation. Only splenic NK cell (but not DC) number was significantly elevated in responders to therapy, suggesting a slight systemic response even 28 days after the last injection. We detected extremely low levels of tumor infiltrating $CD4^+$, $CD8^+$ T lymphocytes and NK cells by immunohistochemistry (Table 21) in every tumor regardless of response to therapy. Effectors of the immune system do not seem to have any effect on the tumors after four weeks of transfection with rFL. Microvascular density and tumor proliferation rate was also unaffected in the surviving tumors; tumors overcame the temporary inhibitory effects provided by the immunotherapy.

4.5.1. The number of APCs/effector cells in the peripheral blood and the spleen

The cause of the limited success of our treatment might have been the inability to increase the number of systemic and/or intratumoral APCs and effector cells for a sufficient period of time. One cause of this could be perhaps the insufficient production of the cytokine. The antitumoral effect of systemic FL treatment is known to be dose and time dependent[105]; dose-dependent increase in the survival of glioma-bearing rats was found after local (adenovirus mediated) rFL treatment as well[107]. It is very likely, that FL production of the transfected tumors decayed after about one week of the last injection as suggested by prior studies describing the kinetic of intratumoral cytokine production after transfection with plasmids coated in cationic liposomes[287]. Achieving higher intratumoral FL level might have improved our results.

It has been documented that changes in leukocyte fractions in peripheral blood may not accompany the FL production of tumor cells after local gene delivery, even in the presence of an augmented immune response[85]. INF-γ and TNF-α levels did not increase in the peripheral blood during our treatment, which also reflects that any change caused by the therapy remained rather locally limited. Plasma cytokine levels may not accompany local administration of FL cDNA, but again, this does necessarily mean that the augmentation of the immune answer is ineffective[85]. Splenocytes reflect

immunological changes better than peripheral blood leukocytes after FL administration[105]. In accordance with these finding, we measured the number of NK cells to be higher only in the spleens of responders to therapy. The kinetic of the cell expansion upon systemic FL treatment has already been described in detail; from these data we think, that not detecting other major changes in splenic leukocyte compartments might have been the result of the decayed immune reaction 4 weeks after the last injection. After cessation of FL injections, the number of DCs in spleen (and peripheral blood) was reported to return shortly to normal level[105]. The increased number of splenic DCs also decayed rapidly after a peak level measured on the 7th day following intramuscular delivery of an FL-coding plasmid by electroporation[133]. In other cases only local recruitment of DCs (1 week post injection) was found with unchanged splenic and blood leukocyte populations (3 weeks post injection) after intramuscular FL cDNA delivery[85]. Our results reinforce the role of NK cells in FL-based therapies, but detecting elevated level of splenic NK cells as late as 4 weeks after cessation of therapy was not yet described by others[103].

NK cells were recently described to effectively interact with DCs, the major cell population affected by FL. The role of NK cells may not confined to the destruction of virus-infected cells or tumors[299, 300] but NK cells interact with other innate immune cells that are present during the early phases of inflammatory responses[301]. These interactions can result in shaping both the innate immune response within inflamed peripheral tissues and the adaptive immune response in secondary lymphoid organs. It has been reported, that an 'NK–DC cross-talk' follows the recruitment of both NK cells and DCs to sites of inflammation[302, 301]. DCs are now known to support the tumoricidal activity of NK cells[64, 66, 303] while cytokine-preactivated NK cells have been demonstrated to activate DCs and to induce their maturation and cytokine production[64, 66]. Such NK–DC interaction promotes the subsequent induction of tumor-specific responses of $CD4^+$ and $CD8^+$ T cells, allowing NK cells to act as "helper" cells in the development of the responses against cancer[66].

4.5.2. The role of intratumoral leukocytes

To elicit effective antitumoral response, APCs have to reach the tumors, acquire antigens, and migrate to lymph nodes; whereas the effectors have to infiltrate again the tumor stroma. The level of infiltration by DCs may be an important factor in tumor

prognosis: elevated level of dendritic cell infiltration correlated well with tumor regression in a murine study where tumors from FL treated animals were analyzed[120]. Enhancing local DC recruitment in pancreatic cancer may be especially important, since these tumors were characterized by the loss of intratumoral DC infiltration, a possible cause of immune tolerance[304]. Lymphocyte infiltration in human pancreatic cancer was recently shown to have prognostic relevance[34]. The major cause of failure of systemic FL treatments may be their inability to enhance the number of tumor infiltrating APCs and effector lymphocytes[117] - DCs did not infiltrate colorectal tumors in humans treated with FL; they remained only in the peritumoral area[126]. These findings provided rationale for experiments aiming to enhance intratumoral FL level.

As shown by previous studies, local application of FL coding vectors may recruit DCs to the injection site[85, 107, 130, 133] without systemic alteration of DC number. Intratumoral application of FL-coding adenoviruses could elicit systemic immune response that had both memory and specificity in two very recent studies[107, 130]. In these works, elevated level of intratumoral DCs, $CD4^+$, $CD8^+$ T lymphocytes and NK cells was described, with the predominance of $CD4^+$ [130] or $CD8^+$[107] T lymphocytes. We did not observe any change in infiltration of tumors with large monocytoid cells or $CD4^+$, $CD8^+$ T lymphocytes and NK cells. We should note, however, that this observation might also have been a consequence of the relatively large time interval between our therapy and the immunohystochemical analysis. The maximal level of infiltration of tumors by $CD8^+$ T lymphocytes and NK cells was described to develop 48 hours post transfection following local adenoviral FL gene-delivery, and this returned to normal level after one week[107]. Depletion of intratumoral CD4+ cells began after one week and was completed at the end of the second week in the same experiment.

Although there is a drop in the tumor infiltrating effector cell number after the cessation of FL treatment, it has been shown that effective antitumoral immunity can still exist after the leukocyte burst, even when the absolute number of tumor infiltrating effector cells is already close to normal[107, 130]. It is not only the number of intratumoral leukocytes, but also their activation/maturational state that determines an effective immune answer. In addition to its questionable effect on intratumoral lymphocyte numbers, FL cDNA therapy in our case might not have lead to such long-term activation.

4.5.3. Maturational status of APCs

Together with the proportional setting of the cells, the markers of leukocyte activation and dendritic cell maturation were found to be unaltered in peripheral blood during our therapy. In contrast, we found some modest changes in the maturational markers of dendritic cells from the spleens of FL treated animals: expression of the DC maturation marker CD80 on splenic DCs (but not the total DC number) was elevated in 38% of rFL treated animals compared to 0% in other groups 4 weeks after the last plasmid injection. The observed moderate level of changes may have been partly attributable to the 4-weeks-long follow up and the subsequent normalization of splenic cell composition. We confirmed the previous finding[305] that splenic DCs in rats express relatively low basal levels of CD80 and higher amount of CD86 (Figure 14, 18). Interestingly, DCs from the spleen of the animal with total tumor regression showed the highest level of CD80 positivity. This may suggest that the immunotherapy has led to improved antigen presentation and activation of DCs in this single case. Elevation of the maturation marker CD86 without change in other co-stimulatory marker levels has already been reported in another study after FL treatment[121]; however, spleens were here collected 24 hours after the last FL injection. We think that other factors may also have relevance, since CD80 elevation alone did not correlate well with response to therapy in our model. Although local FL administration resulted in a proportion of DCs that were more mature in a group of treated animals, effective antitumoral response was rarely seen. There may have been a lack in co-stimulatory signals that should have promoted DC maturation more effectively.

Increasing the number of only mature DCs should be preferred in immunotherapeutical approaches, since this is the population that can initiate cytotoxic T cell responses[49,50,58,59,57,61,104]. Tumor cells themselves usually do not express the co-stimulatory molecules to provide the necessary co-stimulation to T cells, and immature DCs also fail to provide adequate co-stimulation and may, thereby, tolerize T cells recognizing antigens they present[33].

Maturation of tumor infiltrating DCs (TiDCs), the population that has the possibility to aquire tumor antigens is especially important. Only if DCs that interact with tumors become mature is there a hope for therapeutic efficacy. It is possible, that TiDCs remained immature in most of the cases leading to unresponsiveness to therapy in our

study - this can be an explanation of the therapeutical failure in animals where splenic DCs seemed to be more mature but no response to therapy was seen. It has been reported that TiDCs in many solid tumors remain immature[306-310], the phenotype responsible for peripheral tolerance[33]. In their early study Chaux et al[308] showed that tumor infiltrating DCs, while expressing MHC I and MHC II, did not express co-stimulatory B7 molecules and were poor stimulators of primary allogeneic T-cell proliferation and IL-2/INF-γ production compared to splenic dendritic cells. Tumors themselves may be responsible for the suppression of maturation as demonstrated by a variety of studies. Murine prostate cancer inhibited DC generation from bone marrow precursors *in vivo*[311]. DCs infiltrating human breast cancer were characterized as immatures while a more mature population remained in the peritumoral area[306]. It is likely, that significant alterations described in therapeutic effect among several treatment modalities trying to utilize DCs against solid tumors are caused by differences in the maturational state of intratumoral DCs[312, 117] which is strongly influenced by the tumor milieu. As a potential mechanism it has been reported, that most (human) solid-tumors produce VEGF, a cytokine that inhibit $CD34^+$ precursor maturation into functional DCs[313].

FL has been reported to mobilize both mature and immature DCs in mice[105, 111] and humans[126] in many compartments, with varying results concerning the predominance of mature[314] or immature[56, 315, 316] DCs. FL therapy itself may break the tumors capacity to inhibit maturation: it reversed the inhibitory effects of prostate cancer on dendropoiesis in a murine study[311], and the authors suggested the key role of NK-DC interactions in its mechanism. Still, there is enough data to support the ineffectivity of systemic FL therapy to overcome the intratumoral inhibitory conditions on DC maturation alone. These studies are typically the ones presenting limited tumor growth retardation, but not total regression. For example, FL was shown to have limited stimulatory effect on the antigen-presenting capacity of intratumoral and splenic dendritic cells in a colon carcinoma model, where only tumor growth delay could be reached after systemic delivery of the cytokine. In another case, FL administration did not restore the functional level of DCs compared to tumor-free animals, and the measured activation of NK cell toxicity also decreased as tumor mass grew[121]. TiDCs progressively lost MHC II expression during tumor growth after FL therapy in a study[120]. FL-mobilized DCs

could induce either immunity or tolerance against different tumors in animals treated according to the same therapeutic protocol, as shown by adoptive transfer of splenic DCs of the treated group to healthy animals prior to tumor inoculation[111] elegantly proving the importance of different tumor types and their microenvironment on the regulation of DC behavior. Local transfection of tumors with FL may have implication on the maturational status of TiDCs, but the authors did not characterize this in the few studies available in this setting[107, 130].

Enhancing DC maturation *in vivo* in addition to expanding them may be a possibility to improve the effect of our therapy. This has already been proposed in a recent experiment by loading and activating DCs in situ[317]: sufficient numbers of DCs were directed to peripheral tissues using FL, and then tumor-associated antigens and oligonucleotide containing unmethylated CG motifs (an agent known as a strong "danger signal" for DCs) were delivered to these tissues. This method could overcome tolerance in various tumor models and induce effective antitumor immunity.

Another considerable obstacle to the success of DC-based cancer therapies might be the presence of T cells with regulatory function and the potential for DCs to regulate their clonal expansion[59, 318]. Experimental evidence shows that these T cells suppress antitumor immunity and that their removal allows tumor eradication[59]. Increased frequency of Treg cells has been observed in the peripheral blood and tissues of patients with cancer[318]. $DC8^{+}CD62^{-}$ immunosuppressive T cells have also been reported to infiltrate many solid tumors[79]. It has been shown, that Treg preferentially home to tumor sites and locally accumulate with increasing tumor burden in humans – this is thought to be the most important explanation for the failure of local tumor containment while simultaneously an effective concomitant systemic immunity to minimal disease is preserved[25]. We did not find change in any of these cell subsets in the peripheral blood or among splenic lymphocytes. We should note, however, that the mechanism of action of many of these regulatory cell populations may be local and antigen specific[318, 319, 320] and thus they may elicit suppression without bulky changes in systemic compartments.

Similarly to other immuno-gene therapeutical approaches, rFL based therapies need further refinement - immunogene therapies at the current state are inadequate against large tumors[25]. It is very probable that immunogene therapies may yield success only when used in combination with conventional treatments and/or suppressor cell deletion.

4.6. Transferrin receptor expression of human pancreatic tumors

4.6.1. The contrast of in vivo and in vitro findings

The expression of transferrin receptor in human pancreatic tumors has never been analyzed systemically so far. There is only one report which studied the expression of transferrin receptor in normal pancreatic tissue and only in two pancreatic cancer samples[154]. The present study investigated for the first time the expression of TFRC in a significant number of pancreatic cancers and in neuroendocrine tumors of the pancreas. We found that the transferrin receptor was expressed exclusively by malignant cells and in 90% of pancreatic cancers. Primary tumors and metastases showed similar frequency of positive TFRC expression. Tumor cells in three of four neuroendocrine carcinomas expressed the transferrin receptor. Normal pancreatic tissue and benign neuroendocrine tumors were characterized by loss of transferrin receptor staining. This does not necessarily means that there is no expression (TFRC expression is ubiquitous), but the amount of protein is below the detectable level by immunohistochemistry. More sensitive approaches such as Western blotting might quantify the difference in expression. Previous study found a positive staining of TFRC in islets of Langerhans by one of four different monoclonal antibodies[154]. We did not observe any positive reaction in pancreatic exocrine and endocrine tissue by our antibody. We did not analyze samples from chronic pancreatitis at this stage of experiments. However, we suspect that lower TFRC expression of ductal cells would be found in chronic pancreatitis compared to cancer. This hypothesis should ideally be tested in double-blinded setting from FNAB samples; if the theory could be proved it might become a helpful tool in the hand of pathologists.

The present study demonstrated that the expression of transferrin receptor by various proliferating human adenocarcinoma cell was very low under standard conditions *in vitro*: only 2-19% of cells expressed TFRC depending on cell type. This finding is in obvious contrast to the clinical situation, where immunohystochemistry showed strong expression in most cases; although the technique itself is known to be less sensitive than to flow-cytometry. The phenomenon may be explained by the extensive iron supply under cell culture conditions not necessitating upregulation of TFRC. Transferrin receptor expression does not seem to be a direct marker of proliferation, but its expression on malignant cells may be induced by the local microenvironment providing

limited iron supply. Another possibility of TFRC overexpression is tumor hypoxia. There is experimental evidence that tumor hypoxia is a major determinant of TFRC expression. Tacchini et al. showed that expression of the transferrin receptor is strongly dependent on tumor hypoxia[321]. Other study from Bianchi et al. demonstrated that the hypoxia-inducible factor-1 activates the transcription of transferrin receptor in hepatoma cells[322]. Hypoxia is also a known regulator of a class of iron regulator proteins (IRP-2) that plays central role in the up-regulation of cellular TFRC translation[153] (see also 1.6).

4.6.2. Possible implications

The observation that transferrin receptor was expressed in large quantities exclusively by malignant pancreatic cells suggests that high TFRC expression would be a specific marker of malignant phenotype in pancreatic tissue. We suspect, however, that the relatively high sensitivity of the test in adenocarcinomas would be lower for malignant neuroendocrine pancreatic tumors, which are typically characterized by low proliferative and metabolic activity. We believe that our finding may have potential for therapeutic aims: (1) the receptor may be blocked by inhibiting antibodies, and/or (2) a wide range of therapeutic agents may be introduced into cells through the transferrin-TFRC system.

Monoclonal antibodies from various origin have been reported to inhibit cell proliferation or induce apoptosis in a variety of tumor models[152], mostly lymphomas and leukaemias[159]. The effect of antibodies was largely dependent on antibody class and origin. Unfortunately the use of systemic antibodies was often accompanied by intolerable side effects in animals limiting their use, but promising results were obtained with the use of chimeric antibodies[152] and antibody conjugates[323]. A phase I study was performed with a murine anti-TFRC IgA against refractory cancers[324]. Significant side effects were not reported in this human trial, but only haematopoietic malignancies responded to therapy, not solid tumors – this was explained by the rapid clearance of the IgA type antibody.

Transferrin has a high capacity for binding other metals than iron of therapeutic and diagnostic interest - it is only partially saturated by iron *in vivo*. Various metal ions with anticancer properties have been targeted to cancer cells by transferrin in preclinical setting including Ga^{3+}, In^{3+} and Ti^{2+} [153].

Large molecule therapeutic particles may be bound to transferrin itself or to antibodies against TFRC (antibody conjugates) for delivery. Many conventional chemotherapeutic agents have been introduced to cancer cells this way. Conjugation of transferrin with doxorubicin was shown to increase drug uptake, suppress efflux by multidrug resistance transporters, and enhance nuclear accumulation of the drug[325]. In animal models the complex prolonged the lifespan of tumor bearing animals and showed less cardiotoxicity[325, 326]. Therapy with cisplatin-transferrin conjugate resulted in 36% response rate in advanced breast cancer in a Phase I trial with one complete regression[325], which could be increased up to 87% when applied together with iron chelators. Experiments with chlorambucil and mitomycin C conjugates were less promising; but a transferrin-gemcitabine construct increased the drugs toxicity 6-fold against human bladder cancer cells[327].

Naturally occurring toxic proteins have also been targeted to cancer through TFRC. Early studies with the ricin A chain linked to both transferrin and anti-TFRC antibodies proved specific antitumoral toxicity of the construct[323]. After preclinical studies showing tumor reduction or elimination[325], reduction in the tumor volume in patients with leptomeningeal neoplasia was also proven[328] (Phase I). Other plant toxin conjugates (saponin, gelonin) could also kill tumor cells in preclinical studies[325]. Tumor growth delay was also reported after systemic treatment of tumor bearing mice with Pseudomonas exotoxin conjugates[329]. However, the most encouraging results were achieved with the transferrin-CRM 107 toxin conjugate. This construct contains a point mutated form of the diphtheria exotoxin lacking its cell binding capacity but still exerting effective suppressive function on protein translation. Conjugation of CRM 107 with transferrin enhanced its growth inhibitory effect up to 100-fold after intratumoral delivery to experimental gliomas[325]. Transferrin-CRM 107 was delivered by intratumoral infusions to patients with malignant brain tumors resistant to conventional treatments with unexpected success rate: 50% decrease in tumor volume was measured with 2/15 complete regressions[330]. As a consequence, forty-four patients were involved in a next phase II study with recurrent/refractory glioblastomas or anaplastic astrocytomas. Five from the 34 patients who received two injections showed complete, and seven patients partial regressions at one year[331]. These promising results are

currently tested against inoperable refractory/recurrent gliomas in multicenter phase III trials.

TFRC may be used for enhancement of gene delivery[332]. The increased uptake of liposomes containing polyethylene glycol-transferrin inserts in their membrane was accompanied by increased toxicity and prolonged survival in a variety of murine models - these liposomes carried chemotherapeutic agents[333], antisense oligonucleotides[334] or tumor suppressor genes[335]. The transfection rate of tumors expressing low levels of the adenovirus receptor CAR (see also 4.9.1) by adenoviruses could also be effectively enhanced by coupling transferrin to the cysteine residues of genetically modified capsid proteins[336].

4.7. Basal dCK activity and gemcitabine sensitivity of glioma cell lines

The combined effect of dCK overexpression, gemcitabine treatment and irradiation was investigated in two frequently used glioma cell lines. Special emphasize was placed on the level of gemcitabine radiosensitization by dCK overexpression in these tumor models.

Basal dCK expression was found to be relatively low in both glioma cell lines compared to other tumor cell lines from the literature[211, 231, 232, 235, 236]. An interesting finding was the relatively great variation in the basal dCK activity of the U373 and C6 cell lines: about 5-fold difference was found between the specific dCK activity. The low basal dCK activity in our experiments is in accordance with previous data: both rat gliosarcoma[337] and human brain tumors[338, 339] were characterized by low dCK expression/activity formerly.

Interestingly, sensitivity to gemcitabine showed a reverse relation to basal dCK activity in our investigated cell lines. C6 cells that showed lower total and specific dCK activity were more sensitive to gemcitabine, while U373 cells were relatively resistant (Table 25, Figure 23). This observation is in opposition with some reports in the literature that suggest that basal dCK level would be the direct determinant of cellular gemcitabine sensitivity[235, 237, 340]. Other data, in contrary, show that dCK level is not the sole determinant of gemcitabine sensitivity of the cells. It is not rare, that cell lines expressing lower levels of dCK have lower IC50 for gemcitabine[236] – gemcitabine sensitivity is cell type dependent. There may be many causes why specific dCK activity alone can not predict gemcitabine toxicity in a certain cell type. It is suggested that other

enzymes, such as ribonucleotide reductase or deoxycytidine deaminase, which have an important role in regulating the intracellular deoxyribonucleotide pool and indirectly also take part in the intracellular metabolism of gemcitabine, can strongly influence gemcitabine sensitivity[341, 342]. We should also note that big difference between total and specific dCK activity was found in C6 cells, which indicates the presence of an increased mitochondrial TK2 (thymidine kinase) activity. TK2 is expressed in all tissues in mitochondria; it is normally likely to be responsible for the supply of deoxy-ribonucleotides required for mithocondrial DNA synthesis. It was shown, that apart from the dCK, the TK2 enzyme can also phosphorylate gemcitabine[343]. Thus, the increased TK2 enzyme activity in the C6 cells could compensate for the very low basal dCK levels and might explain the discrepancy between the relatively high gemcitabine sensitivity and the low basal dCK activity of the cell line. This hypothesis is also supported by the findings of Sanda et al[344] who showed that gemcitabine, which was phosphorylated in the mitochondria, could incorporate into mitochondrial DNA, which would lead to apoptosis. Still, our experiments clearly proved that adenoviral transfer of the dCK gene into C6 cells substantially increased dCK enzyme activity and improved the activation of gemcitabine, which led to strongly enhanced cytotoxic effects.

The great intercellular variations in gemcitabine sensitivity might be also explained by the multiple mechanisms of action of gemcitabine. Since phosphorylation of the drug by dCK is only one mechanism of drug cytotoxicity, other mechanisms such as incorporation into RNA[178] inhibition of ribonucleotide reductase[345] and DNA polymerase[346] might counteract the importance of low dCK levels in gemcitabine cytotoxicity.

4.8. The effect of dCK overexpression *in vitro*

4.8.1. Ad-HudCK and the intrinsic toxic effect of dCK overexpression

An adenoviral construct, encoding the human dCK enzyme, was used to increase dCK activities in the studied cell lines and in animal tumor models. Transfection with this construct causes transient expression of the protein. Some previous studies showed that there might be species-specific differences in the efficiency of dCK to phosphorylate gemcitabine: the murine dCK could phosphorylate gemcitabine and other nucleoside analogs less efficiently than the human enzyme[347,348,349]. We speculated that the effect of our therapy would be largely dependent on the activity of the enzyme, and the

extrapolation of the results for the treatment of human tumors would be easier in case of human dCK. Therefore, we decided to use the human enzyme in the investigated cell lines and tumor model. We should note, however, that no major differences were noted in the enzyme activities after saturating transfection in the rodent (C6) or human (U373) cell lines in our case (around 8nmol/h per mg protein at high MOI; Table 25). The human dCK enzyme is heterologous for C6 cells, and there is some experimental data suggesting the immunogenity of human dCK in rats [350] (see also 3.8.2). We did not find any effect of human-dCK transfection in the growth of rat tumors. We showed that dCK overproduction (at the level of saturation) by transduction of the cell lines with the Ad-HudCK construct was toxic for U373 cells (~50% toxicity at 100 MOI), but not for C6 rat cells. The toxic effect of the adenovirus vector itself can be excluded, since we did not detect signs of cytotoxicity when cells were transduced with the adenovirus vector encoding the LacZ gene. Moreover, a panel of adenovirus vector constructs encoding various cytokines and drug-sensitizing genes was applied to transduce the Gl261 glioma cells in our laboratory formerly [166, 263, 351], but none of them produced cytotoxic effects comparable to the Ad-HudCK construct.

Manome et al[337] used an adenoviral vector to transfer the dCK gene into 9L cells, but did not discuss the effect of the dCK encoding vector on cell survival. There are few other reports in the literature describing the effect of dCK overproduction in various cells or tumor models, and none of them utilized the cell lines discussed in our study. Hapke et al[352] applied a retroviral vector to transfer the dCK gene into MCF-7, HT-29 and H1437 cell lines. Blackstock et al[353] used the same retroviral construct in HT-29 cells. Beauséjour et al[180] utilized another retroviral construct to transfect the human dCK gene into human A549 lung carcinoma and murine NIH3T3 cell lines. Kawamura et al[350] transduced the Colon 26 mouse colon carcinoma and 9L rat gliosarcoma cell lines with another human dCK encoding retroviral vector. In this latter study tumor growth retardation was experienced *in vivo* after retroviral transfection – but this was not seen in nude mice suggesting an immunological mechanism elicited probably by the heterologous human dCK protein. There was no tumor growth retardation observed in our *in vivo* experiment by human dCK transduced tumors. None of the aforementioned authors mentioned signs of increased toxicity of dCK *in vitro*, but one should take into account that much higher dCK levels can be achieved with adenoviral vectors than with

retroviruses (15 and 44 fold increase in our study with adenovirus vs. 2-7 fold in previous experiments - except the single case of a 42 fold elevation in H1437 small cell lung carcinoma cells by Hapke et al[352]). We think that the strong dCK overexpression compared to the basal activity might produce severe perturbations in the nucleotide pool and metabolism in U373 cells, which could lead to cellular death. Further investigations are necessary to reveal the exact mechanism behind.

4.8.2. Gemcitabine toxicity in dCK overexpressing cells

The *in vitro* toxic and radiosensitizing effect of gemcitabine treatment was assessed next following transfection with Ad-dCK.

Our results show that increased dCK activity upon adenoviral transfer of the gene could sensitize cells to gemcitabine. Deoxycytidine overexpression enhanced gemcitabine toxicity in both investigated cell lines. The rate of sensitization was comparable in C6 and U373 cells. This data is in accordance with the previous hypothesis that dCK activity would be the major determinant of gemcitabine sensitivity (see also 1.13, 4.7).

Several research groups investigated the effect of dCK overexpression on the toxicity of various nucleoside analogs *in vitro*. Most of these groups agree that elevation of the dCK enzyme activity leads to an increase in the drugs toxicity, as well. Hapke et al.[352] investigated the toxicity of three nucleoside analogs after retroviral transfer of dCK into various tumor cell lines. They showed a strong correlation between dCK activity and 1-β-D-arabinofuranosylcytosine (AraC), 2-chloro-2'-deoxyadenosine (CdA) or 2-fluoro-9-β-D-arabinofuranosyladenine (FAraA) toxicity, but a lesser level of correlation existed between dCK activity and gemcitabine toxicity. In contrary we should note, that Beauséjour and co-workers[180] measured unexpected drug resistance against gemcitabine reported after dCK overexpression in human A-549 and murine NIH 3T3 cell lines, while the toxicity of other drugs (AraC, 5-aza-2'-deoxycytidine) was increased. This is a unique phenomenon reported in the literature. The authors hypothesized, that in their model it would be predominantly the mitochondrial TK2-way that is responsible for gemcitabine toxicity. This mechanism has already been described before[344]. According to Beauéjour at al., the overexpression of dCK could lead to competition between the phosphorylating enzymes resulting in relative attenuation of mitochondrial gemcitabine metabolism and thus contributing to enhanced survival. This hypothesis is not supported by our observations and by others[352] (see also 4.7). The mitochondrial apoptosis

mediated by the TK2-pathway is surely not the general way of gemcitabine toxicity: it has been shown that gemcitabine resistance of a cell line was attributable to the loss of dCK function with unaltered TK2 (and various other gemcitabine-related enzyme) activity[234].

4.8.3. The effect of dCK overexpression on gemcitabine radiosensitization

The C6 and U373 cells were relatively sensitive for radiation *in vitro* (approximately 50% survival after 4Gy, Figure 25). This is comparable to the sensitivity of murine glioma cell lines such as Gl261 and RG2[351, 356]; however, human gliomas may be more resistant *in vitro*[357]. Gemcitabine was administered 24 h before irradiation. This scheduling was chosen, because our preliminary experiments (data not shown) in concordance with other reports in the literature confirmed that optimal radiosensitization, both *in vitro* and *in vivo* is achieved if the drug is administered 24–72h prior to irradiation[192, 208] (see also 1.12).

Gemcitabine did not have significant radiosensitizing effect in our cell lines, the effect was only additive. We believe, this is attributable to the low basal dCK activity of the used cell lines. The mechanism of gemcitabine radiosensitization is not fully understood. Most studies show that gemcitabine treatment produces a complete inhibition of DNA synthesis, a depletion of dATP pool and an accumulation of cells in early S phase, which can contribute to misrepair of DNA damage after radiation. Other mechanisms, such as inhibition of chromosome repair after irradiation, might also effectively contribute to radiosensitization[185-190, 192, 195, 205, 206, 216, 219, 221-225, 227, 350, 358] (see also 1.12.2). Our observation is in concordance with other data in the literature which state that gemcitabine radiosensitization is more pronounced in cells with high basal dCK activity[211, 237] – this hypothesis provided the basis of our study aiming to apply GDEPT to enhance the radiosensitizing effect of gemcitabine. The Gl261 murine glioma cell line, which is characterized by relatively higher basal dCK activity (1.3/0.9 total/specific nmol/h/mg protein activity) was found to be radiosensitized later in our laboratory (data not shown). We should here note, that based on a series of *in vitro* assays Pauwels et al.[236] recently observed that the basal specific dCK activity of some cell lines weakly correlated to gemcitabine radiosensitization. One must recognize, however, that the measured basal enzyme activities (8.7±3.2 nmol/h/mg protein activity) together with the measured radiation enhancement rate were almost identical in this

series, which - in our interpretation - makes it impossible to draw a final conclusion. dCK activity of the cells may not be the sole determinant of the level of radiosensitization measured after gemcitabine treatment but seems to be the major factor. The absence of radiosensitization of our untransduced cells is not very surprising in this context: both of our cell lines showed much lower levels of basal dCK activity compared to the usual value of other cell lines.

We showed that dCK overexpression could improve not only the toxic, but also the radiosensitizing effect of gemcitabine. Strong enhancement was found both in C6 and U373 cells (enhancement rate: 2.3 and 3.6, respectively).

Significantly elevated dCK enzyme activity could be measured after transfection in both cell lines – the resulting higher rate of intracellular gemcitabine metabolites could have caused the enhanced radiosensitizing rate; but other explanations also have to be considered. Besides the high intracellular dCK level, another possible cause for the radiosensitizing effect of gemcitabine is that irradiation itself can induce dCK activity (in Gl261 cells) by twofold (G Sáfrány, personal communication) which, in turn might further improve gemcitabine metabolism and amplify its toxic effect. Formerly, Csapo et al.[232] has already described that radiation is a strong dCK activating agent in human lymphocytes. They presumed a two-step mechanism for the radiation-responsive induction of dCK activity: the early dCK activation should be due to post-translational changes rather than modifications in the protein content, while long-term dCK activation was ascribed to adaptive transcriptional changes and elevation in the protein levels, as well.

4.9. The effect of dCK overexpression on *in vivo* gemcitabine- and radiotherapy

Finally, we evaluated the combined *in vivo* therapeutic effect of dCK overexpression, gemcitabine treatment and irradiation in the C6 model. We did not observe signs of spontaneous tumor regression in the control group as was reported before due to the relative immunogenicty of the C6 tumor model[260].

In contrast to the *in vitro* findings C6 tumors were relatively radioresistant *in vivo.* The 4Gy dose 3 day after tumor implantation did not lead to survival in the control groups. Gliomas are known to be one of the most radioresistant tumors[160, 161]. It is also a documented phenomenon, that cell lines that are relatively radiosensitive *in vitro* may

give rise to radioresistant tumors *in vivo*. Intracellular properties of glioma cells may only be partly responsible for their radiation sensitivity[354].

Similarly to the *in vitro* results, dCK overexpression itself was not toxic for C6 cells *in vivo*. Survival enhancement was not seen compared to control – an affective immunologic response due to heterologous HumdCK protein could be excluded. It would be interesting to examine dCK sensitivity in a larger series particularly with cells that show sensitivity to dCK overexpression *in vitro* (Gl261 cells possess such properties – growth retardation was not seen in our laboratory *in vivo,* data not shown).

Few reports exist in the literature that used dCK gene transfer to improve therapeutic responses to nucleoside analogs. One of the earliest reports is that by Manome et al.[337], who showed that viral vector transduction of dCK sensitizes 9L cells to the cytotoxic effects of cytosine arabinoside (AraC) both *in vitro* and *in vivo*. They used both a retroviral and an adenoviral dCK construct. The group showed significantly improved survival rates when tumors were treated with dCK gene transfer and AraC. In another study, Blackstock et al.[353] used a retrovirus vector to introduce the dCK gene in HT29 cells. They showed a substantial regression in the growth rate of HT29 xenografts transduced with dCK encoding vector and treated with gemcitabine. The authors concluded that increased dCK expression enhanced gemcitabine accumulation and prolonged elimination, which, in turn, could augment *in vivo* tumor responses to gemcitabine. Vernejoul et al.[358] generated a fusion gene combining dCK and uridine monophosphate kinase and used it in a suicide gene therapy approach together with gemcitabine to treat human pancreatic cancer established in hamsters. They showed that the fusion gene could efficiently sensitize pancreatic cancer cells to gemcitabine and they also could prove a substantial bystander effect, which was due to apoptosis.

An interesting finding was the modest, but well detectable radiosensitizing effect of dCK overexpression itself; the mechanism is unclear. Gemcitabine alone did not have significant therapeutic effect in the C6 tumor model in the used concentration. dCK overexpression did not enhance survival to gemcitabine treatment significantly *in vivo*. In the *in vitro* setting the used drug concentration itself had substantial cytotoxic effect, which could be enhanced by dCK overexpression. Possibly the used gemcitabine concentration was too low to reach the enhancing concentration in the orthotropic model.

Contrary to this, the strong radiosensitizing effect of gemcitabine that was demonstrated in dCK-overexpressing C6 tumors *in vitro* was translated into a significant improvement in survival *in vivo*. These results strengthen previous reports that gemcitabine is a powerful radiation sensitizer even at doses well below those used to produce cytotoxicity[186, 208, 359].

There might be several ways to further improve the efficiency of our protocol. Significant improvement might be for example the design of dCK mutants, which have an increased efficiency toward gemcitabine phosphorylation[360]. Another possibility is the development of oncolytic viral vectors that replicate specifically in tumors and/or carry multiple suicide genes.

4.9.1. General considerations on viral transfection in a therapeutic setting

Our result, that the dCK-gemcitabine GDEPT might be an effective gene therapeutical approach with considerable radiosensitizing effect is very promising. However, in our *in vivo* experiments Ad-HudCK pre-transduced tumor cells were used. Ad-HudCK is a first generation adenovirus causing transient expression in transfected cells. In our case, about 65–75% of the cells contained the virus at the time of tumor transplantation (Figure 24). The peak of protein expression after transfection is measured at 48h post transfection in these adenoviral systems and remains relatively unaltered for 7 days[351, 361, 362]. This experimental setting reflects an ideal condition, which can seldom be reached in the clinic, where the therapeutic gene should be delivered by direct intratumoral injection of the virus vector. It was the goal of this experiment to show the rational of a new, potentially radiosensitizing GDEPT against gliomas. We did not aim to treat gliomas by intratumoral infection at this step.

The critical point in the further application of this treatment is the appropriate transduction of tumor cells with the viral vectors. It has been shown, that appropriate GDEPT-s may not need more than 10% transfection of all tumor cells to result in tumor elimination[166, 165] – an efficient bystander effect may compensate for the relatively low transduction rates. Still, this limit seems to be a hurdle for current vectors in large tumors. In the clinic, GDEPT may be applied as an adjuvant to conventional treatments first. The infiltration of the tumor bed after tumor resection (like the postsurgical treatment of glioblastomas) is surely not ideal in the adjuvant setting. There may be several possibilities to reach more effective and more specific gene delivery. Choosing

(1) the optimal way of vector delivery (intravenous, intraarterial, intratumoral, intraperitoneal) together with modification of vector tropism (transductional targeting), the use of (2) tissue/tumor-specific viruses or promoters in transgene expression, or (3) replication competent/conditionally replicating oncolytic viruses "armed" with transgenes (armed therapeutic viruses) are all hoped to produce better results[165, 281].

Effective systemic administration could be a key to eliminate potential distant metastases or residual tumors. Adenoviruses, herpes simplex and Newcastle viruses have already been tested for *in vivo* administration in humans without serious toxicity. However, the half life of particles is relatively low by systemic administration. In case of adenoviruses there is an especially rapid hepatic virus uptake that lowers the plasma level, which - together with aspecific plasma protein binding - leads to unsatisfactory transfectional efficiency[165]. The cellular uptake of adenovirus vectors is dependent on interactions between viral capsid proteins, and some target cell surface receptors (Coxsackie and Adenovirus Receptor: CAR, integrins, heparan sulfate glycosaminoglycans)[363]. Transductional retargeting strategies using viral coating, bispecific heterologous retargeting ligands, genetically capsid-modified vectors or chimeric recombinant vectors (capsid protein from different virus) are intensively studied in preclinical models to enhance systemic administration[277].

There is much preclinical and clinical data available about the *in vivo* use of replication-competent oncolytic viruses[281, 165]. Replication competent vectors (encoding prodrug-activating enzymes) have been shown to increase levels and distribution of genomically encoded factors over replication - defective viruses[364]. These agents, with their capacity to amplify their dose through replication at the target site, then spread within the tumor to lyse neoplastic cells and decrease the tumor burden represent theoretically unique anticancer therapeutics. The spread of such replication-competent viruses after local delivery could be effectively stopped by activation of suicide genes with appropriate timing[365].

Many replication- selective oncolytic viruses that performed well in preclinical studies have already been tested in the clinic. These viruses include ONYX-015, Ad5-CD/TK, CV787 and CV706 (Ad5- derived); 1716 and G207 (HSV-1–derived); PV701 and MTH-68/H (Newcastle disease) viruses[165]. Unfortunately at the current state, the clinical experience of these single agent therapies has fallen short of their theoretical

promise - although in a few cases, full and relatively durable cures, or objective responses were achieved in patients with cancers irresponsible to any other treatment[165,366].

The use of replicating viruses in typical GDEPT models affecting DNA synthesis may represent a special problem that has to be considered. Prodrugs whose activated form interferes with DNA replication have been shown to limit the ability of the virus to continue to replicate and spread in the tumor[165] - these reductions in viral burst size mitigate the cytolytic potential of these viruses and potentially compromise the full utility of this approach. Optimized dosing schedules and virus-prodrug pairs would probably help to overcome these problems.

Therapy with viruses encoding multiple suicide genes also improves cytotoxicity[263, 367, 368]. In the first human trial with a replication competent adenovirus a CD/TK gene fusion construct was introduced into locally recurrent prostate cancers in a GDEPT setting - two of 14 patients experienced full tumor regressions, and additional four patients partial regressions (25–80% reduction in PSA levels).

There is evidence, that combined modalities are considerably more successful against cancer than any of the component monotherapies, including gene-therapy[367]. Although not yet successful alone, some current anticancer gene therapies were proved to be effective adjuvants parallel to conventional treatments. With further refinement, these strategies might become a new hope against cancer.

5. Summary and consequences

Gene therapies may serve as adjuvant treatments against tumors. We studied potential immuno-gene and gene-directed enzyme pro-drug (GDEPT) therapy systems in rat models; and examined the presence of transferrin receptor in human pancreatic tumors.

Systemic Flt3-ligand (FL) treatment leads to expansion of dendritic cells with antitumoral effect in animal models. We hypothesized, that intratumoral FL gene transfer would have effect on the antitumoral immune response and tumor growth in experimental DSL6A rat pancreatic cancers. The unknown rat FL cDNA was sequenced, and cloned into a plasmid. Transfection of s.c. growing tumors was augmented by cationic liposomes - 10% transfection rate was achieved. While control tumors grew continuously, 6 times repeated injections of FL-coding plasmids resulted in shrinkage of tumors in half of the treated animals; total regression and tumor size stabilization could also be achieved in some cases. Most treated tumors regained proliferative activity after cessation of treatment. The therapy was accompanied by considerable increase in the expression of CD80 on splenic dendritic cells in some treated animals and increase in splenic NK cell number in therapy responders. The effect of therapy was limited; combinational strategies aiming to activate dendritic cells may be helpful.

We present the first experimental attempt to enhance the radiosensitizing effect of the widely used chemotherapeutic agent gemcitabine by means of GDEPT. Both the cytotoxic and radiosensitizing effect of gemcitabine could be significantly improved by adenovirus mediated overexpression of the dCK enzyme in murine C6 and human U373 glioma cell lines. dCK overexpression in pre-transduced C6 gliomas significantly improved the survival rate of tumor bearing rats in response to chemoradiotherapy by enhancing the radiosensitizing effect of gemcitabine. After further in vivo studies in a therapeutic setting (local transfection), the dCK/gemcitabine GDEPT system might be a candidate of adjuvant gene-therapeutical protocols against tumors, where gemcitabine and radiation is already in clinical use - such as pancreatic cancer and gliomas.

We found that malignant human ductal and neuroendocrine pancreatic tumor cells express considerable amount of transferrin receptor in most cases (90%), while healthy pancreatic tissue and benign tumors do not show expression by immunohistochemistry. This observation may have implication in the diagnosis and (vector-) targeting of these malignancies.

Acknowledgements

I am very grateful to the following persons; without their help this work could not have been accomplished. I would like to say thank you to:

my tutors, Dr. Géza Sáfrány and Dr. Katalin Lumniczky for allowing me to join their team, for teaching me radiation biology, for providing me both material and theoretical support throughout my work - and for letting me performing experiments in a very flexible way necessitated by my daily clinical activity;
my co-authors, especially Dr. Katalin Lumniczky and Tünde Szatmári for their contribution in performing the experiments and in the preparation of the manuscript;
Dr. Hargita Hegyesi for helpful advices about the thesis and presentation;
all the leaders of the National Research Institute for Radiobiology and Radiohygiene for letting me work in the institute;

Prof. Dr. Hans-Günther Sonntag for providing a scholarship for me in Heidelberg;
Prof. Dr. Jan Schmidt for letting me work on projects about pancreatic cancer and for his continuous personal support and help during my stay in Germany;
Dr. Eduard Ryschich for being my closest contact in the everyday work in Heidelberg, for being my tutor and teacher of many laboratory technics, for the contionuous control throughout the project, and for the corrections and advices on the manuscript;
Dr. Nicholas Wentzensen for teaching me molecular biology;
Prof. Dr. Angela Märten for the consultations regarding immunology, and for introducing me flow cytometry;

Ms. Erzsébet Fekete, Ms. Mária Frigyesi, Ms. Rita Lökös, Ms. Claudia Bernardi and Ms. Irina Vöhringer, for their excellent technical assistance.

List of publications

ORIGINAL PUBLICATIONS RELATED TO THE THESIS

First author:

1. Ryschich E, **Huszty G**, Wentzensen N, Schmidt E, Knaebel HP, Märten A, Büchler MW, Schmidt J: Effect of Flt3-ligand-gene transfer in experimental pancreatic cancer. *International Journal of Colorectal Disease.* 2007 Feb;22(2):215-23. ***(shared first authorship) IF:1.918***
2. Szatmári T, **Huszty G**, Désaknai S, Spasokoukotskaja T, Sasvári-Székely M, Staub M, Esik O, Sáfrány G, Lumniczky K.: Adenoviral vector transduction of the human deoxycytidine kinase gene enhances the cytotoxic and radiosensitizing effect of gemcitabine on experimental gliomas. *Cancer Gene Therapy*. 2008 Mar;15(3):154-64. ***(shared first authorship) IF:3.889***

Second author:

3. Ryschich E, **Huszty G**, Knaebel HP, Hartel M, Büchler MW, Schmidt J: Transferrin receptor is marker of malignant phenotype in human pancreatic cancer and in neuroendocrine carcinoma of the pancreas. *European Journal of Cancer.* 2004 Jun; 40(9): 1418-22. ***IF:3.302***

Citable abstract:

4. K. Lumniczky, T. Szatmari, **G. Huszty**, S. Desaknai, M. Sasvari-Szekely, M. Staub, G. Safrany: Combined treatment of experimental gliomas with radiotherapy, radiosensitizing and chemosensitizing gene therapy. Citation: *European Journal of Cancer Supplements* Vol 3, No. 2, October 2005, Page143.

OTHER ORIGINAL PUBLICATIONS

1. **Huszty G**, Mogami K, Sawada T, Seki H, Sakusabe M, Ohuchi S, Kotanagi H: Preoperative evaluation of irreversible bowel ischemia in obturator hernia. *Hepatogastroenterology.* 2007 Apr-May;54(75):775-9. ***IF: 0.904***
2. Hortobagyi L, Kis B, Hrabak A, Horvath B, **Huszty G**, Schweer H, Benyo B, Sandor P, Busija DW, Benyo Z: Adaptation of the hypothalamic blood flow to chronic nitric oxide deficiency is independent of vasodilator prostanoids. *Brain Research* 2007 Feb 2;1131(1):129-37. ***IF:2.218***

Citable abstract:

3. P Krajcsi - D Teknős - **G Huszty** - A Végh: Adenovirus E1B19k protects against calcium-induced cell death. BCI Conference 2004. *Tissue Antigens.* 2004 Oct; 64(4): 317-441.

PRESENTATIONS RELATED TO THE THESIS

2003 Ryschich E, **Huszty G**, Büchler MW, Schmidt, J: Spezifische Expression vom Transferrin Rezeptor beim humanen Pankreaskarzinom. *7. Chirurgischen Forschungstage in Berlin, Berlin, Germany*

2004 K. Lumniczky, **G. Huszty**, M. Staub, M. Sasvári-Székely, G. Sáfrány: Radiosensitizing gene therapy for murine gliomas. *European Radiation Research, Budapest, Hungary*

2005 K. Lumniczky, T. Szatmari, **G. Huszty**, S. Desaknai, M. Sasvari-Szekely, M. Staub, G. Safrany: Combined treatment of experimental gliomas with radiotherapy, radiosensitizing and chemosensitizing gene therapy. *ECCO 13 - European Cancer Conference, Paris*

2005 T. Szatmári, K. Lumniczky, **G. Huszty**, Sz. Désaknai, M. Staub, M. Sasvári-Székely, G. Sáfrány: Treatment of experimental gliomas with the combination of local radiotherapy, radiosensitizing and chemosensitizing gene therapy. The 34th Annual Meeting of the European Society for Radiation Biology, Leicester, UK

2005 Ryschich E, **Huszty G**, Wentzensen N, Weitz J, Knaebel HP, Schmidt E, Büchler MW, Märten A, Schmidt J: Therapeutischer Effekt des Gentransfers von Flt3-Ligand beim experimentellen Pankreaskarzinom. *122. Kongress der Deutschen Gesellschaft für Chirurgie, München, Germany*

2005 Lumniczky K., Szatmári T., **Huszty G**., Désaknai Sz., Sasvári-Székely M., Staub M., Sáfrány G. A gemcitabine kemoterápiás és sugárérzékrnyitı hatásának fokozása a génterápia eszközeivel. A Peptidkémiai Munkabizottság és a Nukleotidkémiai Munkabizottság együttes tudományos ülése. Balatonszemes.

2007 Lumniczky K., Szatmári T., **Huszty G.,** Désaknai Sz., Spasokoukotskaja T., Sasvári-Székely M., Staub M., Sáfrány G. A gemcitabin kemoterápiás és sugárérzékenyítı hatásának fokozása génterápiás eszközökkel kísérletes

agytumorokban. Magyar Onkologusok Társaságának XVII. Jubileumi Kongresszusa. Budapest

2008 **G Huszty**, T Szatmári, S Désaknai, T Spasokoukotskaja, M Sasvári-Székely, M Staub, O Ésik, G Sáfrány, K Lumniczky, E Ryschich, N Wentzensen, E Schmidt, A Märten, J Schmidt: Gene therapy of solid tumors – aspects and two experimental examples. "Keiichi Maruyama" invited honorary lecture at *The 3rd Meeting of the Japanese- Hungarian Surgical Society, Budapest, Hungary*

OTHER PRESENTATIONS AND POSTERS

2001 **Huszty G** -Teknős D – Krajcsi P: The E1B19K protein of the human adenovirus protects the cell against calcium-induced cell death. *Korányi Frigyes Scientific Congress, Budapest, Hungary*

2002 **Huszty G** -Teknős D - Krajcsi P: The E1B19K protein of the human adenovirus protects the cell against Ca^{2+}-induced cell death. *Congress of the Molecular Biological Section of the Hungarian Biochemical Association, Keszthely, Hungary (POSTER)*

2004 **Huszty G** - Teknős D - Dezső P - Végh A – Krajcsi P: Human adenovirus E1B19k protein protects against calcium-induced cell death. *Towards clinical gene therapy: pre-clinical gene transfer assessement, Barcelona, Spain. (POSTER)*

2006 K Mogami, **G Huszty**, T Sawada, H Seki, M Sakusabe, S Ohuchi, H Kotanagi: 閉鎖孔ヘルニア　１８例の検討。(„18 cases of obturator hernia.") *The 2nd Annual Meeting of The Japanese Gastroenterological Assotiation, Tokyo, Japan*

2006 **G Huszty**, K Mogami, T Sawada, H Seki, M Sakusabe, S Ohuchi, H Kotanagi: 閉鎖孔ヘルニアにおける不可逆的腸管虚血の術前評価。(„Preoperative evaluation of irreversible bowel ischemia in obturator hernia.") *The 2nd Meeting of the Japanese- Hungarian Surgical Society, Tokyo, Japan*

2006 J Sandor, A Balint, M Mate, I Irto, M Bereczky, A Solymosi, G Papp, A Dubecz, F Rabai, L Ternyik, **G Huszty**: Postoperative acute acalculous cholecystitis: treatment modalities in the laparoscopic era. *The 2nd Meeting of the Japanese-Hungarian Surgical Society, Tokyo, Japan*

Literature

1. A daganatok sebészete. (1986) Editor: Dr. István Besznyák. Medicina. Page 21.
2. Sabiston textbook of surgery. 17th edition. (2004) Editors: Townsend-Beauchamp-Evers-Mattox. Elsevier Saunders. Page 769.
3. Cross D, Burmester JK. (2006) Gene Therapy for Cancer Treatment: Past, Present and Future. Clinical Medicine & Research; Vol. 4, Number 3: 218-227.
4. Santo E (2004) Pancreatic cancer imaging: which method? JOP 4:253-7
5. Michaud DS (2004) Epidemiology of pancreatic cancer. Minerva Chir 2:99-111
6. Courtney Townsend, Beauchamp RD, Evers BM, Mattox K (1994) Sabiston textbook of surgery. 17th edition. ISBN: 0-7216-0409-9
7. Lim JE, Chien MW, Earle CC (2003) Prognostic factors following curative resection for pancreatic adenocarcinoma. Ann Surg 1:74-85.
8. Neoptolemos JP, Stocken DD, Dunn JA, Almond J, Beger HG, Pederzoli P, Bassi C, Dervenis C, Fernandez Cruz L, Lacaine F, Buell MG, Dean DC, Adab FA, Sutton R, Imrie C, Ihse I, Tihanyi T, Olah A, Pedrazzoli S, Spooner D, Kerr DJ, Friess H, Buchler MW (2001) Influence of resection margins on survival for patients with pancreatic cancer treated by adjuvant chemoradiation and/or chemotherapy in the ESPAC-1 randomized controlled trial. Ann Surg 6:758-768
9. Yeo CJ, Cameron JL, Lillemoe KD Pancreaticoduodenectomy for cancer of the head of the pancreas, 201 patients Ann Surg 22:721-731
10. MacKenzie MJ (2004) Molecular therapy in pancreatic adenocarcinoma. Lancet Oncol 9:541-9
11. Kulke MH (2005) Combination chemotherapy for advanced pancreatic cancer-has its time finally come? Lancet Oncol 6:352-3
12. Van Cutsem E, Aerts R, Haustermans K, Topal B, Van Steenbergen W, Verslype C (2004) Systemic treatment of pancreatic cancer. Eur J Gastroenterol Hepatol 3:265-74
13. Picozzi VJ (2005) Treatment of Localized Pancreas Cancer – An International Perspective. In: US Gastroenterology review – April (2005).

14. Nukui Y, Picozzi VJ, Traverso LW (2000) Interferon-based adjuvant chemoradiation therapy improves survival after pancreaticoduodenectomy for pancreatic adenocarcinoma. Am J Surg 5:367-371
15. Picozzi VJ, Kozarek RA, Traverso LW (2003) Interferon-based adjuvant chemoradiation therapy after pancreaticoduodenectomy for pancreatic adenocarcinoma. Am J Surg 5:476-80
16. Picozzi VJ, Traverso LW (2004) The Virginia Mason approach to localized pancreatic cancer. Surg Oncol Clin N Am 4:663-74
17. Knaebel HP, Marten A, Schmidt J, Hoffmann K, Seiler C, Lindel K, Schmitz-Winnenthal H, Fritz S, Herrmann T, Goldschmidt H, Mansmann U, Debus J, Diehl V, Buchler MW (2005) Phase III trial of postoperative cisplatin, interferon alpha-2b, and 5-FU combined with external radiation treatment versus 5-FU alone for patients with resected pancreatic adenocarcinoma - CapRI: study protocol [ISRCTN62866759]. BMC Cancer 1:37
18. Varadhachary GR, Abbruzzese JL (2005) Treatment of Pancreatic Cancer. In: US Gastroenterology review - April (2005).
19. Steinman RM, Mellman I (2004) Immunotherapy: bewitched, bothered, and bewildered no more. Science 305:197−200
20. Foley, E. J (1953) Antigenic properties of methylcholanthrene-induced tumors in mice of the strain of origin. Cancer Res 13:835−837.
21. Euvrard S, Kanitakis J, Claudy A (2003) Skin cancers after organ transplantation. N Engl J Med 348:1681−1691
22. Mapara MY, Sykes M (2004) Tolerance and cancer: mechanisms of tumor evasion and strategies for breaking tolerance. J Clin Oncol 6:1136-51
23. Goedert JJ, Cote TR, Virgo P (1998) Spectrum of AIDS-associated malignant disorders. Lancet 351:1833-1839
24. Bagley KC. Genetic adjuvant therapy for pancreatic cancer and other solid tumours. Gut. (2008) Mar;57(3):289-91.
25. Tangney M, Casey G, Larkin JO, Collins CG, Soden D, Cashman J, Whelan MC, O'Sullivan GC. (2006) Non-viral in vivo immune gene therapy of cancer: combined strategies for treatment of systemic disease. Cancer Immunol Immunother. Nov;55(11):1443-50.

26. Hakim FT, Flomerfelt FA, Boyiadzis M, Gress RE (2004) Aging, immunity and cancer. Curr Opin Immunol 16:151−156
27. Dighe AS, Richards E, Old LJ, Schreiber RD (1994) Enhanced in vivo growth and resistance to rejection of tumor cells expressing dominant negative IFN receptors. Immunity 1:447−456
28. Morse MA, Lyerly HK, Clay TM, Abdel-Wahab O, Chui SY, Garst J, Gollob J, Grossi PM, Kalady M, Mosca PJ, Onaitis M, Sampson JH, Seigler HF, Toloza EM, Tyler D, Vieweg J, Yang Y (2004) Immunotherapy of surgical malignancies. Curr Probl Surg 1:15-132
29. Burnet FM (1970) The concept of immune surveillance Prog Exp Tumor Res 13:1-27
30. Novellino L, Castelli C, Parmiani G (2005) A listing of human tumor antigens recognized by T cells: March (2004) update. Cancer Immunol Immunother unol Immunother. (2005) Mar;54(3)3:187-207
31. Whiteside TL, Odoux C (2004) Dendritic cell biology and cancer therapy. Cancer Immunol Immunother 3:240-8
32. Falus András (2001) Az immunológia élettani és molekuláris alapjai. 3. edition. Semmelweis Kiadó. ISBN: 9639214175
33. Steinman RM, Hawiger D, Nussenzweig MC. (2003) Tolerogenic dendritic cells. Annu Rev Immunol 21:685-711
34. Ryschich E, Notzel T, Hinz U, Autschbach F, Ferguson J, Simon I, Weitz J, Frohlich B, Klar E, Buchler MW, Schmidt J (2005) Control of T-cell-mediated immune response by HLA class I in human pancreatic carcinoma. Clin Cancer Res 11(2 Pt 1):498-504
35. Zhang L, Conejo-Garcia JR, Katsaros D (2003) Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. N Engl J Med 348:203-213
36. Sinkovics JG, Dreyer DA, Shirato E, Cabiness JR, Shullenberger CC (1971) Cytotoxic lymphocytes. I. Destruction of neoplastic cells by lymphocytes in cultures of human origin. Tex Rep Biol Med 2:227-42
37. Miller JS (2002) Biology of natural killer cells in cancer and infection. Cancer Invest 20:405-19

38. Kronenberg M, Gapin L (2002) The unconventional lifestyle of NKT cells. Nat Rev Immunol 8:557-68
39. Sahin U, Tureci O, Pfreundschuh M (1997) Serological identification of human tumor antigens. Curr Opin Immunol 9:709-716
40. Langerhans P (1868) Ueber die Nerven der menschlichen Haut. Archiv für pathologische Anatomie und Physiologie, und für Klinische Medicin. Berlin. 44:325-337.
41. Steinman RM, Cohn ZA (1973) Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. J Exp Med 137:1142-1162
42. Grouard, G., M.-C. Rissoan, L. Filgueira, I. Durand, J. Banchereau, Y.-J. Liu (1997) The enigmatic plasmacytoid T cells develop into dendritic cells with IL-3 and CD40-ligand. J Exp Med 185:1101-1111
43. Inaba, K., M. Inaba, N. Romani, H. Aya, M. Deguchi, S. Ikehara, S. Muramatsu, R. M. Steinman (1992) Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. J Exp Med 176:1693-1702
44. Lutz, M. B., N. Kukutsch, A. L. J. Ogilvie, S. RoBner, F. Koch, N. Romani, G. Schuler (1999) An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. J Immunol Methods 223:77-92
45. Osugi Y, Vuckovic S, Hart DN (2002) Myeloid blood CD11c+ dendritic cells and monocyte-derived dendritic cells differ in their ability to stimulate T lymphocytes. Blood 100:2858-2866
46. Ratzinger G, Baggers J, de Cos MA, Yuan J, Dao T, Reagan JL, Munz C, Heller G, Young JW (2004) Mature human Langerhans cells derived from CD34+ hematopoietic progenitors stimulate greater cytolytic T lymphocyte activity in the absence of bioactive IL-12p70, by either single peptide presentation or cross-priming, than do dermal-interstitial or monocyte-derived dendritic cells. J Immunol 173:2780-2791
47. Rossi M, Young JW (2005) Human dendritic cells: potent antigen-presenting cells at the crossroads of innate and adaptive immunity. J Immunol 3:1373-81

48. Szabolcs P, Avigan D, Gezelter S, Ciocon DH, Moore MAS, Steinman RM, Young JW (1996) Dendritic cells and macrophages can mature independently from a human bone marrow-derived, post-CFU intermediate. Blood 87:4520-4530
49. Banchereau J, Briere F, Caux C, DavoustJ, Lebecque S, Liu YJ, Pulendran B, Palucka K (2000) Immunobiology of dendritic cells. Annu Rev Immunol 18:767–811
50. Steinman, RM (1991) The dendritic cell system and its role in immunogenicity. Annu Rev Immunol 9:271−296
51. Hawiger, D., K. Inaba, Y. Dorsett, M. Guo, K. Mahnke, M. Rivera, J. V. Ravetch, R. M. Steinman, M. C. Nussenzweig (2001) Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo. J Exp Med 194:769-779
52. Katz SI, K Tamaki, DH Sachs (1979) Epidermal Langerhans cells are derived from cells originating in bone marrow. Nature 282:324-326
53. Silver DF, Hempling RE, Piver MS, Repasky EA (2000) Flt-3 ligand inhibits growth of human ovarian tumors engrafted in severe combined immunodeficient mice. Gynecol Oncol 3:377-82
54. Somers KD, Brown RR, Holterman DA, Yousefieh N, Glass WF, Wright GL Jr, Schellhammer PF, Qian J, Ciavarra RP (2003) Orthotopic treatment model of prostate cancer and metastasis in the immunocompetent mouse: efficacy of flt3 ligand immunotherapy. Int J Cancer 5:773-80
55. Morse MA, Nair SK, Boczkowski D, Tyler D, Hurwitz HI, Proia A, Clay TM, Schlom J, Gilboa E, Lyerly HK (2002) The feasibility and safety of immunotherapy with dendritic cells loaded with CEA mRNA following neoadjuvant chemoradiotherapy and resection of pancreatic cancer. Int J Gastrointest Cancer 1:1-6
56. Ciavarra RP, Holterman DA, Brown RR, Mangiotti P, Yousefieh N, Wright GL Jr, Schellhammer PF, Glass WF, Somers KD (2004) Prostate tumor microenvironment alters immune cells and prevents long-term survival in an orthotopic mouse model following flt3-ligand/CD40-ligand immunotherapy. J Immunother 1:13-26

57. Guermonprez P, Valladeau J, Zitvogel L, Thery C, Amigorena S (2002) Antigen presentation and T cell stimulation by dendritic cells. Annu Rev Immunol 20:621-67
58. Banchereau J, Steinman RM (1998) Dendritic cells and the control of immunity Nature 392:245-252
59. Banchereau J, Palucka AK (2005) Dendritic cells as therapeutic vaccines against cancer Nat Rev Immunol 4:296-306
60. Bell D, Young JW, Banchereau J (1999) Dendritic cells. Adv Immunol 72:255-324
61. Sheng KC, Pietersz GA, Wright MD, Apostolopoulos V (2005) Dendritic cells: activation and maturation-applications for cancer immunotherapy. Curr Med Chem 15:1783-800
62. Caux, C et al (1997) CD34+ hematopoietic progenitors from human cord blood differentiate along two independent dendritic cell pathways in response to granulocyte−macrophage colony-stimulating factor plus tumor necrosis factor: II. Functional analysis. Blood 4:1458−1470
63. Jego, G (2003) Plasmacytoid dendritic cells induce plasma cell differentiation through type I interferon and interleukin 6 Immunity 19:225−234
64. Ferlazzo G, Munz C (2004) NK cell compartments and their activation by dendritic cells. J Immunol 3:1333-9
65. Fernandez, N. C (1999) Dendritic cells directly trigger NK cell functions: cross-talk relevant in innate anti-tumor immune responses in vivo. Nature Med 5:405−411
66. Kalinski P, Giermasz A, Nakamura Y, Basse P, Storkus WJ, Kirkwood JM, Mailliard RB (2005) Helper role of NK cells during the induction of anticancer responses by dendritic cells. Mol Immunol 4:535-9
67. Kadowaki N (2001) Distinct cytokine profiles of neonatal natural killer T cells after expansion with subsets of dendritic cells. J Exp Med 193:1221−1226
68. Rissoan MC (1999) Reciprocal control of T helper cell and dendritic cell differentiation. Science 283:1183−1186
69. Brocker, T (1999) The role of dendritic cells in T cell selection and survival J Leukoc Biol 66:331-35

70. Egilmez NK, Iwanuma Y, Bankert RB (1996) Evaluation and optimization of different cationic liposome formulations for in vivo gene transfer. Biochem Biophys Res Commun 1:169-73
71. Liu K, Iyoda T, Saternus M, Kimura Y, Inaba K, Steinman RM (2002) Immune tolerance following delivery of dying cells to dendritic cells in situ. J Exp Med 196:1091–7
72. Bonifaz, L (2002) Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral CD8+ T cell tolerance. J Exp Med 12:1627−1638
73. Bonifaz, LC (2004). In vivo targeting of antigens to maturing dendritic cells via the DEC-205 receptor improves T cell vaccination. J Exp Med 6:815−824
74. Davey GM, Kurts C, Miller JFAP, Bouillet P, Strasser A (2002) Peripheral deletion of autoreactive CD8 T cells by cross-presentation of self antigen occurs by a Bcl-2-inhibitable pathway mediated by Bim. J Exp Med 196:947–55
75. Terness P, Bauer TM, Rose L, Dufter C, Watzlik A (2002) Inhibition of allogeneic T cell proliferation by indoleamine 2,3-dioxygenase-expressing dendritc cells: mediation of suppression by tryptophan metabolites. J Exp Med 196:447–57
76. Terabe, M (2000) NKT cell-mediated repression of tumor immunosurveillance by IL-13 and the IL-4R−STAT6 pathway. Nature Immunol 1:515−520
77. Gershon RK, Kondo K (1971) Infectious immunological tolerance. Immunology 21:903–913
78. Antony PA, Restifo NP (2005) CD4+CD25+ T Regulatory Cells, Immunotherapy of Cancer, and Interleukin-2. J Immunother 2:120–128
79. Filaci G, Fravega M, Fenoglio D, Rizzi M, Negrini S, Viggiani R, Indiveri F (2004) Non-antigen specific CD8+ T suppressor lymphocytes. Clin Exp Med 2:86-92
80. Yamazaki S (2003) Direct expansion of functional CD25+ CD4+ regulatory T cells by antigen-processing dendritic cells. J Exp Med 198:235−247
81. Jonuleit H, Schmitt E, Schuler G, Knop J, Enk AH (2000) Induction of interleukin 10-producing, nonproliferating CD4+ T cells with regulatory properties by

repetitive stimulation with allogeneic immature human dendritic cells. J Exp Med 192:1213−1222

82. Coley WB. The treatment of malignant tumors by repeated inoculations of erysipelas. With a report of ten original cases. 1893. Clin Orthop Relat Res (1991):3–11.
83. Davis ID, Jefford M, Parente P, Cebon, J (2003) Rational approaches to human cancer immunotherapy. J Leukoc Biol 73:3−29
84. Rosenberg SA, Yang JC, Restifo NP (2004) Cancer immunotherapy: moving beyond current vaccines. Nature Med 10:909−915
85. Sang H, Pisarev VM, Munger C, Robinson S, Chavez J, Hatcher L, Parajuli P, Guo Y, Talmadge JE (2003) Regional, but not systemic recruitment/expansion of dendritic cells by a pluronic-formulated Flt3-ligand plasmid with vaccine adjuvant activity. Vaccine 21:3019-29
86. Nemunaitis J. (2003) J GVAX (GMCSF gene modified tumor vaccine) in advanced stage non small cell lung cancer. Control Release 91:225-231.
87. Laheru D, Yeo C, Biedrzycki B (2007): A safety and efficacy trial of lethally irradiated allogeneic pancreatic tumor cells transfected with the GM-CSF gene in combination with adjuvant chemoradiotherapy for the treatment of adenocarcinoma of the pancreas. Gastrointestinal Cancers Symposium, Orlando, FL, (2007) Jan 19-21. Abstract 106.
88. Kikuchi T. (2006) Genetically modified dendritic cells for therapeutic immunity. Tohoku J Exp Med ;208:1-8.
89. Ugen KE, Kutzler MA, Marrero B, (2006) Regression of subcutaneous B16 melanoma tumors after intratumoral delivery of an IL-15-expressing plasmid followed by in vivo electroporation. Cancer Gene Ther;13:969–74.
90. Kutzler MA, Weiner DB. (2004) Developing DNA vaccines that call to dendritic cells. J Clin Invest 114:1241–4.
91. Cunningham CC, Chada S, Merritt JA, Tong A, Senzer N, Zhang Y, Mhashilkar A, Parker K, Vukelja S, Richards D, Hood J, Coffee K, Nemunaitis J. (2005) Clinical and local biological effects of an intratumoral injection of mda-7 (IL24; INGN 241) in patients with advanced carcinoma: a phase I study. Mol Ther; 11:149-159.

92. McLoughlin JM, McCarty TM, Cunningham C, Clark V, Senzer N, Nemunaitis J, Kuhn JA. (2005) TNFerade, an adenovector carrying the transgene for human tumor necrosis factor alpha, for patients with advanced solid tumors: surgical experience and long-term follow-up. Ann Surg Oncol;12:825-830.
93. Posner M, Chang KJ, Rosemurgy A, Stephenson J, Khan M, Reid T, Fisher WE, Waxman I, Von Hoff D, Hecht R Jr (2007) Multicenter phase II/III randomized controlled linical trial using TNFerade combined with chemoradiation in patients with locally advanced pancreatic cancer (LAPC). J Clin Oncol (Meet Abstracts); 25: 4518.
94. Shurin MR, Esche C, Lotze MT (1998) FLT3: receptor and ligand. Biology and potential clinical application. Cytokine Growth Factor Rev 1:37-48
95. Yarden Y, Ullrich A (1988) Growth factor receptor tyrosine kinases. Annu Rev Biochem 57:443-78
96. Antonysamy MA, Thomson AW (2000) Flt3 ligand (FL) and its influence on immune reactivity. Cytokine 2:87-100
97. Karsunky H, Merad M, Cozzio A, Weissman IL, Manz MG (2003) Flt3 ligand regulates dendritic cell development from Flt3+ lymphoid and myeloid-committed progenitors to Flt3+ dendritic cells in vivo. J Exp Med 2:305-13
98. Lyman SD, James L, Vanden Bos T, de Vries P, Brasel K, Gliniak B, Hollingsworth LT, Picha KS, McKenna HJ, Splett RR (1993) Molecular cloning of a ligand for the flt3/flk-2 tyrosine kinase receptor: a proliferative factor for primitive hematopoietic cells. Cell 6:1157-67
99. Lyman SD, James L, Escobar S, Downey H, de Vries P, Brasel K, Stocking K, Beckmann MP, Copeland NG, Cleveland LS (1995) Identification of soluble and membrane-bound isoforms of the murine flt3 ligand generated by alternative splicing of mRNAs. Oncogene 1:149-57
100. McKenna HJ, de Vries P, Brasel K, Lyman SD, Williams DE (1995) Effect of flt3 ligand on the ex vivo expansion of human CD34+ hematopoietic progenitor cells. Blood 9:3413-20
101. Fernandez NC, Lozier A, Flament C, Ricciardi-Castagnoli P, Bellet D, Suter M, Perricaudet M, Tursz T, Maraskovsky E, Zitvogel L (1999) Dendritic cells

directly trigger NK cell functions: cross-talk relevant in innate anti-tumor immune responses in vivo. Nat Med 4:405-11

102. Lynch DH, Andreasen A, Maraskovsky E, Whitmore J, Miller RE, Schuh JC (1997) Flt3 ligand induces tumor regression and antitumor immune responses in vivo. Nat Med 6:625-31
103. Shaw SG, Maung AA, Steptoe RJ, Thomson AW, Vujanovic NL (1998) Expansion of functional NK cells in multiple tissue compartments of mice treated with Flt3-ligand: implications for anti-cancer and anti-viral therapy. J Immunol 6:2817-24
104. Dong J, McPherson CM, Stambrook PJ (2002) Flt-3 ligand: a potent dendritic cell stimulator and novel antitumor agent. Cancer Biol Ther 5:486-9
105. Maraskovsky E, Brasel K, Teepe M, Roux ER, Lyman SD, Shortman K, McKenna HJ (1996) Dramatic increase in the numbers of functionally mature dendritic cells in Flt3 ligand-treated mice: multiple dendritic cell subpopulations identified. J Exp Med 5:1953-62
106. Metcalf DK, Shortman D, Vremec S, Mifsud L Di Kago (1996) Effects of excess GM-CSF levels on hematopoiesis and leukemia development in GM-CSF/max 41 double transgenic mice. Leukemia 10:713-719
107. Ali S, Curtin JF, Zirger JM, Xiong W, King GD, Barcia C, Liu C, Puntel M, Goverdhana S, Lowenstein PR, Castro MG (2004) Inflammatory and anti-glioma effects of an adenovirus expressing human soluble Fms-like tyrosine kinase 3 ligand (hsFlt3L): treatment with hsFlt3L inhibits intracranial glioma progression. Mol Ther 6:1071-84
108. Braun SE, Chen K, Blazar BR, Orchard PJ, Sledge G, Robertson MJ, Broxmeyer HE, Cornetta K (1999) Flt3 ligand antitumor activity in a murine breast cancer model: a comparison with granulocyte-macrophage colony-stimulating factor and a potential mechanism of action Hum Gene Ther 13:2141-51
109. Dong J, Bohinski RJ, Li YQ, Van Waes C, Hendler F, Gleich L, Stambrook PJ (2003) Antitumor effect of secreted Flt3-ligand can act at distant tumor sites in a murine model of head and neck cancer. Cancer Gene Ther 2:96-104

110. Chen K, Braun S, Lyman S, Fan Y, Traycoff CM, Wiebke EA, Gaddy J, Sledge G, Broxmeyer HE, Cornetta K (1997) Antitumor activity and immunotherapeutic properties of Flt3-ligand in a murine breast cancer model. Cancer Res 16:3511-6
111. Miller G, Pillarisetty VG, Shah AB, Lahrs S, DeMatteo RP (2003) Murine Flt3 ligand expands distinct dendritic cells with both tolerogenic and immunogenic properties. J Immunol 7:3554-64
112. Yanagi K, Nagayama Y, Nakao K, Saeki A, Matsumoto K, Ichikawa T, Ishikawa H, Hamasaki K, Ishii N, Eguchi K (2003) Immuno-gene therapy with adenoviruses expressing fms-like tyrosine kinase 3 ligand and CD40 ligand for mouse hepatoma cells in vivo. Int J Oncol 2:345-51
113. Averbook BJ, Schuh JL, Papay R, Maliszewski C (2002) Antitumor effects of Flt3 ligand in transplanted murine tumor models J Immunother 1:27-35
114. Brasel K, McKenna HJ, Morrissey PJ, Charrier K, Morris AE, Lee CC, Williams DE, Lyman SD (1996) Hematologic effects of flt3 ligand in vivo in mice Blood Sep 6:(2004)-12
115. Peron JM, Esche C, Subbotin VM, Maliszewski C, Lotze MT, Shurin MR (1998) FLT3-ligand administration inhibits liver metastases: role of NK cells. J Immunol 11:6164-70
116. Esche C, Subbotin VM, Maliszewski C, Lotze MT, Shurin MR (1998) FLT3 ligand administration inhibits tumor growth in murine melanoma and lymphoma. Cancer Res 3:380-3
117. Furumoto K, Soares L, Engleman EG, Merad M (2004) Induction of potent antitumor immunity by in situ targeting of intratumoral DCs. J Clin Invest Mar 5:774-83
118. Ciavarra RP, Somers KD, Brown RR, Glass WF, Consolvo PJ, Wright GL, Schellhammer PF (2000) Flt3-ligand induces transient tumor regression in an ectopic treatment model of major histocompatibility complex-negative prostate cancer. Cancer Res 8:2081-4
119. Chomarat P, Dantin C, Bennett L, Banchereau J, Palucka AK (2003) TNF skews monocyte differentiation from macrophages to dendritic cells. J Immunol 5:2262–2269

120. Ciavarra RP, Brown RR, Holterman DA, Garrett M, Glass WF 2nd, Wright GL Jr, Schellhammer PF, Somers KD (2003) Impact of the tumor microenvironment on host infiltrating cells and the efficacy of flt3-ligand combination immunotherapy evaluated in a treatment model of mouse prostate cancer. Cancer Immunol Immunother 9:535-45
121. Favre-Felix N, Martin M, Maraskovsky E, Fromentin A, Moutet M, Solary E, Martin F, Bonnotte B (2000) Flt3 ligand lessens the growth of tumors obtained after colon cancer cell injection in rats but does not restore tumor-suppressed dendritic cell function. Int J Cancer 86(6):827-34
122. Freedman RS, Vadhan-Raj S, Butts C, Savary C, Melichar B, Verschraegen C, Kavanagh JJ, Hicks ME, Levy LB, Folloder JK, Garcia ME (2003) Pilot study of Flt3 ligand comparing intraperitoneal with subcutaneous routes on hematologic and immunologic responses in patients with peritoneal carcinomatosis and mesotheliomas. Clin Cancer Res 14:5228-37
123. Higano CS, Vogelzang NJ, Sosman JA, Feng A, Caron D, Small EJ (2004) Safety and biological activity of repeated doses of recombinant human Flt3 ligand in patients with bone scan-negative hormone-refractory prostate cancer. Clin Cancer Res 4:1219-25
124. Maraskovsky E, Daro E, Roux E, Teepe M, Maliszewski CR, Hoek J, Caron D, Lebsack ME, McKenna HJ (2000) In vivo generation of human dendritic cell subsets by Flt3 ligand. Blood 96:878-884
125. McNeel DG, Knutson KL, Schiffman K, Davis DR, Caron D, Disis ML (2003) Pilot study of an HLA-A2 peptide vaccine using flt3 ligand as a systemic vaccine adjuvant. J Clin Immunol 1:62-72
126. Morse MA, Nair S, Fernandez-Casal M, Deng Y, St Peter M, Williams R, Hobeika A, Mosca P, Clay T, Cumming RI, Fisher E, Clavien P, Proia AD, Niedzwiecki D, Caron D, Lyerly HK (2000) Preoperative mobilization of circulating dendritic cells by Flt3 ligand administration to patients with metastatic colon cancer. J Clin Oncol 23:3883-93
127. Dunphy EJ, McNeel DG (2005) Antigen-specific IgG elicited in subjects with prostate cancer treated with flt3 ligand. J Immunother 3:268-75

128. Juan TS, McNiece IK, Van G, Lacey D, Hartley C, McElroy P, Sun Y, Argento J, Hill D, Yan XQ, Fletcher FA (1997) Chronic expression of murine flt3 ligand in mice results in increased circulating white blood cell levels and abnormal cellular infiltrates associated with splenic fibrosis. Blood 1:76-84
129. Wang A, Braun SE, Sonpavde G, Cornetta K (2000) Antileukemic activity of Flt3 ligand in murine leukemia. Cancer Res 7:1895-900
130. Mosca PJ, Hobeika AC, Colling K, Clay TM, Thomas EK, Caron D, Lyerly HK, Morse MA. (2002) Multiple signals are required for maturation of human dendritic cells mobilized in vivo with Flt3 ligand. J. Leukoc. Biol. 72: 546–553.
131. Wang H, Dai J, Hou S, Qian W, Li B, Ma J, Fan X, Zhao J, Yang S, Sang H, Yang Q, Wang R, Guo Y (2005) Treatment of hepatocellular carcinoma with adenoviral vector-mediated Flt3 ligand gene therapy. Cancer Gene Ther 9:769-77
132. Hung CF, Hsu KF, Cheng WF, Chai CY, He L, Ling M, Wu TC (2001) Enhancement of DNA vaccine potency by linkage of antigen gene to a gene encoding the extracellular domain of Fms-like tyrosine kinase 3-ligand. Cancer Res 3:1080-8
133. Peretz Y, Zhou ZF, Halwani F, Prud'homme GJ (2002) In vivo generation of dendritic cells by intramuscular codelivery of FLT3 ligand and GM-CSF plasmids. Mol Ther 3:407-14
134. Shimao K, Takayama T, Enomoto K, Saito T, Nagai S, Miyazaki J, Ogawa K, Tahara H (2005) Cancer gene therapy using in vivo electroporation of Flt3-ligand. Int J Oncol 2:457-63
135. Yang Q, Yang G, Wei L, Jia F, Wu M, Guo Y (2002) Gene transfer of murine Flt3 ligand mediated by adenoviral vector efficiently induces growth inhibition of murine liver cancer. Zhonghua Yi Xue Za Zhi 11:775-9
136. Kawakami Y, Okada T, Akada M (2004) Development of immunotherapy for pancreatic cancer. Pancreas. 28(3):320-5
137. Ko AH (2007) Future strategies for targeted therapies and tailored patient management in pancreatic cancer. Semin Oncol 34:354-364
138. Laheru D, Jaffee EM. Immunotherapy for pancreatic cancer - science driving clinical progress. Nat Rev Cancer. (2005) Jun;5(6):459-67.

139. Märten A, Büchler MW. Immunotherapy of pancreatic carcinoma. Curr Opin Investig Drugs. (2008) Jun;9(6):565-9.
140. Wong HH, Lemoine NR. Biological approaches to therapy of pancreatic cancer. Pancreatology. (2008);8(4-5):431-61.
141. Gjertsen MK, Buanes T, Rosseland AR, Bakka A, Gladhaug I, Soreide O, Eriksen JA, Moller M, Baksaas I, Lothe RA, Saeterdal I, Gaudernack G (2001) Intradermal ras peptide vaccination with granulocyte-macrophage colony-stimulating factor as adjuvant: Clinical and immunological responses in patients with pancreatic adenocarcinoma. Int J Cancer 3:441-50.
142. Pecher G, Haring A, Kaiser L, Thiel E (2002) Mucin gene (MUC1) transfected dendritic cells as vaccine: results of a phase I/II clinical trial. Cancer Immunol Immunother 11-12:669-73
143. Zalatnai A. Pathologic diagnosis of pancreatic cancer--facts, pitfalls, challenges. Orv Hetil. (2001) Sep 2;142(35):1885-90.
144. Jones S, Zhang X, Parsons DW, Lin JC, Leary RJ, Angenendt P, Mankoo P, Carter H, Kamiyama H, Jimeno A, Hong SM, Fu B, Lin MT, Calhoun ES, Kamiyama M, Walter K, Nikolskaya T, Nikolsky Y, Hartigan J, Smith DR, Hidalgo M, Leach SD, Klein AP, Jaffee EM, Goggins M, Maitra A, Iacobuzio-Donahue C, Eshleman JR, Kern SE, Hruban RH, Karchin R, Papadopoulos N, Parmigiani G, Vogelstein B, Velculescu VE, Kinzler KW. Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. Science. (2008) Sep 26;321(5897):1801-6.
145. Tada M, Komatsu Y, Kawabe T, Sasahira N, Isayama H, Toda N, Shiratori Y, Omata. Quantitative analysis of K-ras gene mutation in pancreatic tissue obtained by endoscopic ultrasonography-guided fine needle aspiration: clinical utility for diagnosis of pancreatic tumor. M. Am J Gastroenterol. (2002) Sep;97(9):2263-70.
146. Brentnall TA, Chen R, Lee JG, Kimmey MB, Bronner MP, Haggitt RC, Kowdley KV, Hecker LM, Byrd DR. Microsatellite instability and K-ras mutations associated with pancreatic adenocarcinoma and pancreatitis. Cancer Res (1995);55:4264-4267.
147. Gerdes B, Ramaswamy A, Ziegler A, Lang SA, Kersting M, Baumann R, Wild A, Moll R, Rothmund M, Bartsch DK. p16INK4a is a prognostic marker in resected

ductal pancreatic cancer: an analysis of p16INK4a, p53, MDM2, an Rb. Ann Surg (2002);235:51-59.

148. Konner J, O'Reilly E. Pancreatic cancer: epidemiology, genetics, and approaches to screening. Oncology (Huntingt) (2002);16:1615-2.
149. Kleeff J, Friess H, Simon P, Susmallian S, Buchler P, Zimmermann A, Buchler MW, Korc M. Overexpression of Smad2 and colocalization with TGF-beta1 in human pancreatic cancer. Dig Dis Sci (1999);44:1793-1802.
150. Aho U, Zhao X, Löhr M, Andersson R. (2007) Molecular mechanisms of pancreatic cancer and potential targets of treatment. Scand J Gastroenterol. Mar;42(3):279-96.
151. Goggins M. (2007) Identifying molecular markers for the early detection of pancreatic neoplasia. Semin Oncol. Aug;34(4):303-10.
152. Daniels TR, Delgado T, Helguera G, Penichet ML (2006) The transferrin receptor part I: Biology and targeting with cytotoxic antibodies for the treatment of cancer Clin Immunol 121:144—158.
153. Quian ZH, Li H, Sun H, HO K. (2002) Targeted drug delivery via the transferrin receptor-mediated endocytosis pathway. Pharmacol Rev 54:561–587.
154. Gatter KC, Brown G, Trowbridge IS, Woolston RE, Mason DY. (1983) Transferrin receptors in human tissues: their distribution and possible clinical relevance. J Clin Pathol;36:539-545.
155. Niitsu Y, Kohgo Y, Nishisato T, Kondo H, Kato J, Urushizaki Y, Urushizaki I. (1987) Transferrin receptors in human cancerous tissues. Tohoku J Exp Med;153:239-243.
156. Prior R, Reifenberger G, Wechsler W.(1990)Transferrin receptor expression in tumours of the human nervous system: relation to tumour type, grading and tumour growth fraction. Virchows Arch A Pathol Anat Histopathol;416:491-496.
157. Tanaka N, Sugihara K, Odajima T, Mimura M, Kimijima Y, Ichinose S. (2002)Oral squamous cell carcinoma: electron microscopic and immunohistochemical characteristics. Med Electron Microsc;35:127-138.
158. Shinohara H, Fan D, Ozawa S, Yano S, Van Arsdell M, Viner JL, Beers R, Pastan I, Fidler IJ. (2000)Site-specific expression of transferrin receptor by human colon

cancer cells directly correlates with eradication by antitransferrin recombinant immunotoxin. Int J Oncol;17:643-651.

159. Kemp JD. Iron deprivation and cancer: a view beginning with studies of monoclonal antibodies against the transferrin receptor. (1997)Histol Histopathol;12:291-296.
160. Wen PY, Kesari S. (2008)Malignant gliomas in adults. The New England Journal of Medicine, 359 (5): 492–507.
161. Stupp R, Mason WP, van den Bent MJ. (2005)Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. The New England Journal of Medicine;352:987-96
162. Cairncross JG, Ueki K, Zlatescu MC. (1998)Specific genetic predictors of chemotherapeutic response and survival in patients with anaplastic oligodendrogliomas. J Natl Cancer Inst;90:1473-9.
163. Lumniczky K, Safrany G. (2006)Cancer gene therapy: combination with radiation therapy and the role of bystander cell killing in the anti-tumor effect. Pathol Oncol Res; 12: 118-24.
164. Altaner C. (2008) Prodrug cancer gene therapy. Cancer Lett. Nov 8;270(2):191-201.
165. Hermiston TW, Kuhn I. (2002) Armed therapeutic viruses: strategies and challenges to arming oncolytic viruses with therapeutic genes. Cancer Gene Ther. Dec;9(12):1022-35.
166. Desaknai S, Lumniczky K, Esik O, Hamada H, Safrany G. (2003)Local tumor irradiation enhances the anti-tumour effect of a double-suicide gene therapy system in a murine glioma model. J Gene Med; 5: 377-85.
167. Dilber MS, Gahrton G. (2001) Suicide gene therapy: possible applications in haematopoietic disorders. J Intern Med. Apr;249(4):359-67.
168. Freeman SM, Abboud CN, Whartenby KA, Packman CH, Koeplin DS, Moolten FL, Abraham GN. (1993) The "bystander effect": tumor regression when a fraction of the tumor mass is genetically modified. Cancer Res. Nov 1;53(21):5274-83.
169. J Gene Med Clinical Trial Site: www.wiley.co.uk/genetherapy/clinical/

170. Hamel W, Westphal M. (2003) Gene therapy of gliomas. Acta Neurochir. [Suppl] 88:125-135.
171. Rainov NG. (2000)A phase III clinical evaluation of herpes simplex virus type 1 thymidine kinase and ganciclovir gene therapy as an adjuvant to surgical resection and radiation in adults with previously untreated glioblastoma multiforme.. Hum Gene Ther 20:2389-2401.
172. Harsh GR, Deisboeck TS, Louis DN, Hilton J, Colvin M, Silver JS, Qureshi NH, Kracher J, Finkelstein D, Chiocca EA, Hochberg FH(2000). Thymidine kinase activation of ganciclovir in recurrent malignant gliomas: a gene-marking and neuropathological study.. J Neurosurg 92:804-811.
173. Immonen A, Vapalahti M, Tyynelä K, Hurskainen H, Sandmair A, Vanninen R, Langford G, Murray N, Ylä-Herttuala S. (2004) AdvHSV-tk gene therapy with intravenous ganciclovir improves survival in human malignant glioma: a randomised, controlled study. Mol Ther. Nov;10(5):967-72.
174. Shinoura N, Koike H, Furitu T, Hashimoto M, Asai A, Kirino T, Hamada H. (2000) Adenovirus-mediated transfer of caspase 8 auguments cell death in gliomas: implication for gene therapy. Hum Gene Ther 11:1123-1137.
175. Markert JM, Parker JN, Gillespie GY, Whitley RJ. (2001)Genetically engineered human herpes simplex virus in the treatment of brain tumors.. Herpes 8:17-22.
176. Hertel LW, Kroin JS, Misner JW, Tustin JM. (1988) Synthesis of 2-deoxy-2,2-difluoro-D-ribose and 2-deoxy-2,2-difluoro-D-ribofuranosyl nucleotides. J Org Chem; 53: 2406-9.
177. Plunkett W, Huang P, Searcy CE, Gandhi V. (1996) Gemcitabine: preclinical pharmacology and mechanisms of action. Semin Oncol. Oct;23(5 Suppl 10):3-15.
178. Ruiz van Haperen VW, Veerman G, Vermorken JB, Peters GJ. (1993)20,20-Difluoro-deoxycytidine (gemcitabine) incorporation into RNA and DNA of tumour cell lines. Biochem Pharmacol; 46: 762–766.
179. Berk AJ, Clayton DA (1973) A genetically distinct thymidine kinase in mammalian mitochondria. Exclusive labeling of mitochondrial deoxyribonucleic acid. J. Biol. Chem.: 248 (8): 2722–9.
180. Beauséjour CM, Gagnon J, Primeau M, Momparler RL. (2002)Cytotoxic activity of 2',2'-difluorodeoxycytidine, 5-aza-2'- deoxycytidine and cytosine arabinoside

in cells transduced with deoxycytidine kinase gene. Biochem Biophys Res Commun; 293: 1478–1484.

181. Hertel LW, Boder GB, Kroin JS, Rinzel SM, Poore GA, Todd GC. (1990)Evaluation of the antitumor activity of gemcitabine. Cancer Res; 50: 4417-22.
182. Eisbruch A, Shewach DS, Bradford CR (2001) Radiation concurrent with gemcitabine for locally advanced head and neck cancer: A phase I trial and intracellular drug incorporation study. J Clin Oncol;19:792–799.
183. Trodella L, GrAnone P, Valente S(2002)Phase I trial of weekly gemcitabine and concurrent radiotherapy in patients with inoperable non-small-cell lung cancer. J Clin Oncol;20:804–810.
184. Sigmond J, Honeywell RJ, Postma TJ, Dirven CM, de Lange SM, van der Born K, Laan AC, Baayen JC, Van Groeningen CJ, Bergman AM, Giaccone G, Peters GJ. (2008)Gemcitabine uptake in glioblastoma multiforme: potential as a radiosensitizer. Ann Oncol. Aug 12.
185. Shewach DS, Lawrence TS. (1996)Gemcitabine and radiosensitization in human tumor cells. Invest New Drugs;14:257–263.
186. Lawrence TS, Chang EY, Hahn TM (1996)e Radiosensitizationof pancreatic cancer cells by 2, 2,difluoro-2-deoxycytidine.Int J Radiat Oncol Biol Phys;34:867–872.
187. Lawrence TS, Eisbruch A, Shewach DS. (1997)Gemcitabine-mediatedradiosensitization. Semin Oncol;24:24–28.8.
188. Rosier JF, Beauduin M, Bruniaux M (1999) The effect of 2-2difluorodeoxycytidine (dFdC, gemcitabine) on radiation-induced cell lethality in two human head and neck squamouscarcinoma cell lines differing in intrinsic radiosensitivity. Int JRadiat Biol;75:245–251.
189. Shewach DS, Hahn TM, Chang E, (1994) Metabolism of 2,2-difluoro-2-deoxycytidine and radiation sensitization of humann colon carcinoma cells. Cancer Res;54:3218–3223.
190. Shewach DS, Lawrence TS. (1996)Radiosensitization of human solid tumor cell lines with gemcitabine. Semin Oncol;23:65–71.

191. Joschko MA, Webster LK, Groves J (1997) Enhancement of radiation-induced regrowth delay by gemcitabine in a human tumor xenograft model. Radiat Oncol Invest 5:62–71.
192. Milas L, Fujii T, Hunter N (1999) Enhancement of tumor radioresponse in vivo by gemcitabine. Cancer Res 59: 107–114.
193. Mason KA, Milas L, Hunter NR (1999) Maximizing therapeutic gain with gemcitabine and fractionated radiation. Int JRadiat Oncol Biol Phys 44:1125–1135.
194. Lawrence TS, Davis MA, Hough A, Rehemtulla A. (2001)The role of apoptosis in 2',2'-difluoro-2'-deoxycytidine (Gemcitabine)-mediated radiosensitization. Clin Cancer Res; 7: 314-9.
195. Horsman MR, Bohm L, Margison GP, Milas L, Rosier JF, Safrany G (2006)Tumor radiosensitizers-current status of development of various approaches: report of an international atomic energy agency meeting. Int J Radiat Oncol Biol Phys; 64: 551-61.
196. Gertler SZ, MacDonald D, Goodyear M, Forsyth P, Stewart DJ, Belanger K. (2000)NCIC-CTG phase II study of gemcitabine in patients with malignant glioma (IND94). Ann Oncol; 11: 315-8.
197. Weller M, Streffer J, Wick W, Kortmann RD, Heiss E, Kuker W(2001) Preirradiation gemcitabine chemotherapy for newly diagnosed glioblastoma. A phase II study. Cancer; 91: 423-7.
198. Maraveyas A, Sgouros J, Upadhyay S, Abdel-Hamid AH, Holmes M, Lind M. (2005)Gemcitabine twice weekly as a radiosensitizer for the treatment of brain metastases in patients with carcinoma: a phase I study. Br J Cancer; 92: 815-9.
199. Fabi A, Mirri A, Felici A, Vidiri A, Pace A, Occhipinti E, Cognetti F, Arcangeli G, Iandolo B, Carosi MA, Metro G, Carapella CM. (2008) Fixed dose-rate gemcitabine as radiosensitizer for newly diagnosed glioblastoma: a dose-finding study. J Neurooncol. Mar;87(1):79-84. Epub (2007) Nov 7.
200. Coleman CN (1989) Modification of radiotherapy by radiosensitizers and cancer chemotherapy agents. I. Radiosensitizers. Semin Oncol. Jun;16(3):169-75.
201. Szybalski W (1974) X-ray sensitization by halopyrimidines.Cancer Chemother Rep. Jul-Aug;58(4):539-57.

202. A radiológia alapfogalmai. Szerk. Horváth Ferenc. (1999), Medicina. ISBN 9632426037.
203. Wilson GD, Bentzen SM, Harari PM. (2006) Biologic basis for combining drugs with radiation. Semin Radiat Oncol.16(1):2-9.
204. Valerie K, Brust D, Fransworth J, Amir C, Taher MM, Hershey C, Feden J. (2000)Improved radiosensitization of rat glioma cells with adenovirus-expressed mutant herpes simplex virus – thimidine kinase in combination with acyclovir. Cancer Gene Ther.. 7:879-884.
205. Lawrence TS, Blackstock AW, McGinn C. (2003) The mechanism of action of radiosensitization of conventional chemotherapeutic agents. Semin Radiat Oncol. 13(1):13-21.
206. Overgaard J. (1994)Clinical evaluation of nitroimidazoles as modifiers of hypoxia in solid tumors. Oncol Res.;6(10-11):509-18.
207. Rischin D, Peters L, Fisher R, Macann A, Denham J, Poulsen M, Jackson M, Kenny L, Penniment M, Corry J, Lamb D, McClure B. (2005) Tirapazamine, Cisplatin, and Radiation versus Fluorouracil, Cisplatin, and Radiation in patients with locally advanced head and neck cancer: a randomized phase II trial of the Trans-Tasman Radiation Oncology Group (TROG 98.02). J Clin Oncol. Jan 1;23(1):79-87.
208. Cividalli A, Livdi E, Ceciarelli F, Fontana G, Altavista P, Cruciani G, Danesi DT. (2001) Combined use of gemcitabine and radiation in mice Anticancer Res. Jan-Feb;21(1A):307-12.
209. Lawrence TS, Chang EY, Hahn TM, Shewach DS. (1997) Delayed radiosensitization of human colon carcinoma cells after a brief exposure to 2',2'-difluoro-2'-deoxycytidine (Gemcitabine). Clin Cancer Res. May;3(5):777-82.
210. Pauwels B, Korst AE, de Pooter CM, Lambrechts HA, Pattyn GG, Lardon F, Vermorken JB. (2003) The radiosensitising effect of gemcitabine and the influence of the rescue agent amifostine in vitro. Eur J Cancer. 39(6):838-46.
211. Grégoire V, Rosier JF, De Bast M, Bruniaux M, De Coster B, Octave-Prignot M, Scalliet P. (2002) Role of deoxycytidine kinase (dCK) activity in gemcitabine's radioenhancement in mice and human cell lines in vitro. Radiother Oncol. Jun;63(3):329-38.

212. Fields MT, Eisbruch A, Normolle D, Orfali A, Davis MA, Pu AT, Lawrence TS. (2000) Radiosensitization produced in vivo by once- vs. twice-weekly 2'2'-difluoro-2'-deoxycytidine (gemcitabine). Int J Radiat Oncol Biol Phys. 1;47(3):785-91.
213. Crane CH, Wolff RA, Abbruzzese JL, Evans DB, Milas L, Mason K, Charnsangavej C, Pisters PW, Lee JE, Lenzi R, Lahoti S, Vauthey JN, Janjan NA. (2001) Combining gemcitabine with radiation in pancreatic cancer: understanding important variables influencing the therapeutic index. Semin Oncol. 28(3 Suppl 10):25-33.
214. Gandhi V. (2007)Radiosensitization by gemcitabine fixed-dose-rate infusion versus bolus injection in a pancreatic cancer. J Clin Oncol;25: 5691.
215. Morgan MA, El Shaikh M, Abu-Isa E, Davis MA, Lawrence TS. (2008)Radiosensitization by gemcitabine fixed-dose-rate infusion versus bolus injection in a pancreatic cancer model. Transl Onco;1:449.
216. Morgan MA, Parsels LA, Maybaum J, Lawrence TS. (2008) Improving gemcitabine-mediated radiosensitization using molecularly targeted therapy: a review. Clin Cancer Res. 1;14(21):6744-50.
217. Girard N, Mornex F. (2007)Gemcitabine and radiotherapy in non-small cell lung cancer: dolce vita at last! Bull Cancer.;94 Spec No Actualites:S127-33.
218. Robinson BW, Im MM, Ljungman M, Praz F, Shewach DS. (2003) Enhanced radiosensitization with gemcitabine in mismatch repair-deficient HCT116 cells. Cancer Res. Oct 15;63(20):6935-41.
219. Pauwels B, Korst AE, Pattyn GG, Lambrechts HA, Van Bockstaele DR, Vermeulen K, Lenjou M, de Pooter CM, Vermorken JB, Lardon F. (2003) Cell cycle effect of gemcitabine and its role in the radiosensitizing mechanism in vitro. Int J Radiat Oncol Biol Phys. Nov 15;57(4):1075-83.
220. Ostruszka LJ, Shewach DS. (2000) The role of cell cycle progression in radiosensitization by 2',2'-difluoro-2'-deoxycytidine. Cancer Res. Nov;60(21):6080-8.
221. Matthews DJ,Yakes FM, Chen J. (2007) Pharmacological abrogation ofS -phase checkpoint enhances the anti-tumor activity ofgem citabine in vivo. Cell Cycle;6.

222. GregoireV, Beauduin M, Bruniaux M, De Coster B, Octave Prignot M, Scalliet P. Radiosensitization of mouse sarcoma cells by fludarabine (F-ara-A) or gemcitabine (dFdC), two nucleoside analogues, is notmediated by an increased induction or a repair inhibition ofDNA double-strand breaks as measured by pulsedfield gel electrophoresis. (1998)Int J Radiat Biol;73: 511-20.
223. Jeggo P, Lobrich M. (2006)Radiation-induced DNA damage responses. Radiat Prot Dosimetry;122: 124-7.
224. van Putten JWG, Groen HJM, Smid K, Peters GJ, Kampinga HH. (2001)End-joining deficiency and radiosensitization induced by gemcitabine. Cancer Res;61:1585-91.
225. Wachters FM, van Putten JW, Maring JG, Zdzienicka MZ, Groen HJ, Kampinga HH. (2003)Selective targeting of homologous DNA recombination repair by gemcitabine. Int JRadiat Oncol Biol Phys;57:553-62.
226. van Bree C, Rodermond HM, de Vos J, Haveman J, Franken NA(2005). Mismatch repair proficiency is not required for radioenhancement by gemcitabine. Int J Radiat Oncol Biol Phys. Aug 1;62(5):1504-9.
227. Dewey WC, Ling CC, Meyn RE. (1995) Radiation-induced apoptosis: relevance to radiotherapy. Int J Radiat Oncol Biol Phys. Nov 1;33(4):781-96.
228. Robinson BW, Shewach DS. (2001) Radiosensitization by gemcitabine in p53 wild-type and mutant MCF-7 breast carcinoma cell lines. Clin Cancer Res. Aug;7(8):2581-9.
229. Chen DJ, Nirodi CS. (2007)The epidermal growth factor receptor: a role in repair ofrad iation-induced DNA damage. Clin Cancer Res;13:6555-60.
230. Morgan MA, Parsels LA, Kollar LE, Normolle DP, Maybaum J, LawrenceTS. (2008)The combination ofE GFR inhibitors with gemcitabine and radiation in pancreatic cancer. Clin Cancer Res;14:5142-9.
231. Hatzis P, Al-Madhoon AS, Jullig M, Petrakis TG, Eriksson S, Talianidis I. (1998)The intracellular localization of deoxycytidine kinase. J Biol Chem; 273: 30239-43.
232. Csapo Z, Keszler G, Sáfrány G, Spasokoukotskaja T, Talianidis I, Staub M. (2003) Activation of deoxycytidine kinase by gamma-irradiation and inactivation by hyperosmotic shock in human lymphocytes. Biochem Pharmacol; 65: 2031–2039.

233. Spasokoukotskaja T, Arnér ES, Brosjö O, Gunvén P, Juliusson G, Liliemark J, Eriksson S. (1995) Expression of deoxycytidine kinase and phosphorylation of 2-chlorodeoxyadenosine in human normal and tumour cells and tissues. Eur J Cancer.;31A(2):202-8.
234. Willett CG, Safran H, Abrams RA, Regine WF, Rich TA. (2003)Clinical research in pancreatic cancer: the Radiation Therapy Oncology Group trials. Int J Radiat Oncol Biol Phys;56:31-37.
235. Kroep JR, Loves WJP, van der Wilt CL, Alvarez E, Talianidis I, Boven E. (2002). Pretreatment deoxycytidine kinase levels predict in vivo gemcitabine sensitivity. Mol Cancer Ther; 1: 371–376.
236. Pauwels B, Korst AEC, Pattyn GGO, Lambrechts HAJ, Kamphuis JAE, De Pooter (2006) CMJ The relation between deoxycytidine kinase activity and the radiosensntizing effect of gemcitabine in eight different human tumor cell lines. BMC Cancer; 6: 142.
237. van Bree C, Kreder NC, Loves WJP, Franken NA, Peters GJ, Haveman J. (2002) Sensitivity to ionizing radiation and chemotherapeutic agents in gemcitabine-resistant human tumor cell lines. Int J Radiat Oncol Biol Phys; 54: 237–244.
238. Vernejoul F, Ghénassia L, Souque A, Lulka H, Drocourt D, Cordelier P, Pradayrol L, Pyronnet S, Buscail L, Tiraby G. (2006) Gene therapy based on gemcitabine chemosensitization suppresses pancreatic tumor growth. Mol Ther. Dec;14(6):758-67. Epub (2006) Sep 25.
239. Short SC. External beam and conformal radiotherapy in the management of gliomas. (2003) Acta Neurochir Suppl; 88: 37-43.
240. Irie N, Matsuo T, Nagata I. (2004) Protocol of radiotherapy for glioblastoma according to the expression of HIF-1. Brain Tumor Pathol; 21: 1-6.
241. Frohman MA, Dush MK, Martin GR (1988) Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. Proc Natl Acad Sci USA 85:8998-9002
242. Longnecker DS, Roebuck BD, Yager JDJ, Lilja HS, Siegmund B (1981) Pancreatic carcinoma in azaserine-treated rats: induction, classification and dietary modulation of incidence. Cancer 47:1562-1572

243. Pettengill OS, Faris RA, Bell RH Jr, Kuhlmann ET, Longnecker DS (1993) Derivation of ductlike cell lines from a transplantable acinar cell carcinoma of the rat pancreas. Am J Pathol 1:292-303
244. Schmidt J, Ryschich E, Maksan SM, Werner J, Gebhard MM, Herfarth C, Klar E (2000) Influence of gastrointestinal hormones on tumor microcirculation of experimental pancreatic cancer in the rat. Dig Surg 3:250-5
245. Lehr HA, van der Loos CM, P Teeling, Gown AM (1999) Complete Chromogen Separation and Analysis in Double Immunohistochemical Stains Using Photoshop-based Image Analysis. The Journal of Histochemistry & Cytochemistry 1:119–125
246. Lowry, OH, NJ Rosbrough, AL Farr, and RJ Randall (1951) Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265-275
247. Ryschich E, Werner J, Gebhard MM, Klar E, Schmidt J (2003) Angiogenesis inhibition with TNP-470, 2-methoxyestradiol and paclitaxel in experimmental pancreatic carcinoma. Pancreas 26:166-172
248. Giese M, Kirchner H (1988) Interferons and their effects. Onkologie 4:151-4
249. Farrar MA, Schreiber RD (1993) The molecular cell biology of interferon-gamma and its receptor. Annu Rev Immunol 11:571–611
250. Fiers W (1995) Biologic therapy with TNF: preclinical studies. In: Biologic therapy of cancer. 2nd ed. Philadelphia: JB Lippincott Company; p. 295-327 (editors: DeVita VT Jr, Hellman S, Rosenberg SA)
251. Lee JK, Sayers TJ, Brooks AD, Back TC, Young HA, Komschlies KL, Wipsgginton JM, Wiltrout RH (2000) IFN-γ-dependent delay of in vivo tumor progression by Fas overexpression on murine renal cancer cells. J Immunol 164:231–239
252. Ryschich E, Jesnowski R, Ringel J, Harms W, Fabian OV, Saller R, Schrewe M, Engel A, Schmidt J, Lohr M (2005) Combined therapy of experimental pancreatic cancer with CYP2B1 producing cells: low-dose ifosfamide and local tumor irradiation. Int J Cancer 4:649-53
253. Lanier LL (1998) NK cell receptors. Annu Rev Immunol 16:359-93
254. Springer TA (1994) Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell 76:301-314

255. O'Sullivan B, Thomas R (2003) CD40 and dendritic cell function. Crit Rev Immunol 1-2:83-107

256. Hall PA (1990) Proliferating cell nuclear antigen (PCNA) immunolocalization in paraffin sections, an index of cell proliferation with evidence of deregulated expression in some neoplasms. J Pathol 162:285

257. "Eisold S, Ryschich E, Linnebacher M, Giese T, Nauheimer D, Wild A, Bartsch DK, Büchler MW, Schmidt J. Characterization of FAMPAC, a newly identified human pancreatic carcinoma cell line with a hereditary background. (2004) Cancer. May 1;100(9):1978-86."

258. Kucharczak J, Pannequin J, Camby I, Decaestecker C, Kiss R, Martinez J. (2001) Gastrin induces over-expression of genes involved in human U373 glioblastoma cell migration. Oncogene. Oct 25;20(48):7021-8.

259. Massa A, Barbieri F, Aiello C, Arena S, Pattarozzi A, Pirani P, Corsaro A, Iuliano R, Fusco A, Zona G. (2004) The expression of the phosphotyrosine phosphatase DEP-1/PTP dictates the responsivity of glioma cells to somatostatin inhibition of cell proliferation. J Biochem Chem.. Vol. 279, No. 28, pp. 29004–29012.

260. Vince GH, Bendszus M, Schweitzer T, Goldbrunner RH, Hildebrandt S, Tilgner J, Klein R, Solymosi L, Christian Tonn J, Roosen K. (2004) Spontaneous regression of experimental gliomas: an immunohistochemical and MRI study of the C6 glioma spheroid implantation model. Exp Neurol, 190: 478–485.

261. Benda. P.. Lightbody, J., Sato, G., Levine, L., and Sweet, W. (1968) Differentiated rat glial cell strain in tissue culture. Science (Wash. DC), 161: 370-371..

262. Sibenaller ZA, Etame AB, Ali MM, Barua M, Braun TA, Casavant TL, Ryken TC. (2005) Genetic characterization of commonly used glioma cell lines in the rat animal model system. Neurosurg Focus. Oct 15;19(4):E1.

263. Lumniczky K, Desaknai S, Mangel L, Szende B, Hamada H, Hidvegi EJ. (2002) Local tumor irradiation augments the antitumor effect of cytokine-producing autologous cancer cell vaccines in a murine glioma model. Cancer Gene Ther; 9: 44-52.

264. Arner ESJ, Spasokoukotskaja T, Eriksson S. (1992) Selective assays for thymidine kinase 1 and 2 and deoxycytidine kinase and their activities in extracts from human cells and tissues. Biochem Biophys Res Commun; 188:712–718.

265. Longnecker DS (1993) Experimental models of exocrine pancreatic tumors Go VLW (Hrsg): The pancreas: biology, pathobiology, and disease. Raven Press, NY. 27, 551--564.
266. Banuls, M.P., A. Alvarez, I. Ferrero, A. Zapata, C. Ardavin (1993) Cell-surface marker analysis of rat thymic dendritic cells. Immunology 79:298-304
267. Hannum C, Culpepper J, Campbell D, McClanahan T, Zurawski S, Bazan JF, Kastelein R, Hudak S, Wagner J, Mattson J (1994) Ligand for FLT3/FLK2 receptor tyrosine kinase regulates growth of haematopoietic stem cells and is encoded by variant RNAs. Nature 368 (6472):643-648
268. Yang S, Sim GK (2000) Molecular cloning of canine and feline flt3 ligand reveals high degree of similarity to the human and mouse homologue but uniquely long cytoplasmic domain. DNA Seq. 1-2:163-6
269. Wentzensen N (2002) Charakterisierung viral-zellulärer Fusionstranskripte aus Papillomvirus-positiven anogenitalen Läsionen. Med. Dissertation, Universität Heidelberg
270. Zuhorn IS, Hoekstra D (2002) On the mechanism of cationic amphiphile-mediated transfection. To fuse or not to fuse: is that the question? J Membr Biol 3:167-79
271. Nomura T, Nakajima S, Kawabata K, Yamashita F, Takakura Y, Hashida M (1997) Intratumoral pharmacokinetics and in vivo gene expression of naked plasmid DNA and its cationic liposome complexes after direct gene transfer. Cancer Res 13:2681-6
272. Wolff JA, Budker V. (2005) The mechanism of naked DNA uptake and expression. Adv Genet 54:3-20
273. Ferber D (2001) Gene therapy. Safer and virus-free? Science 294:1638-42
274. Ma CH, Zhang Y, Wang XY, Gao LF, Liu H, Guo C, Liu SX, Cao YL, Zhang LN, Sun WS (2004) Human endostatin gene transfer, either naked or with liposome, has the same inhibitory effect on growth of mouse liver tumor cells in vivo. World J Gastroenterol 19:2874-7
275. Shi F, Rakhmilevich AL, Heise CP, Oshikawa K, Sondel PM, Yang NS, Mahvi DM (2002) Intratumoral injection of interleukin-12 plasmid DNA, either naked or in complex with cationic lipid, results in similar tumor regression in a murine model. Mol Cancer Ther 11:949-57

276. Yang JP, Huang L (1996) Direct gene transfer to mouse melanoma by intratumor injection of free DNA. Gene Ther 6:542-8
277. Mathis JM, Stoff-Khalili MA, Curiel DT. (2005) Oncolytic adenoviruses - selective retargeting to tumor cells. Oncogene. 21;24(52):7775-91.
278. MacLachlan I, Cullis P, Graham RW (1999) Progress towards a synthetic virus for systemic gene therapy. Curr Opin Mol Ther 2:252-9
279. Ilan Y, Droguett G, Chowdhury NR, Li Y, Sengupta K, Thummala NR, Davidson A, Chowdhury JR, Horwitz MS (1997) Insertion of the adenoviral E3 region into a recombinant viral vector prevents antiviral humoral and cellular immune responses and permits long-term gene expression. Proc Natl Acad Sci USA 6:2587-92
280. Rubanyi GM (2001) The future of human gene therapy. Mol Aspects Med 3:113-42
281. Green NK, Seymour LW. (2002) Adenoviral vectors: Systemic delivery and tumor targeting. Cancer Gene Therapy. 9, 1036 – 1042.
282. JO Radler, I Koltover, T Salditt, CR Safinya (1997) Structure of DNA-cationic liposome complexes: DNA intercalation in multilamellar membranes in distinct interhelical packing regimes. Science 275:810– 814
283. Ito I, Ji L, Tanaka F, Saito Y, Gopalan B, Branch CD, Xu K, Atkinson EN, Bekele BN, Stephens LC, Minna JD, Roth JA, Ramesh R (2004) Liposomal vector mediated delivery of the 3p FUS1 gene demonstrates potent antitumor activity against human lung cancer in vivo. Cancer Gene Ther 11:733-9
284. El-Aneed A (2004) An overview of current delivery systems in cancer gene therapy. J Control Release 1:1-14
285. Schatzlein AG (2001) Non-viral vectors in cancer gene therapy: principles and progress. Anticancer Drugs 4:275-304
286. Hottiger MO, Dam TN, Nickoloff BJ, Johnson TM, Nabel GJ (1999) Liposome-mediated gene transfer into human basal cell carcinoma. Gene Ther 6:1929-35
287. Clark PR, Stopeck AT, Ferrari M, Parker SE, Hersh EM (2000) Studies of direct intratumoral gene transfer using cationic lipid-complexed plasmid DNA. Cancer Gene Ther 6:853-60

288. Egilmez NK, Cuenca R, Yokota SJ, Sorgi F, Bankert RB (1996) In vivo cytokine gene therapy of human tumor xenografts in SCID mice by liposome-mediated DNA delivery. Gene Ther 7:607-14.
289. Saffran DC, Horton HM, Yankauckas MA, Anderson D, Barnhart KM, Abai AM, Hobart P, Manthorpe M, Norman JA, Parker SE (1998) Immunotherapy of established tumors in mice by intratumoral injection of interleukin-2 plasmid DNA: induction of CD8+ T-cell immunity. Cancer Gene Ther 5:321-30
290. Hui KM, Ang PT, Huang L, Tay SK (1997) Phase I study of immunotherapy of cutaneous metastases of human carcinoma using allogeneic and xenogeneic MHC DNA±liposome complexes. Gene Ther 4:783-90
291. Plautz GE, Yang ZY, Wu BY, Gao X, Huang L, Nabel GJ (1993) Immunotherapy of malignancy by in vivo gene transfer into tumors. Proc Natl Acad Sci USA 10:4645-9
292. Takakuwa K, Fujita K, Kikuchi A, Sugaya S, Yahata T, Aida H, Kurabayashi T, Hasegawa I, Tanaka K (1997) Direct intratumoral gene transfer of the herpes simplex virus thymidine kinase gene with DNA-liposome complexes: growth inhibition of tumors and lack of localization in normal tissues. Jpn J Cancer Res 2:166-75
293. Porteous DJ, Dorin JR, McLachlan G, Davidson-Smith H, Davidson H, Stevenson BJ, Carothers AD, Wallace WA, Moralee S, Hoenes C, Kallmeyer G, Michaelis U, Naujoks K, Ho LP, Samways JM, Imrie M, Greening AP, Innes JA (1997) Evidence for safety and efficacy of DOTAP cationic liposome mediated CFTR gene transfer to the nasal epithelium of patients with cystic fibrosis. Gene Ther 3:210-8
294. Alsheikhly AR, Zweiri J, Walmesley AJ, Watson AJ, Christmas SE (2004) Both soluble and membrane-bound forms of Flt3 ligand enhance tumor immunity following "suicide" gene therapy in a murine colon carcinoma model. Cancer Immunol Immunother 11:946-54
295. Ryschich E (1996) Mikrozirkulation und Angiogenese beim experimentellen Pankreaskarzinom. Med. Dissertation, Universität Heidelberg

296. Borges L, Miller RE, Jones J, Ariail K, Whitmore J, Fanslow W, Lynch DH (1999)Ota T, Maeda M, Tatsuka M (2002) Cationic liposomes with plasmid DNA influence cancer metastatic capability. Anticancer Res 6C:4049-52
296. Borges L, Miller RE, Jones J, Ariail K, Whitmore J, Fanslow W, Lynch DH (1999)Synergistic action of fms-like tyrosine kinase 3 ligand and CD40 ligand in the induction of dendritic cells and generation of antitumor immunity in vivo. J Immunol 3:1289-97
298. Chakravarty PK, Alfieri A, Thomas EK, Beri V, Tanaka KE, Vikram B, Guha C (1999) Flt3-ligand administration after radiation therapy prolongs survival in a murine model of metastatic lung cancer. Cancer Res 24:6028-32
299. Biron CA (1997) Activation and function of natural killer cell responses during viral infections. Curr Opin Immunol 9:24-34.
300. Janeway CA Jr, Medzhitov R (2002) Innate immune recognition. Annu Rev Immunol 20:197-216
301. Moretta A (2005) The dialogue between human natural killer cells and dendritic cells. Curr Opin Immunol (2005) 3:306-11.
302. Ferlazzo G, Munz C (2004) NK cell compartements and their activation by dendritic cells. J Immunol 3:1333-1339
303. Fernandez, N. C., A. Lozier, C. Flament, P. Ricciardi-Castagnoli, D. Bellet, M. Suter, M. Perricaudet, T. Tursz, E. Maraskovsky, L. Zitvogel (1999) Dendritic cells directly trigger NK cell functions: cross-talk relevant in innate anti-tumor immune responses in vivo. Nat Med 4:405-11
304. Dallal RM, Christakos P, Lee K, Egawa S, Son YI, Lotze MT (2002) Paucity of dendritic cells in pancreatic cancer. Surgery 2:135-8
305. Inaba K, Witmer-Pack M, Inaba M, Hathcock KS, Sakuta H, Azuma M, Yagita H, Okumura K, Linsley PS, Ikehara S (1994) The tissue distribution of the B7-2 costimulator in mice: abundant expression on dendritic cells in situ and during maturation in vitro. J Exp Med 5:1849-60
306. Bell D, Chomarat P, Broyles D, Netto G, Harb GM, Lebecque S, Valladeau J, Davoust J, Palucka KA, Banchereau J (1999) In breast carcinoma tissue, immature dendritic cells reside within the tumor, whereas mature dendritic cells are located in peritumoral areas. J Exp Med 10:1417-26.

307. Chaux P, Favre N, Martin M, Martin F (1997) Tumor-infiltrating dendritic cells are defective in their antigen-presenting function and inducible B7 expression in rats. Int J Cancer Aug 4:619-24
308. Chaux P, Hammann A, Martin F, Martin M (1993) Surface phenotype and functions of tumor-infiltrating dendritic cells: CD8 expression by a cell subpopulation. Eur J Immunol 10:2517-25
309. Gabrilovich DI, Corak J, Ciernik IF, Kavanaugh D, Carbone DP (1997) Decreased antigen presentation by dendritic cells in patients with breast cancer. Clin Cancer Res 3:483-90
310. Menetrier-Caux C, Montmain G, Dieu MC, Bain C, Favrot MC, Caux C, Blay JY (1998) Inhibition of the differentiation of dendritic cells from CD34(+) progenitors by tumor cells: role of interleukin-6 and macrophage colony-stimulating factor. Blood 12:4778-91
311. Tourkova IL, Yamabe K, Chatta G, Shurin GV, Shurin MR (2003) NK cells mediate Flt3 ligand-induced protection of dendritic cell precursors in vivo from the inhibition by prostate carcinoma in the murine bone marrow metastasis model. J Immunother 266:468-72
312. Cerundolo V, Hermans IF, Salio M (2004) Dendritic cells: a journey from laboratory to clinic. Nat Immunol 1:7-10
313. Ohm JE, Shurin MR, Esche C, Lotze MT, Carbone DP, Gabrilovich DI (1999) Effect of vascular endothelial growth factor and FLT3 ligand on dendritic cell generation in vivo. J Immunol 6:3260-8
314. Masten BJ, Olson GK, Kusewitt DF, Lipscomb MF (2004) Flt3 ligand preferentially increases the number of functionally active myeloid dendritic cells in the lungs of mice. J Immunol 7:4077-83
315. Teleshova N, Jones J, Kenney J, Purcell J, Bohm R, Gettie A, Pope M (2004) Short-term Flt3L treatment effectively mobilizes functional macaque dendritic cells. J Leukoc Biol 6:1102-10
316. Westermann J, Nguyen-Hoai T, Mollweide A, Richter G, Schmetzer O, Kim HJ, Blankenstein T, Dorken B, Pezzutto A (2004) Flt-3 ligand as adjuvant for DNA vaccination augments immune responses but does not skew TH1/TH2 polarization. Gene Ther 13:1048-56

317. Okano F, Merad M, Furumoto K, Engleman EG (2005) In vivo manipulation of dendritic cells overcomes tolerance to unmodified tumor-associated self antigens and induces potent antitumor immunity. J Immunol 5:2645-52.
318. Wang HY (2004) Tumor-specific human CD4+ regulatory T cells and their ligands: implications for immunotherapy. Immunity 20:107−118
319. Wang HY, Wang RF (2005) Antigen-specific CD4+ regulatory T cells in cancer: implications for immunotherapy. Microbes Infect. 7(7-8):1056-62.
320. Jones E, Golgher D, Simon AK, Dahm-Vicker M, Screaton G, Elliott T, Gallimore A. (2004) The influence of CD25+ cells on the generation of immunity to tumour cell lines in mice. Novartis Found Symp. 256:149-52.
321. Tacchini L, Bianchi L, Bernelli-Zazzera A, Cairo G. (1999) Transferrin receptor induction by hypoxia. HIF-1-mediated transcriptional activation and cell-specific post-transcriptional regulation. J Biol Chem;274:24142-24146.
322. Bianchi L, Tacchini L, Cairo G. (1999) HIF-1-mediated activation of transferrin receptor gene transcription by iron chelation. Nucleic Acids Res;27:4223-4227.
323. Griffin TW, Pagnini PG, McGrath JJ, McCann JC, Houston LL. (1988) In vitro cytotoxicity of recombinant ricin A chain- antitransferrin receptor immunotoxin against human adenocarcinomas of the colon and pancreas. J Biol Response Mod;7:559-567.
324. Brooks D, Taylor C, Dos Santos B, Linden H, Houghton A, Hecht TT, Kornfeld S, Taetle R. (1995) Phase Ia trial of murine immunoglobulin A antitransferrin receptor antibody 42/6. Clin Cancer Res. 1995 Nov;1(11):1259-65.
325. Daniels TR, Delgado T, Helguera G, Penichet ML (2006) The transferrin receptor part II: The transferrin receptor part II: Targeted delivery of therapeutic agents into cancer cells. Clin Immunol 121:159—176.
326. Li H, Sun H, Qian ZM. (2002) The role of the transferrin-transferrin-receptor system in drug delivery and targeting. Trends Pharmacol Sci. 2002 May;23(5):206-9
327. Mayers GL, Raghavan D, Hitt S, Glaves D. (1998) Transferrin-gemcitabine conjugate: application to chemotherapy. Proceedings of the 89th Annual Meeting of the American Association for Cancer Researc, New Orleans, Louisiana, USA. p63.

328. Laske DW, Muraszko KM, Oldfield EH, DeVroom HL, Sung C, Dedrick RL, Simon TR, Colandrea J, Copeland C, Katz D, Greenfield L, Groves ES, Houston LL, Youle RJ. (1997) Intraventricular immunotoxin therapy for leptomeningeal neoplasia. Neurosurgery. Nov;41(5):1039-49; discussion 1049-51.
329. Debinski W, Pastan I. (1992) Monovalent immunotoxin containing truncated form of Pseudomonas exotoxin as potent antitumor agent. Cancer Res;52:5379-5385.
330. Laske DW, Youle RJ, Oldfield EH. (1997) Tumor regression with regional distribution of the targeted toxin TF-CRM107 in patients with malignant brain tumors. Nat Med. 3:1362-1368.
331. Weaver M, Laske DW. (2003) Transferrin receptor ligand-targeted toxin conjugate (Tf-CRM107) for therapy of malignant gliomas. J Neuro-oncol. 65. 3-13.
332. Xu L, Huang CC, Huang W, Tang WH, Rait A, Yin YZ, Cruz I, Xiang LM, Pirollo KF, Chang EH. (2002) Systemic tumor-targeted gene delivery by anti-transferrin receptor scFv-immunoliposomes. Mol Cancer Ther;1:337-346.
333. Iinuma H, Maruyama K, Okinaga K, Sasaki K, Sekine T, Ishida O, Ogiwara N, Johkura K, Yonemura Y. (2002) Intracellular targeting therapy of cisplatin-encapsulated transferrin-polyethylene glycol liposome on peritoneal dissemination of gastric cancer, Int J Cancer 99: 130– 137.
334. Jhaveri MS, Rait AS, Chung KN, Trepel JB, Chang EH. (2004) Antisense oligonucleotides targeted to the human alpha folate receptor inhibit breast cancer cell growth and sensitize the cells to doxorubicin treatment. Mol. Cancer Ther. 3: 1505– 1512.
335. Xu L, Frederik P, Pirollo KF, Tang WH, Rait A, Xiang LM, Huang W, Cruz I, Yin Y, Chang EH. (2002) Self-assembly of a virusmimicking nanostructure system for efficient tumor-targeted gene delivery, Hum. Gene Ther. 13: 469– 481.
336. Kreppel F, Gackowski J, Schmidt E, Kochanek S. (2005) Combined genetic and chemical capsid modifications enable flexible and efficient de- and retargeting of adenovirus vectors, Mol Ther 12:107–117.
337. Manome Y, Wen PY, Dong Y, Tanaka T, Mitchell BS, Kufe DW. (1996) Viral vector transduction of the human deoxycytidine kinase cDNA sensitizes glioma

cells to the cytotoxic effects of cytosine arabinoside in vitro and in vivo. Nat Med; 2: 567–573.

338. Hubeek I, Peters GJ, Broekhuizen AJ, Talianidis I, Sigmond J, Gibson BE, Creutzig U, Giaccone G, Kaspers GJ. (2005) Immunocytochemical detection of deoxycytidine kinase in haematological malignancies and solid tumours. J Clin Pathol.58(7):695-9.
339. Hubeek I, Peters GJ, Broekhuizen AJF, Talianidis I, Schouten van Meeteren AY, van Wering ER (2004) Immunocytochemical detection of deoxycytidine kinase in pediatric malignancies in relation to in vitro cytarabine sensitivity. Nucleosides Nucleotides Nucleic Acids; 23: 1351–1356.
340. Bergman AM, Pinedo HM, Jongsma AP, Brouwer M, Ruiz van Haperen VW, Veerman G. (1999) Decreased resistance to gemcitabine (2',2'-difluoro-deoxycytidine) of cytosine arabinoside-resistant myeloblastic murine and rat leukemia cell lines: role of altered activity and substrate specificity of deoxycytidine kinase. Biochem Pharmacol; 57: 397–406.
341. Goan YG, Zhou B, Hu E, Mi S, Yen Y. (1999) Overexpression of ribonucleotide reductase as a mechanism of resistance to 2-2-difluorodeoxycytidine in the human KB cancer cell line. Cancer Res; 59: 4204–4207.
342. Eliopoulos N, Cournoyer D, Momparler RL. (1998) Drug resistance to 5-aza-2'-deoxycytidine, 2',2'-difluorodeoxycytidine, and cytosine arabinoside conferred by retroviral-mediated transfer of human cytidine deaminase cDNA into murine cells. Cancer Chemother Pharmacol; 42: 373–378.
343. Wang L, Munch-Petersen B, Herrstrom SA, Hellman U, Bergman T, Jornvall H. (1999) Human thymidine kinase 2: molecular cloning and characterisation of the enzyme activity with antiviral and cytostatic nucleoside substrates. FEBS Lett; 443: 170–174.
344. Sanda A, Zhu C, Johanson M, Karlsson A. (2001) Bystander effects of nucleoside analogs phosphorylated in the cytosol or mitochondria. Biochem Biophys Res Commun; 287: 1163–1166.
345. Heinemann V, Xu YZ, Chubb S, Sen A, Hertel LW, Grindey GB. (1990) Inhibition of ribonucleotide reduction in CCRF-CEM cells by 2',2'-difluorodeoxycytidine. Mol Pharmacol; 38: 567–572.

346. Huang P, Chubb S, Hertel LW, Grindex GB, Plunkett W. (1991) Action of 20,20-difluorodeoxycytidine on DNA synthesis. Cancer Res; 51: 6110–6117.
347. Habteyesus A, Nordenskjö ld A, Bohman C, Eriksson S. (1991) Deoxynucleoside phosphorylating enzymes in monkey and human tissues show great similarities, while mouse deoxycytidine kinase has a different substrate specificity. Biochem Pharmacol; 42: 1829–1836.
348. Karlsson A, Johansson M, Erikson S. (1994) Cloning and expression of mouse deoxycytidine kinase. Pure recombinant mouse and human enzymes show differences in substrate specificity. J Biol Chem; 269: 24374–24378.
349. Reichelova´ V, Juliusson G, Spasokoukotskaja T, Eriksson S, Liliemark J. (1995) Interspecies differences in the kinetic properties of deoxycytidine kinase elucidate the poor utility of a phase I pharmacologically directed dose-escalation concept for 2-chloro-20-deoxyadenosine. Cancer Chemother Pharmacol; 36: 524–529.
350. Kawamura K, Namba H, Bahar R, Miyauchi M, Maeda T, Hamada H. (2000) Transduction of the human deoxycytidine kinase gene in rodent tumor cells induces in vivo growth retardation in syngeneic hosts. Cancer Lett; 156: 151–157.
351. Szatmári T, Lumniczky K, Désaknai S, Trajcevski S, Hidvegi EJ, Hamada H. (2006) Detailed characterization of the mouse glioma 261 tumor model for experimental glioblastoma therapy. Cancer Sci; 97: 546-53.
352. Hapke DM, Stegmann APA, Mitchell BS. (1996) Retroviral transfer of deoxycytidine kinase into tumor cell lines enhances nucleoside toxicity. Cancer Res; 56: 2343–2347.
353. Blackstock AW, Lightfoot H, Case LD, Tepper JE, Mukherji SK, Mitchell BS. (2001) Tumor uptake and eliminatin of 2',2'-difluoro-2'-deoxycytidine (gemcitabine) after deoxycytidine kinase gene transfer: correlation with in vivo tumor response. Clin Cancer Res; 7: 3263–3268.
354. Weizsacker M, Nagamune A, Winkelstroter R, Vieten H, Wechsler W. (1982) Radiation and drug response of the rat glioma RG2. Eur J Cancer Clin Oncol: 18: 891–895.
355. Yount GL, Levine KS, Kuriyama H, Haas-Kogan DA, Israel MA. (1999) Fas (APO- 1/CD95) signaling pathway is intact in radioresistant human glioma cells. Cancer Res.: 59: 1362–1365.

356. Rosier JF, Bruniaux M, Husson B, Octave-Prignot M, Beauduin M, Grégoire V. (2004) Role of 2'-2' difluorodeoxycytidine (gemcitabine)-induced cell cycle dysregulation in radioenhancement of human head and neck squamous cell carcinomas. Radiother Oncol; 70: 55–61.
357. Taghian A, Dubois W, Budach W, Baumann M, Freeman J, Suit H. (1995) In vivo radiation sensitivity of glioblastoma multiforme. Int J Radiat OncolBiol Phys.. 32: 99– 104.
358. Vernejoul F, Ghe´nassia L, Souque A, Lulka H, Drocourt D, Cordelier P. (2006) Gene therapy based on gemcitabine chemosensitization suppresses pancreatic tumor growth. Mol Ther; 14: 758–767.
359. Hsu YF, Reitz J. (1997) Gemcitabine. A cytidine analog active against solid tumors. Am J HealthSyst Pharm; 54: 162–170.
360. Sabini E, Ort S, Monnerjahn C, Konrad M, Lavie A. (2003) Structure of human dCK suggests strategies to improve anticancer and antiviral therapy. Nat Struct Biol; 10: 513–519.
361. Xing Z, Braciak T, Jordana M, Croitoru K, Graham FL, Gauldie J. (1994) Adenovirus-mediated cytokine gene transfer at tissue sites. Overexpression of IL-6 induces lymphocytic hyperplasia in the lung. J Immunol.. 153 (9): 4059-4069.
362. De Martin R, Raidl M, Hofer E, Binder BR. (1997) Adenovirus-mediated expression of green fluorescent protein. Gene Ther.. 4: 493–495.
363. Hemmi S, Geertsen R, Mezzacasa A, Peter I, Dummer R. (1998) The presence of human coxsackievirus and adenovirus receptor is associated with efficient adenovirus-mediated transgene expression in human melanoma cell cultures. Hum Gene Ther. 1;9(16):2363-73.
364. Ichikawa T, Chiocca EA. (2001) Comparative analyses of transgene delivery and expression in tumors inoculated with a replication- conditional or defective viral vector. Cancer Res.;61:5336–5339.)
365. Nanda D, Vogels R, Havenga M, Avezaat CJ, Bout A, Smitt PS. (2001) Treatment of malignant gliomas with a replicating adenoviral vector expressing herpes simplex virus-thymidine kinase. Cancer Res. 61(24):8743-50.

366. Nemunaitis J, Khuri F, Ganly I. (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.;19:289–298.
367. Freytag SO, Movsas B, Aref I, Stricker H, Peabody J, Pegg J, Zhang Y, Barton KN, Brown SL, Lu M, Savera A, Kim JH. (2007) Phase I trial of replication-competent adenovirus-mediated suicide gene therapy combined with IMRT for prostate cancer. Mol Ther. 15(5):1016-23.
368. Freytag SO, Khil M, Stricker H, Peabody J, Menon M, DePeralta-Venturina M, Nafziger D, Pegg J, Paielli D, Brown S, Barton K, Lu M, Aguilar-Cordova E, Kim JH. (2002) Phase I study of replication-competent adenovirus-mediated double suicide gene therapy for the treatment of locally recurrent prostate cancer. Cancer Res. 62(17):4968-76.