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Treatment of cancer with combined chemo-gene therapy based on TNFα polyplexes and liposomal doxorubicine

vorgelegt von

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Erklärung

Diese Dissertation wurde im Sinne von § 13 Abs. 3 bzw. 4 der Promotionsordnung vom 29. Januar 1998 von Professor Dr. Ernst Wagner betreut.

Ehrenwörtliche Versicherung

Diese Dissertation wurde selbständig, ohne unerlaubte Hilfe erarbeitet.

München, am …….

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.....dedicated to my parents
in love and gratitude

Table of Contents

Abstract

Tumor necrosis factor alpha (TNFα) is a potent antitumoral cytokine, but its high intrinsic toxicity precludes systemic treatment. Here we demonstrated localized antitumor activity of TNF concomitant with decreased vessel density (CD31 expression) after systemic injection of TNF coding polyplexes using a non viral, biodegradable gene carrier (G3-HD-OEI) exhibiting intrinsic tumor affinity in vivo in murine neuroblastoma model. We used a novel plasmid vector with CpG free backbone and an optimized promoter-enhancer combination led to sustained and high TNF-α transgene expression in vitro on Neuro2A murine neuroblastoma cells. Secreted TNF-α induced high cytotoxicity on L929 indicator cells and increased endothelial cell permeability in a transwell system. Synthetic gene carriers allowed tumor restricted transgene expression after intravenous injection either by passive accumulation of the gene carrier within the tumor or by specifically targeting the epidermal growth factor receptor. Systemic TNF gene delivery promoted tumor accumulation of liposomally encapsulated doxorubicine (Caelyx®) in subcutaneous Neuro2A murine neuroblastoma and HUH7 human hepatocellular carcinoma by enhancing tumor endothelium permeability. TNFα gene therapy was combined with liposomal doxorubicine (Caelyx®) for the treatment of subcutaneous and metastatic murine neuroblastoma and metastatic human colon adenocarcinoma. The combined chemo- and gene therapy synergized in antitumoral activity: both, subcutaneous tumors and tumor metastases responded to the treatment with significant tumor growth delay. Also tumors re-growing after initial treatment were successfully treated in a second cycle pointing at the absence of resistance mechanisms. This schedule opens the possibility for the treatment of solid tumors otherwise not accessible for macromolecular drug carrier.

1. Introduction

1.1. Gene therapy

1.1.1. Introduction to gene therapy

Genes - the biological units of heredity, which recent have been better understood and manipulated well, have set the stage for scientists to alter patients' genetic material by this experimental technique to fight or prevent disease. Since late 1970s, gene therapy rather quickly developed. Gene therapy offers a new treatment paradigm for curing human disease. Gene therapy is a promising treatment option for a number of diseases, such as cancer, peripheral vascular disease, arthritis and other acquired diseases, but the technique is still highly experimental and under study to make sure that it will be safe and effective. Up to now, there are still many problems should be overcome before gene therapy becomes a common technique for treating disease. The success of gene therapy is largely dependent on an efficient and safe gene transfer to the host cells or tissue and the appropriate expression of the introduced gene. In order to treat cancer and other diseases effectively with gene therapy, researchers have to develop gene delivery vectors that can specifically focus on the target cells located throughout the body and without any seriously side effect and the related gene expression system. There will be no “universal vector”, and each clinical indication may require a specific set of technical hurdles to overcome, such as cell-based gene delivery technologies and so on (M.Rubanyi 2001).

1.1.2. How is gene therapy being studied in the treatment of cancer?

Normally, to keep our body healthy, cells grow and divide as part of the normal process of cell regeneration. However, sometimes the genetic material (DNA) of a cell are damaged, producing mutations that affect normal cell growth and division and the cells in our body do not die when they supposed to, but more cells are formed. These extra cells may develop into tumor. Nearly all cancers are caused by genetic material mutation in cells.

Surgery, radiotherapy and chemotherapy are three conventional modalities to treat cancer, but they are often unsuccessful in treating cancer. As the development of gene therapy, it is becoming the fourth modality for cancer treatment. In one approach, researchers replace missing or altered genes (e.g., p53 and some other cancer suppressing or apoptosis inducing genes) (Schmitt; 2002). Another approach is to improve a patient's immune response to cancer by stimulating the body's natural ability to attack cancer cells, since cancer patients generally have lowered immune response. Various genes, mainly encoding for cytokines, co-stimulatory molecules and growth factor can modify tumor cells as well as immune effector cells. Genes coding for cytokines have also been used to enhance the immune response against the tumor cells (Kikuchi 2000). Cytokines currently being tested in cancer vaccine trials include IL-2, IL-4, IL-7, tumor necrosis factor (Wagner 2004), interferon-γ and GM-CSF. A constant supply of oxygen, nutrients, hormones and growth factors can be provided after formation of new blood vessels or angiogenesis for the growth and dissemination of tumors. It has been shown that inhibiting angiogenesis regresses experimental tumors thus being a suitable target for gene therapy (Huang 2001). Also, to make them more sensitive to chemotherapy, radiation therapy, or other treatments, scientists are studying the insertion of genes into cancer cells. Besides, DNA vaccination, which means that the naked plasmids DNA which contains genes coding for tumor-specific antigens were injected and commence synthesizing the protein intracellularly, is also one of the approaches of gene therapy for the treatment of cancer (Benton 1998).

Cancer gene therapy approaches could be either applied as single-agent therapy or combined with an established conventional treatment regimen such as chemotherapy or radiotherapy, which might lead to greater therapeutic effect. Up to now, gene therapy for cancer is still a challenge and the success of this treatment modality will ultimately depend upon the ability to target delivery to cancer cells, express the gene of interest at high levels and minimize toxicity by targeting transgene expression to specific cells. The concept of cancer-specific

gene therapies are becoming more viable and promising since the development in cell and molecular biology and the advances in bioinformatics.

1.2. Tumor necrosis factor (TNFα)

1.2.1. Introduction to TNFα

Tumor necrosis factor (TNF, structure shown in **fig. 1**), is primarily produced as a 212-amino acid-long type II transmembrane protein arranged in stable homotrimers, which upon cleavage by the metalloprotease TACE produces a soluble trimer of 157 amino acids (Killar L 1999; Lejeune 2006). Both isoforms of TNF (membrane and soluble TNF) bind two distinct receptors that are ubiquitous, TNFR-1, and TNFR-2 (Dembic Z 1990). As show in **fig. 2,** evidences for two opposing pathways were found in endothelial cells: one is leading to caspase activation (Lin 1999), which is dependent on the activation of TNFR-1 (Wong 1994). It is an apoptotic pathway initiated by the clustering of death domain containing proteins (Tartaglia 1993; Grell 1994; Rath 1999). The other one is a proliferation and survival pathway involving the activation of nuclear factor NF–κB (Hsu 1996; Rath 1999; Horssen 2006). As a major inflammatory cytokine involved in systemic inflammation, the primary role of TNF is the regulation of immune cells. However, TNF is also believed to induce rapid haemorrhagic necrosis of experimental cancers. TNF is able to induce apoptotic cell death, induce inflammation, and to inhibit tumorigenesis and viral replication. A variety of human diseases, including major depression (Dowlati 2010), Alzheimer's disease (Swardfager 2010) and cancer (Locksley 2001) could be resulted due to dysregulation of TNF production.

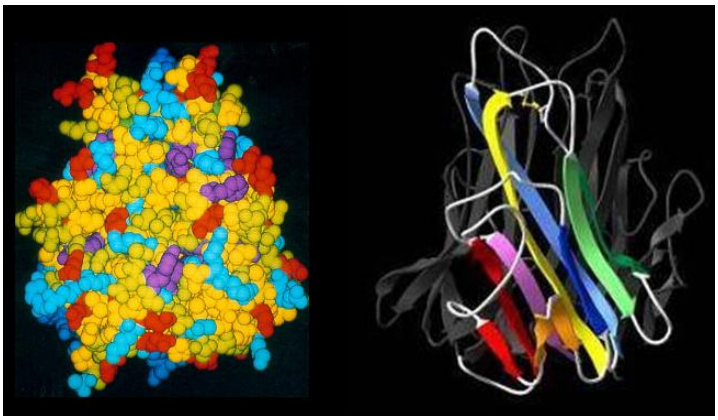

Fig. 1, the structure of TNF protein (Figure from (Lejeune March 2006))

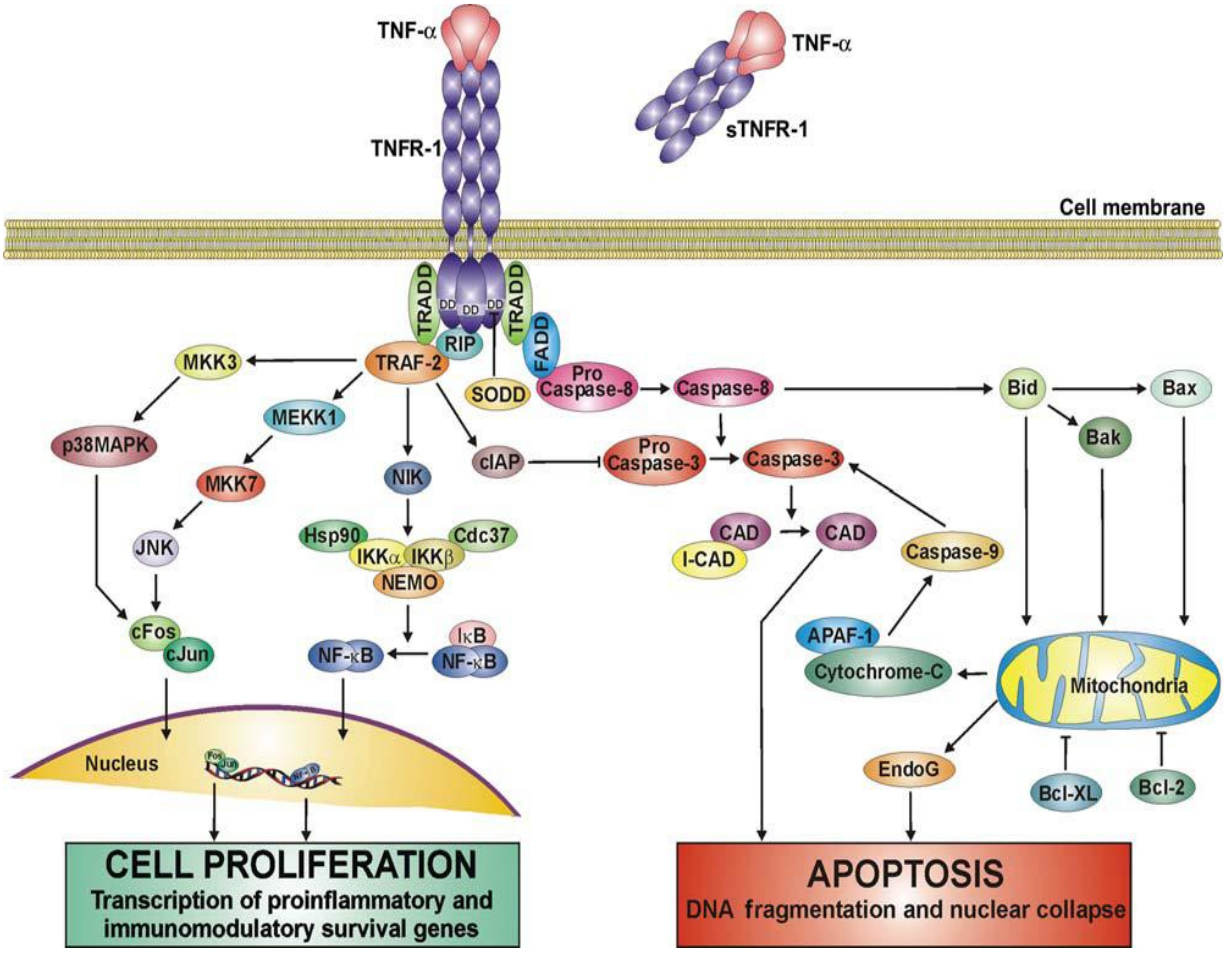

Fig. 2: Tumor necrosis factor receptor 1 (TNFR-1) signaling pathway, (figure from (Horssen 2006)).

1.2.2. The timeline of the work on TNFα and cancer treatment

In around 1890s, Dr. Coley and other investigators developed a treatment for malignant tumors by injections of bacterial toxins and found that intercurrent bacterial infections were said to have elicited profound effects upon malignant

tumors (Nauts 1945; Balkwill 2009). In 1944 Shear *et al.* isolated lipopolysaccharide from bacterial extracts and showed that this was responsible for tumor regression in a mouse model of cancer (Shear 1944; Balkwill 2009). In around 1962, to reduce the often lethal effects of endotoxin or other bacterial products, O'Malley *et al.* isolated serum from endotoxin treated animals to treat animals with experimental cancers. The serum also caused tumors necrosis, leading to the conclusion that it contained a "tumor necrotizing factor" (O'Malley 1962; Balkwill 2009). In 1975 came a major advance, since Dr. Carswell *et al* reported that it was an endotoxin-induced serum factor which then was named "tumor necrosis factor" that causes necrosis of tumors (Carswell 1975). From 1984 until present, identification and characterization of other members of the TNF and TNF receptor families were performed by scientists around the world which enabled the development of a number of research tools, including gene-deleted mice (Gray 1984; Locksley 2001; Ashkenazi 2002). In 1987, first clinical trials with TNF were performed in advanced cancer (Blick 1987; Kimura 1987; Selby 1987; Creagan 1988). In 1984 and 1985, human and mouse TNF genes were cloned (Pennica 1984; Marmenout 1985). Between 1984 and 1988, scientists found that local treatment with recombinant TNF causes tumor necrosis in a range of mouse models (Pennica 1984; Balkwill 1986; Brouckaert 1986; Talmadge 1988). However, locally and repeatedly injection of TNF should be perfomed for optimal activity, even though there still was a risk of regrowth at the periphery of the lesion. However, when recombinant mouse TNF was given to mice, it caused symptoms similar to high doses of endotoxin (Brouckaert 1986; Kettlehut 1987; Havell 1988). To decrease this toxicity, in 1992s surgeons Ferdy Lejeune, Alexander Eggermont and their colleagues performed a local approach of TNF therapy by isolated limb perfusion (ILP), to deliver high doses of TNF in combination with IFN γ and melphalan locoregionally to patients with cancers of the extremities. Specific destruction of tumor vasculature, haemorrhagic necrosis and complete tumor disappearance were observed in

patients with advanced soft tissue sarcomas or melanoma due to this treatment (Lienard 1992; Grunhagen 2006). As the results of further refinements and studies into mechanisms of action in animal models suggested, with mild hyperthermia optimizing the anti-tumor effect, TNF synergized with melphalan chemotherapy for cancer treatment in a rat osteosarcoma ILP model (de Wilt 1999), while TNF alone was ineffective in this setting. A combination of TNF and doxorubicin had comparable effects in rat sarcoma models (van der Veen 2000). It appeared that tumor blood vessel permeability was enhanced upon TNF treatment, leading to tissue concentrations of chemotherapy augmenting and the tumor vasculature destroying (Seynhaeve 2007).

1.2.3. Clinical trials with recombinant TNFα

It was the expectation of many scientists that recombinant human TNF would be an important new treatment for cancer patients. In 1987, First clinical trials with TNF were performed in advanced cancer (Blick 1987; Kimura 1987; Selby 1987; Creagan 1988). Unfortunately, systemic TNF administration always causes severe toxicity. Thus, local administration of TNF is necessary instead of systemic treatment. As mentioned in section **1.2.2 ,** to decrease this toxicity, in 1992 a local approach of TNF therapy isolated limb perfusion (ILP) to deliver high doses of TNF in combination with IFNγ and melphalan locoregionally, to patients with cancers of the extremities were performed by surgeons Ferdy Lejeune, Alexander Eggermont and their colleagues. Base on encouraging results of research and clinical trials, in Europe in 1999, TNF (tasonermin) was licensed specific for the treatment of irresectable soft tissue sarcoma of the limbs used in combination with melphalan via mild hyperthermic ILP.

1.2.4. If TNFα could be a treatment of cancer, how does it work?

The mechanism how TNF function in tumor therapy might be complicated. At first it was thought that TNF was also directly killing the malignant cells in the animal models of cancer. TNF is considered as a cytokine involved in a

receptor-triggered signaling pathway that leads to apoptosis (Urs von Holzen 2005). As mention in section **1.2.1**, evidences for two opposing pathways were found in endothelial cells: one leading to caspase activation(Lin 1999), which is dependent on the activation of TNFR-1(Wong 1994), it is an apoptotic pathway initiated by the clustering of death domain containing proteins (Tartaglia LA 1993; Grell M 1994). Recombinant TNF was reported to be toxic for cancer cells in tissue culture studies (Colotta 1984). However, many of these data suggest that the cytotoxic potential of TNF is unmasked only in combination with metabolic inhibitors such as mitomycin C, cyclohexamide or actinomycin D (Colotta 1984; Sugarman 1985; Dealtry 1987) or in combination with interferon-γ (IFN γ) (Williamson 1983; Fransen 1986). The asscociated mechanism could be that the metabolic inhibitors inactivate the intracellular survival and inflammatory pathways downstream of TNF signaling, thus allowing apoptosis to proceed. A large number of evidences suggest that the antitumor activity of TNF depends on selective destruction of the tumor-associated vascular bed and TNF-induced alteration of endothelial barrier function and of immune mechanisms rather than having toxic effects directly on tumor cells (Nawroth 1986; Palladino 1987; Nawroth 1988; Clauss 1990; Gasparri 1999). One explanation of the mechanism shared by Horssen is shown in **fig 3.** (Figure and legends from (Horssen 2006)). The double-induced hyperpermeability (by tumor endothelium and by TNF mediate permeability enhancement), along with the dual targeting—the tumor-associated vasculature (TAV) (by TNF-α) and the tumor cells (by the chemotherapy drug)— might explain the observed synergy between TNF and chemotherapy in locoregional treatment of patients with advanced tumors of the limb (Lienard 1999; Horssen 2006). Thereof, after patients were given isolated limb perfusion with TNF in combination with interferon-γ and melphalan, it was observed that the macro- and microvasculature of tumors, but not of normal tissue, to be extensively damaged (Eggermont 1996). Alberto Mantovani's group reported that TNF changed the perception of the tumor vasculature by activating

endothelial cells in a gene expression-dependent way (Mantovani 1989). Besides, the mouse experiments did reveal a role for T cells in the anti-tumor actions of TNF. There was a diminished anti-tumor effect of TNF in T cell-deficient mice (Havell 1988) and T cell mediated immunity developed in animals cured of the Meth A sarcoma by TNF (Palladino 1987). TNF is an important effector molecule in stimulation of the cellular immune response and direct cytotoxicity to tumor cells (Urs von Holzen 2005), like CD8+ T cell and natural killer (NK) cell killing of immunogenic tumor cells (Kashii 1999; Prevost-Blondel 2000).

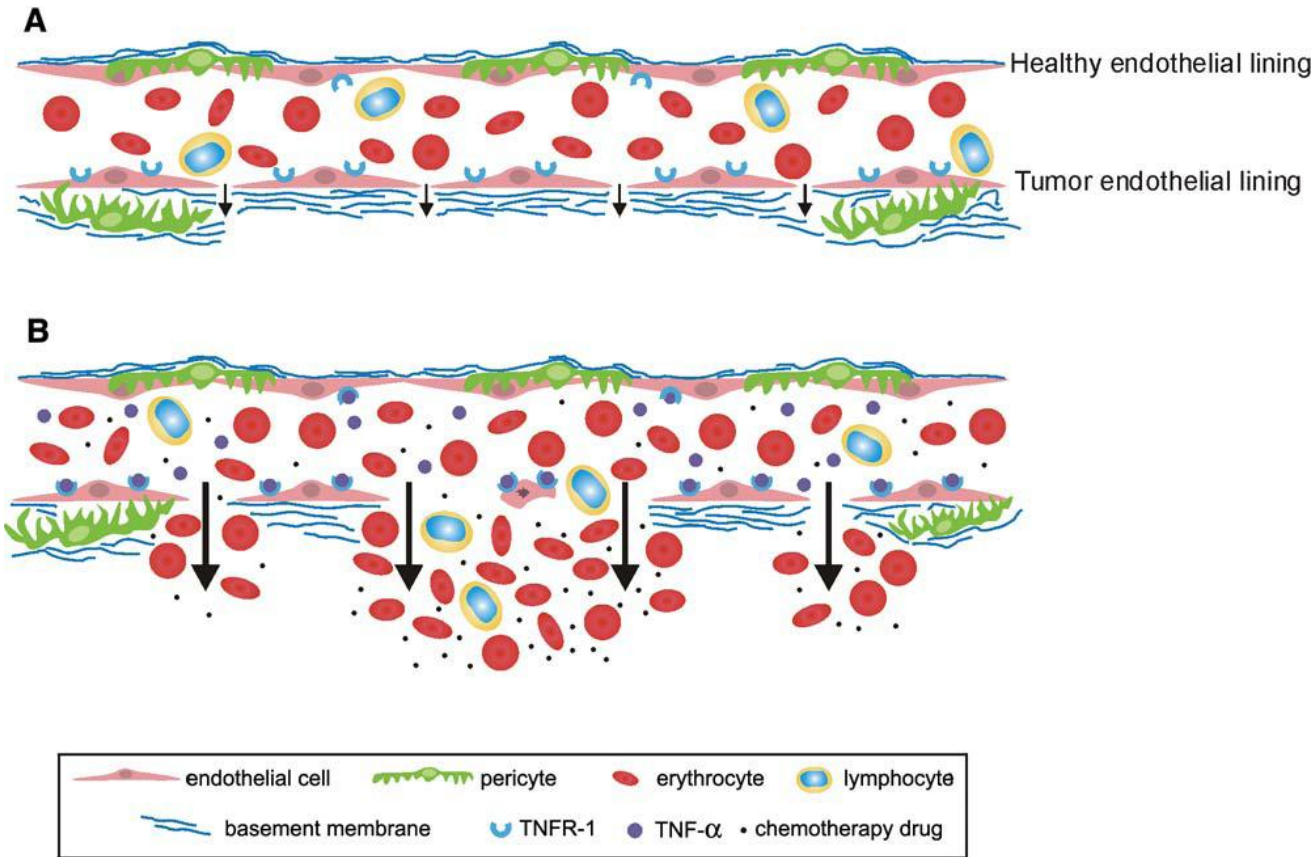

Fig. 3: A proposed mechanism of the tumor necrosis factor alpha (TNF-α) effect
(figure and description from (Horssen 2006))
(A): Scheme of a vessel with healthy endothelial lining (upper) and tumor endothelial lining (lower). There are differences between healthy vascular and tumor vascular. The healthy endothelium is with low permeability and extravasation tightly organized since it is consists with a continuous lining of endothelial cells, pericytes and a thin basement membrane. In contrast, tumor endothelium is greater permeability, since it is consists with uncontinuous lining of endothelial cells, lacks pericyte coverage while the basement membrane is thickened. Besides, a higher TNFR-1 expression level induced by TNFR-1–upregulating factors (produced by vessel-surrounding cells) is exhited on tumor endothelium. So, the tumor vessels are specific target for TNF-α treatment due to the high level of TNFR and the specific architecture of the endothelial lining. **Fig. B**: Upon TNF-α treatment, tumor endothelium binds TNF-α, which induces apoptosis in some endothelial cells leading to an enormous induction of vessel permeability. As a result, the chemotherapy drug is well

distributed throughout the tumor, and destroys the tumor cells and the tumor develops hemorrhagic necrosia due to a strong extravasation of erythrocytes (figure and legend from (Horssen 2006)).

1.2.5. TNFα in cancer: target or treatment?

Although many researchers found the powerful ability of TNF as cancer treatment, we still have to ask: was the research of the previous 40 years correct? Did recombinant TNF cause tumor necrosis in mouse cancer models? However, since 1990, many adverse findings emerged in large number. In 1989–1993, scientists found that TNF may increase experimental cancer growth and spread (Malik 1989; Malik 1990; Orosz 1993). By the mid 1990s it was becoming clear that macrophage-derived TNF play a crucial role in inflammation and tumor development (Oguma 2008; Popivanova 2008). It was found in an *in vitro* model of colorectal cancer that TNF in the tumor microenvironment may also induce the epithelial – mesenchymal transition of malignant cells (Bates 2003), which may partly explain the ability of TNF to increase the metastastic activity of tumor cells first reported in the 1990s. In 1999, scientists found that TNF-knockout mice are resistant to skin carcinogenesis. In 2003–2008, scientists found anti-TNF antibodies or antagonists inhibit murine cancer growth involving carcinogen-induced, transplantable xenograft and genetic models of common epithelial cancers (Scott 2003; Rao 2006; Egberts 2008; Popivanova 2008). In 2004, a first clinical trial with TNF antagonists was performed in cancer patients (Madhusudan 2004). The accumulating evidences were that TNF was not only made by cancer cells in tissue culture but was also present in the tumor microenvironment of many cancers, raising the possibility that it might actually be enhancing cancer growth.

Is TNF the key to this endeavour? It could be like many scientists indicated, low concentrations of TNF-a have been shown to promote proliferation of endothelial cells (Frater-Schroder 1987; Fajardo 1992) while higher concentrations are cytotoxic to, and result in apoptosis of, endothelial cells both *in vitro* and *in vivo* (Frater-Schroder 1987; Sato 1987; Fajardo 1992;

Polunovsky 1994). Anyway, as Dr. Frances Balkwill has suggested in *Nature Reviews on Cancer*, TNF probably holds greater promise for further clinical development when used in combination with other cancer treatments. Besides, whether pro- or anti-tumoral, there is no doubt that TNF is important to cancer biology and treatment (Balkwill 2009).

1.3. Novel plasmids for gene therapy

1.3.1. pTNF - the novel plasmid with CpG free backbone and human CMV as enhancer

Here we used pTNF, a novel TNF encoding plasmid with CpG free back bone and hCMV as enhancer. Viral promoters and enhancers are common features in plasmid design, and the most extensively used originates from the cytomegalovirus (CMV). The close interrelation and still distinct functionality of the CMV enhancers has been demonstrated by replacing the enhancer of the murine CMV (mCMV) with the hCMV enhancer *in vitro* without changing the wild-type characteristic growth of mCMV in murine cells (Angulo A. 1998). However, replacing the enhancer of hCMV with the murine analogue resulted in less efficient replication in human cells (Isomura 2003). The CMV promoter might still be the most commonly used promoter since it renders strong and ubiquitous expression. But because of the reported fast inactivation by promoter methylation (Collas 1998; Brooks 2004) other ubiquitous promoters are being investigated, for example the promoter of the elongation factor 1α (EF-1) or β-actin gene (Alexopoulou A.N. 2008). Thus, the plasmid vectors here carry a hCMV enhancer and an EF-1 promoter. Some transgene vectors are silenced by the host cell, or even disposed of by the immune system, although they own strong and efficient promoter and enhancer element. These are caused by specific unmethylated CpG dinucleotides commonly found in bacterial DNA, which can elicit a direct inflammatory response. This effect has been harnessed to improve the immune response after vaccination by using CpG-motifs as adjuvants (McCluskie 2000; Kumagai 2008) or for cancer

immune therapy approaches (Wooldridge 2003). It was shown that CpG-dinucleotides in the plasmid DNA have a strong negative effect on the duration of transgene expression (Yew 2000; Yew 2002; Hodges 2004; Mitsui 2009). Even as few as one CpG dinucleotide in the plasmid DNA sequence can lead to increased levels of pro-inflammatory cytokines (TNF-α, IL-12, IFN-γ) and infiltrating neutrophils; additional CpG´s increase these levels incrementally, whereas CpG-depleted plasmids shows no measurable elevation of the cytokine levels (Hyde 2004). Stephen C Hyde *et al* demonstrated that retention of even a single CpG in pDNA is sufficient to elicit an inflammatory response, whereas CpG-free pDNA vectors do not. Using a CpG-free pDNA expression vector led to sustained (≥56 d) *in vivo* transgene expression in the absence of lung inflammation (Hyde 2004). The mechanism by which unmethylated CpG-sequences reduces transgene expression is not entirely clear yet. Because of the disadvantageous effects of CpG´s in the plasmid vector such as activation of the immune system or silencing of the transgene and in the context of gene therapy where a long-term transgene expression is desired, CpG-free backbone design is helpful, thus we tried to design a plasmid with CpG-free backbone.

1.3.2. pRGD-TNF - plasmid targeting TNF expression by employing sequence of vasculature-targeting motif RGD

The vascular effects of TNF mentioned above in section **1.2.4** provide the rationale for developing vasculature-targeting strategies aimed at increasing the therapeutic index of this cytokine. Recently several peptides that can bind receptors expressed within angiogenic vessels have been selected by in vivo phage display. Among the various peptides identified so far, CNGRC and CDCRGDCFC, containing Asn-Gly-Arg (NGR) or Arg-Gly-Asp (RGD) motifs, have been used for targeting chemotherapeutic drugs, proapoptotic peptides, and cytokines to tumors in experimental models (Arap 1998; Zarovni 2004). Flavio Curnis and colleagues report that coupling TNF to CNGRC, a CD13

ligand capable of “homing” to tumor vessels (Curnis 2000) enhances the immunotherapeutic properties of TNF and improves its therapeutic index in lymphoma and melanoma animal models. Natasa Zarovni also showed that by fusing the TNF sequence with those of peptides able to target tumor vessels such as CNGRCG or ACDCRGDCFCG, the therapeutic properties of the TNF gene are improved, while the treatment did not result in any side effect (Zarovni 2004), which indicated vascular targeting with TNF coupled to tumor-homing peptides is rationale. Thus, here we try to investigate the therapeutic potential of a plasmids encoding TNF fused to the C terminus of ACDCRGDCFCG (pRGD-TNF) and compare them with plasmid pTNF.

1.3.3. pTNF-miR143 - plasmid selective TNF expression by employing miRNA-143 targeting sequence

MicroRNAs (miRNAs) represent a class of small nucleotides (20–25 bp) which are typically excised from 60–110 nucleotide hairpin RNA precursor structures. They regulate gene expression by repressing translation of target cellular transcripts and play a crucial role in regulating many cellular events, including the balance between proliferation and differentiation during tumorigenesis and organ development (Calin 2006; Kloosterman 2006; Zhao 2007; Kimberly 2009). In May 2006, a group led by Dr. Luigi Naldini and Dr. Brian Brown in Milan, Italy reported that they developed a way to prevent the immune system from rejecting a newly delivered transgene by utilizing the natural function of miRNA to selectively turn off the expression of their therapeutic gene in cells of the immune system and prevent the gene product from being found and destroyed. Their results provide novel evidence of miRNA regulation and demonstrate a new paradigm in vector design with applications for genetic engineering and therapeutic gene transfer (Brown 2006). It was also reported that miR-143 is the most enriched miRNA during differentiation of mouse embryonic stem (mES) cells into multipotent cardiac progenitors (Ivey 2008), but down regulated in various cancer cell lines, colon cancers, and lung

cancers (Budker 1996; Kimberly 2009). In view of the natural function of microRNA, pTNF-mir143 was constructed by Dr. Rudolf Haase in our lab by inserting miR-143 targeting sequence (5 copies of sequence GAGCTACAGTGCTTCATCTCA) into plasmid pTNF (at 3' untranslated region of gene TNF). Thus, the expression of the therapeutic gene should be selectively turned off in cells which are rich in miR-143 (almost all organs are miR-143 rich) through miRNA specifically repression, while not affecting the transgene expession in the tissues low miR-143, such as various cancer cells.

1.4. Approaches for gene delivery

How are genes transferred into cells so that gene therapy can take place? In the past few years during the development of gene therapy, various methods have evolved to transfer genes to the target cells. In focus have been physical, chemical, and biological principles to develop safe and efficient method that delivers a transgene into target cells for appropriate expression. For the gene transfer basically two major branches exist: viral and non-viral vectors.

1.4.1. Viral vectors for gene transfer

Viruses are the most commonly used vectors in gene therapy. So far, the most widely used and also perhaps efficient method of gene transfer is by means of viral vectors. Over the years, viruses have evolved to enter the cell and efficiently used the cells to make its own viral proteins. Viruses include retroviruses lentiviruses, poxviruses, adenoviruses, herpes viruses and adeno-associated viruses are ubiquitous used as vectors for the desired gene delivery in many researches and early clinical trials for gene therapy. Since these viruses differ in characteristics, researchers may use different vectors according to the specific characteristics and requirements of the study. Viruses are quite effecint in gene delievery, however, even the most efficient gene transfer vectors in use today, have certain disadvantages. One of the major disadvantages is decreased effectiveness during repeated treatments in vivo

due to immunogenicity of viral vectors. Thus, many non-viral approaches for gene delivery are developing to conquer these problems.

1.4.2. Non-viral approaches for gene delivery

Despite efficient transgene transfection potential of viruses, their utility in clinic is severely limited due to several disadvantages such as immunogenicity of viral proteins, risk of oncogenesis, insertional mutagenesis, dose limiting hepatotoxicity and inadvertent creation of infectious viral particles (Gao 2007; Russ 2007; Pathak 2009). Hence, to avoid these disadvantages of the viral vectors for gene delivery, the non-viral approaches have been extensively studied. Nonviral gene delivery approaches consist of physical approaches (carrier-free gene delivery) and chemical approaches (synthetic vector-based gene delivery).

Physical approaches is charactered by permeating the cell membrane by a physical force which then facilitates the intracellular gene transfer by diffusion (Gao 2007). Physical approaches include naked DNA injection, electroporation, gene gun, ultrasound, Jet injection, Magnetofection, Photochemical internalization (PCI), and hydrodynamic delivery (Wagner 2004).

The chemical approaches use synthetic or naturally occurring compounds as carriers to deliver the transgene into cells. Till now, encapculation of DNA into condensed particles by using cationic lipids or cationic polymers are the most frequently studied strategy for nonviral gene delivery.

Several polymers have been explored as non-viral vectors for delivery of DNA. The polymer can package DNA into nanoparticles, protect it from degradation and enhance its binding to cells and transport into the cytoplasm. Over the years, a significant number of cationic polymers, such as polyamidoamine (Haensler 1993), polyethylenimine (PEI) (Boussif 1995), polyallylamine, and polypropylamine dendrimers (Schatzlein 2005), chitosan (Venkatesh 1997), cationic proteins (polylysine, protamine, and histones) (Balicki 1997), cationic dextran (Hosseinkhani 2004), and cationic peptides (Balicki 2002; Park 2003)

have been explored as carriers for in vitro and in vivo gene delivery. Cationic polymers differ dramatically in their transfection activity and toxicity, while sharing the function of delievering the DNA into the cell. PEI, which is perhaps the most active and most studied polymer for gene delivery, was first introduced in 1995 (Boussif 1995). The non-biodegradable nature of PEI might be one disadvantage for its use as a transfection reagent (Fischer 2003). PEI oligomers owing high transfection efficiency can be generated through cross-linking of low-molecular weight PEI with bifunctional reagents. It was shown that transfection activity can been significant increased by conjugation of lytic peptides (melittin) to 25 kDa PEI (Ogris 2001 ; Boeckle 2006). The surface charge of PEI can be dramatically reduced by surface modification with PEG, thus the tendency to form large aggregates in the serum is also reduced. To provide shielding to polyplexes, scientists try to conjugate PEI with a ligand, such as transferrin, antibody, sugar moieties, or avidin, and the conjugation also leads to the possibility of target-specific gene delivery (Kichler 2004). To reduce the toxicity associated with cationic polymer-based gene delivery systems, more biodegradable cationic polymers have been designed.

1.4.3. Polymer G3-HD-OEI/G2-HD-OEI and polymer LPEI-PEG-GE11

The challenge for in vivo gene delivery is to pinpoint the limiting factors and implementation strategies to enhance gene delivery efficiency with minimal tissue damage. A major challenge for non-viral gene delivery systems is the efficient targeting of these genes to the specific tissue such as tumor to reduce side effects like tissue damage. When developing non-viral vectors, scientists found that oligocations transfected as efficient as PEI 25 k but were significantly less toxic to cells after cross-linking of small PEI or PPI with a biodegradable bond such as a disulfide or ester bond (Gosselin 2001; Forrest 2003). Careful modifications by conjugation of relatively bulky groups (proteins, such as EGF, transferrin; peptides, such as GE11; PEG) or smaller groups (sugar, small polymers, acyl group) to reduce a portion of the positive charges have yielded

PEI derivatives that are more efficient in transfection and less toxic to cells (Kichler 2004).

Several grafted polypropylenimine dendrimers were synthesized by Dr. Verena Russ in our lab through modifying either polypropylenimine (PPI) dendrimer generation 2 (G2) or generation 3 (G3) via 1.6-hexandioldiacrylate with branched oligoethylenimine 800Da (OEI) or PPI dendrimer G2-, as the structure shown in **fig. 4** (Russ 2008). All grafted dendrimers display an advantageous biocompatibility profile compared to LPEI or BPEI, since no erythrocyte aggregation after incubation with plain polymer occurred while OEI-grafted dendrimers are moreover biodegradable resulting in low toxic metabolites. Transgene expression was predominantly found in the (subcutaneous) tumors in tumor-bearing mice upon i.v. injection of OEI-grafted dendrimer polyplexes (the transfection level for G3-HD-OEI is 12-fold higher) (Russ 2008). Importantly, the tumor gene expression levels significantly increased with the higher dendrimer core generation. All these results show that OEI-grafted polypropylenimine dendrimers is a degradable and biocompatible vectors which is very attractive for further investigations. G2-HD-OEI was used as systemic nonviral delivery agent of the sodium iodide symporter gene for targeted radioiodine therapy of neuroblastoma tumors, and exhibits high transfection efficiency in tumors (Klutz 2009).

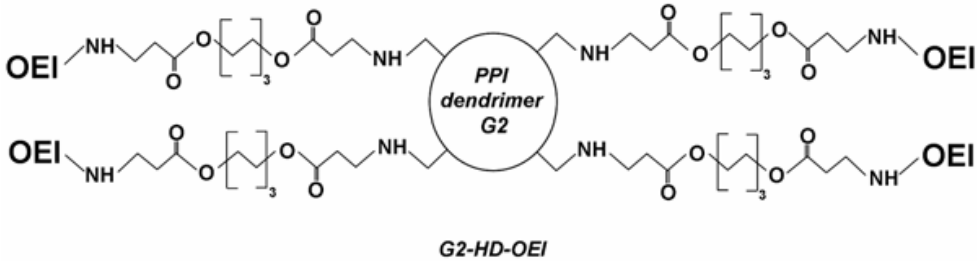

Fig. 4: Schematic structure of G2-HD-OEI (Russ 2008)

Targeted therapy for cancer has gained momentum during the past decade. Many cancer cells such as glioblastoma and hepatocellular carcinoma cell lines show epidermal growth factor receptor (EGFR) over-expression. Thus, EGF could be a targeting part of vectors to specifically deliver the therapeutic genes to cancer cells. Dr. David Schaffert in our lab found that the optimized

LPEI-PEG2kDa-EGF conjugate displays reduced chemical complexity and efficient poly(I:C)-mediated killing of EGFR overexpressing tumors in vitro and in vivo (Schaffert 2011). One simplification was done in our lab with the vector is that EGF is replaced by a 12 amino acid peptide (GE11), which binds to the EGFR with 40 nM affinity and is able to internalize with the receptor (Li 2005). LPEI-PEG-GE11 is based on linear polyethylenimine carrying a peptide (GE11) selectively binding to the epidermal growth factor receptor, the efficacy of these conjugates in poly (I:C)-mediated cell killing was evaluated in vitro using two human U87MG glioma cell lines and showed that the GE11 vector has a similar or even better performance as EGF vector. It will simplify vector production, reduce markedly production costs, and may have fewer side effects. LPEI-PEG-GE11 was used as systemic nonviral delivery agent of the sodium iodide symporter gene for targeted radioiodine therapy of hepatocellular carcinoma (HCC) (HuH7), and exhibits high transfection efficiency in tumors (Klutz 2011). So here, we employed LPEI-PEG-GE11 as gene carrier in the HUH7 tumor model.

1.5. Chemotherapeutics

1.5.1. Chemotherapy

Chemotherapy is the treatment of cancer with anticancer drugs. Chemotherapy with the main purpose of killing cells is usually used in patients with cancer that has already metastasized. Chemotherapy can cure some types of cancer, while in some cases it is used to slow the growth of cancer cells or to prevent the metastasis of the tumor cells after surgery. Most chemotherapy drugs interfere with the ability of cells to grow or multiply. Chemotherapy is usually given as an adjuvant in addition to other cancer treatments, such as surgery and radiation therapy. The use of two or more drugs together often works better than a single drug for treating cancer. This is called combination chemotherapy.

1.5.2. Doxorubicin and Liposomal formulation

Doxorubicin (trade name Adriamycin; also known as hydroxydaunorubicin) is used to treat a wide range of cancers, such as breast cancer, stomach cancer, lymphoma, leukemia, multiple myeloma, bone cancer, and ovarian cancer (Ozturk 2011). It is an antitumor antibiotic, which are made from natural substances such as fungi in the soil. In the 1950's, the drug was originally isolated from bacteria found in soil samples taken from an Italian castle - Castel del Monte. Doxorubicin slows or stops the growth and spread of cancer cells in the body. Doxorubicin's most serious adverse effect is life-threatening heart damage (Ozturk 2011). Doxorubicin showed good activity against murine tumors, especially solid tumors. Although it is thought to interact with DNA by intercalation, the exact mechanism of action of doxorubicin is complicated and still somewhat unclear (Fornari 1994).

To reduce systemic toxicity caused by anticancer agents while retaining or even improving in vivo efficacy, scientists try to encapsulate them into pegylated, long circulating liposomes (Papahadjopoulos 1991; Lasic 1996; Hagen 2002; Seynhaeve 2007). These liposomes tend to accumulate in tumors by leaking through the often-compromised tumor vasculature since their small size, long circulation time, and reduced interaction with serum proteins (Gabizon 1992; Wu 1993; Seynhaeve 2007). There are two liposomal doxorubicin drugs that work in slightly different ways and are used to treat different types of cancer, Caelyx® and Myocet®.

Caelyx® (Doxil) is a novel pegylated liposomal formulation of the first-generation anthracycline, doxorubicin made by Ben Venue Laboratories for Johnson & Johnson in the United States. It was developed to treat some types of cancer, among which, the most commonly ones are ovarian cancer and Kaposi's sarcoma, an AIDS-related cancer that causes lesions to grow under the skin. Recently its therapeutic effect in the treatment of metastatic breast cancer, as well as recurrent squamous cell cervical carcinoma, soft tissue sarcoma, prostate cancers and others was investigated (Tejada-Berges 2002). The length of the treatment and the number of cycles the patients are treated

with depend on the type of cancer. The substitution liposome-encapsulated doxorubicin for doxorubicin would be desirable because liposome-encapsulated doxorubicin are less cardiotoxic than unencapsulated doxorubicin and also have enhanced efficacy in some solid tumors compared with free doxorubicin, due to their higher accumulation in the target solid tumors by passing the tumor asscoatied vasculature which exhibit higher permeability than healthy vasculature (Kimberley 2005). Doxil is also approved by the FDA for treatment of ovarian cancer and multiple myeloma. Outside the United States, Doxil is known as Caelyx and is marketed by Janssen. However, the polyethylene glycol coating usually results in a side effect called palmar plantar erythrodysesthesia (PPE), more commonly known as hand-foot syndrome. The result of the leakage of drugs from capillaries in the palms of the hands and soles of the feet after administration of Doxil is redness, tenderness, and peeling of the skin that can be painful (Lorusso 2007). The prevalence of this side effect limits the Doxil dose and thereby limits potential application. Myocet is a non- pegylated liposomal formulation of doxorubicin. Unlike Doxil, since Myocet liposome does not has a polyethylene glycol coating, it does not result in the same prevalence of hand-foot syndrome, which may allow for its substitution for doxorubicin in the same treatment regimen, thereby improving safety with no loss of efficacy.

1.5.3. The combination of TNFα with chemotherapy for cancer therapy

Conventional anticancer strategies are destruction of tumor cells directly by surgery, radiotherapy or chemotherapy, while recently indirect attacks of tumors by means of agents destroying intratumoral vessels are suggested to be efficient, since cancer growth depends on the formation of a vascular supply, which is referred to as tumor angiogenesis (Lejeune 2006). It is well recognized that the pathophysiology of the tumor vasculature and stromal compartment presents an important obstacle which induce inadequate drug delivery leading to poor response and regrowth of tumors. Thus, delivery of drugs into solid

tumors is still a major problem faced in chemotherapy; adequate levels of chemotherapeutics reaching the tumor cells of solid tumors are indispensable for a successful chemotherapy. Methods to enhance vascular abnormality, such as augmenting leakage of the tumor-associated vasculature, may improve tumor response to chemotherapeutics as well. It was believed that recombinant humanTNFα has pleiotropic properties; it has strong effects on angiogenic vessels in tumors, and the ability to cause apoptosis of tumor-associated endothelial cells which leads to the complete destruction of the tumor vasculature. In the study of ten Hagen's group, they found that that addition of TNFα rendered more tumor vessels permeable, thus augmented the tumor accumulation of these liposomes 5- to 6-fold, and also induced a more homogeneous distribution of the liposomes throughout the tumor, which induced strongly enhanced tumor response. It was believed that vasoactive drugs like TNFα can augment the vascular leakage, leading to delivery of nanoparticulate drug formulations to solid tumor (Seynhaeve 2007). The mechanism involved in these events could include the rapid (in the order of minutes) perturbation of cell-cell adhesive junctions and inhibition of αvβ3 integrin signalling in tumor-associated vessels, and the subsequently massive death of endothelial cells and tumor vascular collapse 24 hours later (Seynhaeve 2007). In addition, in the isolated limb perfusion, addition of TNFα results in increased accumulation of drug inside the tumor leading to improved response rates (van der Veen 2000; Hoving 2006). Studies on the mechanism show that TNF alone was ineffective, however, when combined with melphalan chemotherapy in a rat osteosarcoma ILP model together with mild hyperthermia, these treatments synergize resulting in a very high objective response rate (de Wilt 1999). TNFα treatment leads to an increase in endothelium permeability which subsequently facilitates augmented accumulation and distribution of the drug in the tumor; besides, killing of angiogenic endothelial cells results in tumor vessel destruction. As mention in section **1.2.4**, the TNFα induced hyperpermeability, along with the dual

targeting—the tumor-associated vasculature (TAV) (by TNF-α) and the tumor cells (by the chemotherapy drug)— might explain the observed synergy between TNF and chemotherapy in locoregional treatment of patients with advanced tumors of the limb (Lienard 1999).

1.6. Existing TNFα gene therapy approaches for cancer therapy

As mention above, gene therapy which can be used either alone or as an adjuvant to other treatment modalities is the emerging modality for the treatment of cancer. Exhibit tumor specificity, efficiency and safety are three indispensable factors for the development of vectors which can lead to success of cancer gene therapy. Particularly attractive for cancer treatment is the potential of gene therapy to target the expression of therapeutic genes to the desired target cells. Potential to improve local tumor control through the delivery of therapeutic DNA constructs that encode cytotoxic or immunomodulating proteins have demonstrated in the preclinical gene therapy studies (Staba MJ 1998). The pleiotropic cytokine tumor necrosis factor TNFα was originally known for its ability to induce hemorrhagic tumor necrosis and tumor regression. However, the need to target TNFα activity to the tumor is indispensable due to the fact that the therapeutic application of TNFα is hampered by its high systemic toxicity (Kircheis R 2003). In the past decades, many researchers have put emphasis on employing viral or non-viral gene carriers to target delivery TNFα-coding plasmid to the tumor, to express the TNFα gene at high levels with minimum toxicity. TNFα gene therapy has been applied either alone or as an adjuvant to other treatment modalities such as ionizing radiation or chemotherapy. Till now, many related encouraging results have been achieved.

1.6.1. Adenoviral TNFα gene therapy

In the 1990s, scientists from Ralph R. Weichselbaum’s lab in Harvard University investigated the interactive effects of adenoviral TNFα gene therapy

and radiotherapy in human malignant glioma (D54) xenografts (Staba MJ 1998) and human epidermoid carcinoma (SQ-20B) xenografts (Hallahan DE 1995). They administered Ad.Egr-TNF, an adenoviral vector (Ad5) which contains DNA sequences of the radiation-inducible early growth response gene (Egr-1) promoter linking to a cDNA encoding the TNFα gene) and radiation, to both human malignant glioma (D54) xenografts (Staba MJ 1998) and human epidermoid carcinoma (SQ-20B) xenografts (Hallahan DE 1995) and observed significant tumor regression, tumor growth delay, compared with xenografts treated with either agent alone or Ad.null with radiation (Hallahan DE 1995; Staba MJ 1998). They took advantage of both the killing effect and the targeting potential of ionizing radiation to achieve spatial and temporal regulation of TNFα gene transcription and enhance tumor cell killing (Hallahan DE 1995).

In the 2000s, based on these researches, a new drug named TNFerade that employs a replication–deficient adenoviral vector carrying the gene for human tumor necrosis factor TNFα , regulated by radiation-sensitive promoter , was under development by Genvec and has applied in the phase I/ II/III clinical trials for the potential treatment of cancer including pancreas cancer, Esophagus tumor, sarcoma, solid tumor and so on (Sharma A. 2001; Kircheis R 2003). Results of clinical trials demonstrated that most of patients TNFerade treated showed objective tumor response (tumor shrinkage) which is dose dependency. Meanwhile, TNFerade was well tolerated by patients with mild side effect (Sharma A. 2001; Kircheis R 2003), which suggests that it has promising potential for development into a new anticancer agent.

1.6.2. Cationic liposomes mediated TNFα gene therapy

Besides the works of adenovector–mediate TNFα gene therapy, cationic liposomes–mediated TNFα gene transfection has been utilized in *in vitro* and *in vivo* experiments. In 1997, scientists from Dr. Ohno's lab administered the liposome–DNA–HMG-1,2 complex intraperitoneally to tumor bearing nude mice and demonstrated the median survival of tumor bearing nude mice was

prolonged by the administration of a human tumor necrosis factor-alpha (TNFα) gene inserted into a eukaryotic strong expression vector (pcagTNFα) and exogenously added interferon-γ (INF-γ), compared with groups receiving the TNF-α gene inserted in the reverse orientation (pcagTNF-αR) and normal saline (NS), pcagTNF-αR and INF-γ, and pcagTNF-α and NS, respectively (Namiki Y 1998).

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1.6.3. Nonviral cationic polymers mediated TNFα gene therapy

Moreover, many nonviral cationic polymers, such as Transferrin-PEG-PEI was employed as gene carrier of TNFα gene and showed the efficient targeting of TNFα genes to tumor to reduce side effects like tissue damage (Kircheis R 2002; Kursa 2003).

Scientists from our lab found that repeated systemic application of surface -shielded Tf–PEI complexes with the tumor necrosis factor (TNFα) gene, or an optimum formulation for in vivo application, PEI22/Tf-PEG-PEI/PEI22-PEG5, containing plasmid DNA encoding for the TNFα gene, resulted in preferential expression of TNFα in the tumor which led to pronounced hemorrhagic tumor necrosis and inhibition of tumor growth in three murine tumor models of different tissue origins, Neuro2a neuroblastoma, MethA fibrosarcoma, and M-3 melanoma. No systemic TNFα related toxicity was observed due to the localization of the TNF- activity to the tumor (Kircheis R 2002; Kursa 2003).

In 2009, Christine Dufès's lab reported that the intravenous administration of a novel delivery system DAB-Tf (transferrin-bearing generation 3- diaminobutyric polypropylenimine dendrimer) complexed to a TNFα expression plasmid driven by a tumor-specific promoter led to a rapid and sustained tumor regression over one month, with long term survival of 90% complete response, 10% partial response of the animals (Koppu S. 2010). To sum up, these results might suggest that cationic polymer–mediated targeted gene therapy may be an attractive strategy applicable to highly active, yet toxic, molecules such as TNFα.

1.6.4. Preliminary work of TNFα gene therapy combined with liposomal doxorubicine for cancer therapy

Combined gene therapy with chemo- or radiation therapy is one alternative to overcome the drawbacks of sigle therapy and to gain a better therapeutic effect in cancer treatment; especially when the different molecular actions of genes and classical chemotherapeutics or radiation synergize in their therapeutic effects. Examples include combination of p53 viral or nonviral gene formulations with chemo- (Nemunaitis J 2000; Gautam A 2002) or radiation therapy, or TNF gene plus radiation (Hallahan DE 1995; Staba MJ 1998). As another example, scientists from our lab have observed enhanced therapeutic effects in a murine B16F10 melanoma model by the combination of tumor-targeted TNF gene therapy with DOXIL®, a liposomal doxorubicin formulation that passively accumulates within tumors (Wagner E. 2004). These findings were encouraging, but had preliminary character. Especially the applied TNFα plasmid construct were not optimized, leading to suboptimum gene expression. There were hopes that the therapeutic strategy can be further improved.

1.7. Aim of the thesis

The aim of this thesis is to combine systemic TNF gene therapy with liposomal doxorubicine in the treatment of different tumor models in mice.

The clinical use of TNF as an anti-cancer drug is limited to local treatments because of its dose-limiting systemic toxicity (Curnis 2000). C57B1 mice normally tolerated up to 1 x $I0^7$U (4.91 µg) per kg TNF i.p. (Jones 1990). The problem of its dose-dependent toxicity has been particularly apparent in human trials, wherein its maximal tolerated dose was 40 times less than that used in mice (Xiang 2003). The maximum tolerated intravenous dose in phase I studies was 9 x $I0^5$ U (440µg) per m^2. Doses at or above 9 x 10^5 units (400µg) m^2 were associated with hypotension, abnormal liver enzymes, leucopenia and mild

renal impairment in a substantial proportion of patients (Xiang 2003). Thus, the high systemic toxicity induced by recombinant TNFα indicates need to target TNFα activity selectively to the tumor.

Here we want to investigate localized antitumor activity of TNF after systemic injection of a non viral, biodegradable gene carrier (G3-HD-OEI) exhibiting intrinsic tumor affinity in vivo in murine Neuroblastoma model (Russ 2008). Thus, when we employ G3-HD-OEI as gene carrier of optimized TNF coding plasmid, it is supposed that most of TNF is expressed in tumor tissue. The influrence of pTNF polyplexes application on the accumulation of liposomal doxorubicine are evaluated, the pTNF polyplexes are either based on the biodegradable polymer G3-HD-OEI in murine neuroblastoma model in A/J mice or based on linear polyethylenimine carrying a peptide (GE11) selectively binding to the epidermal growth factor receptor (which is highly overexpressed in HuH7) (Klutz 2011) in hepatoma xenografts in SCID mice.

Moreover, a novel TNF coding plasmid with CpG free back bone and hCMV as enhancer was cloned and its ability to express TNF is at high and sustained levels, since Stephen C Hyde *et al* observed that CpG-free plasmids with the hCMV/EF1α combination confer reduced inflammation and increase the magnitude and duration of gene expression in vivo (Hyde 2004).

Another aim was to investigate any synergistic effects of systemic TNF alpha gene therapy together with liposomal doxorubicine in the treatment of subcutaneous and metastatic murine neuroblastoma model in A/J mice and intrasplenic liver metastatic colon adenocarcinoma model in NMRI-nude mice. pTNF containing polyplexes had to be applied using either G3-HD-OEI as gene carrier in murine neuroblastoma or LPEI-PEG-GE11 in human hepatocellular carcinoma and human colon adenocarcinoma. Results of our studies show that combining liposomal chemotherapeutic with TNF gene therapy leads to a synergistic effect on tumor reduction in vivo.

2. Materials and methods

2.1. Chemicals, kits, and other materials

The synthesis of polymers G2-HD-OEI and G3-HD-OEI was described previously (Russ 2008). Several grafted polypropylenimine dendrimers were synthesized by modifying either polypropylenimine (PPI) dendrimer generation 2 (G2) or generation 3 (G3) via 1.6-hexandioldiacrylate with branched oligoethylenimine 800Da (OEI) or PPI dendrimer G2. LPEI-PEG-GE11 is based on linear polyethylenimine carrying a peptide (GE11) selectively binding to the epidermal growth factor receptor coupled to LPEI via a heterobifunctional polyethyleneglycol linker (2kDa) (Klutz 2011).

Actinomycin D was purchased from Sigma (Saint Louis, USA) and diluted into 5mg/ml. Caelyx (liposomal doxorubicin) 2mg/ml is commercially available from Schering-Plough (Kenilworth, USA). Corning Transwell polycarbonate membrane inserts (pore size 0.4um, membrane diam. 6.5mm were purchased from Corning (NY, USA). Mouse TNFα (Tumor Necrosis Factor alpha) ELISA kit was purchased from eBioscience (San Diego, CA).

2.2. Cells and animals

Murine neuroblastoma (Neuro2A, N2A, ATCC CCL-131) and Neuro2A-PGK-EGFLuc (Neuro2A lenti Luc cell line transduced with a lentivral vector PGK-EGFPLuc for stable luciferase expression, generated by Arzu Cengiceroglu (our lab) were cultured with Dulbecco's modified Eagle's medium (DMEM, Biochrom) supplemented with 10% fetal bovine serum, 2 mM of L-glutamine. The murine fibroblast cell line L929 (ATCC CCL-1) and U87MG, a human glioblastoma-astrocytoma cell line (ATCC HTB 14), were incubated in RPMI-1640 (Biochrom) complete medium with 10% fetal bovine serum, 2 mM of L-glutamine. Human hepatocellular carcinoma cell line HUH7 (JCRB 0403, Tokyo, Japan) was incubated in Ham's F-12 complete medium with 10% fetal bovine serum, 2 mM of L-glutamine. BT549 , a human breast cancer cell line (CLS, #300132) was incubated in RPMI 1640 medium with 0.023 IU/ml insulin,

10%; fetal bovine serum. MBA-MD-231, a human breast carcinoma (kindly provided by A. Ullrich, Max-Planck Institute, Munich, Germany), was incubated in DMEM: Ham's F12 (1:1 mixture) supplemented with 2 mM L-glutamine and 5% fetal bovine serum. MDA-MB-453 ,a human breast cancer cell line (kindly provided by A. Ullrich, Max-Planck Institute, Munich, Germany) was cultivated in DMEM/Ham's F-12 medium 1:1 supplemented with 10% FCS and 2mM stable glutamine and 1% penicillin and streptomycin. Primary porcine endothelial cells (PECs) were cultured in M199 medium supplemented with 10% FCS, and 10ng/ml bFGF (basic fibroblast growth factor) and 100 U/ml of penicillin and 100 m g/ml of streptomycin. The human breast adenocarcinoma cell line MCF-7 (kindly provided by A. Ullrich, Max-Planck Institute, Munich, Germany) was cultured in DMEM medium (5g glucose/l) supplemented with 20% FCS. Primary porcine smooth muscle cells (PSMC) were isolated by Dr. Terese Magnusson according to a protocol by Pelisek et al (Pelisek 2001) and grown in Ham´s F12 (Biochrom, Berlin, Germany)/Waymouth (Gibco, Darmstadt, Germany) medium 1:1, 37,5 ‰ sodium bicarbonate (Gibco), 0,01M HEPES, 10% bovine calf serum (FCS) (Gibco), 40mM stable glutamine (Biochrom), 100U/ml penicillin and 100µg/ml streptomycin (Biochrom), 0,25 µg/ml amphotericin B (Sigma Aldrich, Steinheim, Germany). PC3 (ATCC CRL-1435) human prostate carcinoma was cultured in Eagle's minimal essential medium supplemented with 10% FCS.

LS174T PGK-EGFPLuc (LS174t, human colon adenocarcinoma (ATCC CCL188) transduced with a lentivral vector PGK-EGFPLuc for stable luciferase expression, generated by Arzu Cengiceroglu (our lab) were cultured in Minimum essential medium (Eagle) in Earle's BSS supplemented with 2 mM L-glutamine, 1% non-essential amino acids and 10% fetal bovine serum. All cultured cells were grown at 37 °C in 5% CO_2 humidified atmosphere.

A/J mice (6-7 weeks, female) were purchased from Harlan (Bicester, UK). NMRI-nude mice (6-7 weeks, female) were purchased from Janvier, France. Mice were kept with a 12 h night/day cycle with water and chow provided ad

libitum. All animal experiments were approved by the local ethics committee and carried out according to the guidelines of the German law of protection of animal life.

2.3. Cloning and propagation of plasmids

Murine TNFα cDNA based on the published sequence (nucleotide ID NM_013693.2) was synthesized by Geneart (Regensburg, Germany). The sequence was designed for optimized codon usage in mouse, cryptic splicing sites were removed. TNFα cDNA was obtained within the vector pMA flanked by restriction sites for *BglII* and *NehI*. The luciferase gene was excised from pCpG-hCMV-Luc plasmid (short: pLuc, details see (Navarro 2010)) by *BglII-NehI* double restriction enzymes digestion and was then ligated with the TNFα CDNA excised from the pMA-TNFα plasmid with the same enzymes to construct pCpG-hCMV-TNFα (short: pTNF). pdeTNF was constructed by Dr. Rudolf Haase (Ludwig-Maximilians-University, Munich) by deleting the EF1a promoter of pTNF plasmid, and used as the control plasmid. pRGDTNF, encoding the RGD-TNF protein , was obtained by fusing the TNF sequence to the C terminus of peptides able to target tumor vessels-----ACDCRGDCFCG, which containing Arg-Gly-Asp (RGD) motifs, the detail methods could refer to Zarovni's paper (Zarovni Apr. 2004), briefly, sequence encoding ACDCRGDCFCG was inserted, by polymerase chain reaction (PCR), upstream of the mature TNF-coding region, to generate pRGD-TNF. The strategy for obtaining pRGD-TNF included two PCRs, aimed at generating two overlapping fragments containing complementary parts of the ACDCRGDCFCG sequence and the *Sac*II restriction site were generated by PCR, using the following primers:

5-primer-1: TTGAATTCAGACACCATGAGCACAGAAAGC combined with 3-primer-1a:CGCCCGCGGCAATCGCATGCTGTGAGGGTCTGGGCCATAG AAA, and 5-primer-2a GCCCCGCGGTGATTGCTTCTGTGGCCTCAGATCATCTTCTCAAAATT

Combined with 3-primer-2: GGATCCTCACAGAGCAATGACTCCAAAG.

In view of the natural function of microRNA, pTNF-mir143 was constructed by Dr. Rudolf Haase (our lab) by inserting miR-143 targeting sequence (5 copies of sequence GAGCTACAGTGCTTCATCTCA) synthesized by Geneart (Regensburg, Germany) into plasmid pTNF (at 3' untranslated region of gene TNF), thus, the expression of the therapeutic gene should be selectively turned off in cells which are enrich in miR-143 through microRNA specifically repression, while do not affect the transgene expession in the tissue which are low miR-143, such as various cancer cells. All plasmids were amplified in *E. coli* DB3.1λpir, and isolated using a Qiagen (Hilden, Germany) Plasmid Giga or Maxi Prep.

2.4. In vitro studies

2.4.1. Preparation of transfection polyplexes

DNA polyplexes were prepared as described (Kircheis R. 2001; Kircheis R 2002). In vitro, plasmid DNA (40 µg/mL) was flash-mixed with G3-HD-OEI or G2-HD-OEI at c/p=1, or with LPEI-PEG-GE11 at c/p=0.78. For in vivo application, 125 µl plasmid DNA of concentration 400 µg/ml (to final dose 50 µg DNA/mouse) was flash-mixed with 125 µl related polymer at certain c/p ratio respectively. Complexes were prepared in 20 mM HEPES (pH=7.4) /5% glucose.

2.4.2. In vitro transfection in the Neuro2A cell

Neuro2A cell was seeded at 7 $\times 10^4$ cells/well in 24-well tissue culture plates, cultured overnight at 37°C and then transfected with G3-HD-OEI/ DNA polyplexes (1ug pDNA per well). Four hours after transfection, the medium were replaced with the fresh one. Then, from 24 hours till 6 days thereafter, the conditioned medium was collected for the L929 cytotoxicity bioassay and ELISA and replaced it with the fresh one every 24 hours.

2.4.3. TNFα cytotoxicity bioassay

TNFα cytotoxicity was analyzed by modifying the methods from Ming-Yuh Shiau (Shiau 2001). Briefly, 1.25×10^4 cells/well of L929 cells were seeded in a 96-well tissue culture plate and were cultured overnight at 37°C. The medium was replaced by 100 µl of the conditioned medium of Neuro2A transfected with pTNF/G3-HD-OEI which was collected at different time points as described above. After L929 cells were exposed in the conditioned medium with 1µg/ml actinomycin D for 24h, 10 u l of 5mg/ml MTT was added into each well of L929 cells. After 4 h of incubation at 37°C, the supernatants in the wells were carefully discarded and 100 µl/well of dimethylsulfoxide (DMSO) was added into the plate to dissolve Formazan crystals for 10min. The absorbance was read and recorded at 570 nm, with reference absorbance at 630nm in the microplate reader. Results were represented as the percentage of L929 cytotoxicity. The percentage of L929 cytotoxicity was calculated by following formula: cytotoxicity = (absorbance of 100% viable cell control wells –absorbance of test wells) divided absorbance of 100% viable cell control wells.

2.4.4. Endothelial permeability (transwell assay)

As method mentioned in other papers (BRETT 1989; Nooteboom 2002), we seeded PEC at 20,000 per well in the inner chamber of the transwell (coated with collagen A), and waited about 72 hours for the confluence of cells. Then, the medium in the inner chamber was exchanged for medium containing TNF protein (1ng/ml) or conditioned medium from Neuro2A cells transfected with G3-HD-OEI/pTNF polyplexes or G3-HD-OEI/pLuc polyplexes (as control), together with 1µg/ml actinomycin D. After incubation for another 24 hours, FITC-dextran (MW=40,000, Sigma) was added to a final concentration of 1mg/ml in the inner chamber. 50 min later, the medium in both chambers were collected and the fluorescence of dextran were measured in a Cary Eclipse fluorescence spectrometer (Lexington, USA) at Ex=490nm, Em=530nm and compared with a standard curve prepared with known concentration of

FITC-dextran diluted in medium. The percent of transfer was calculated as amount of FITC-dextran in outer chamber divide amount of total dextran applied in the inner chamber.

2.5. In vivo studies

2.5.1. Evaluation of luciferase reporter gene expression after systemic application

On day 0 (8 days after subcutaneous inoculation of 1×10^6 Neuro2A tumor cells,, when tumors reached 5-8 mm in size), day 3 and day 6 respectively, pLuc/G3-HD-OEI polyplexes at c/p=1 (50µg DNA/ mouse) were intravenously injected into A/J mice bearing subcutaneous Neuro2A tumors. Bioluminescence imaging (BLI) was carried out under a CCD camera (Xenogen IVIS) 10 minutes after intraperitoneal injection of 100 µl luciferin (60 mg/ml) every 24 hours. And luciferase activity was quantified in the tumor area by Bioluminescence imaging (BLI) and expressed as photons/second/region of interest (ROI) (n=3 + stddev). Mice were kept anaesthetised by inhalation of isoflurane (Forene, Abbott, Wiebaden-Delkenheim, Germany) by a gas manifold with a flow rate of 2% during live Bioluminescence imaging (BLI).

Two days after last polyplexes injection, mice were sacrificed and organs were resected and homogenized with homogenizer (Ultra-turrax 8, Germany) in 1× cell lysis buffer (Promega, Mannheim, Germany) The bioluminescence in supernatant of organs lysate was measured in a tube luminometer (Lumat LB 9507, Berthold Technologies, Bad Wildbad, Germany) with an integration time of 30 seconds following a 2 seconds lag time after the injection of luciferase assay reagent (LAR; 20mM glycylglycine, 1 mM MgCl2, 0,1mM EDTA, 3mM DTT (Sigma-Aldrich), 0,55mM ATP (Roche, Mannheim, Germany), 0,3mM CoA (Sigma-Aldrich), 0,5 mM dluciferin (Promega), pH 8,5).

2.5.2. Assessment of Caelyx uptake and in tumor and tissue distribution

Mice were injected intravenously with pTNF/G3-HD-OEI polyplexes or

pLuc/G3-HD-OEI polyplexes at c/p=1. 48 hours after polyplexes treatment, Caelyx (2 mg/ml) was applied intravenously at a dose of 100 µg/ mouse (diluted in 250 µl PBS buffer). 24 hours after Caelyx application, at which time maximal tumor localization by Stealth liposomes is expected (van der Veen 1998; HAGEN 2000), mice were sacrificed and the tumors and other organs excised and weighted. Tumors were analyzed for Caelyx and its fluorescent metabolites as described (Mayer 1989; HAGEN 2000). Briefly, after incubation of tissue in acidified isopropanol (0.075 N HCl in 90% isopropanol) for 24 hours at 4°C, tumors and indicated organs were homogenized (using an IKA Homogenizer), centrifuged for 30 min at 4000 rpm and supernatants were harvested. Samples were measured in a Cary Eclipse fluorescence spectrometer (Lexington, USA) at excitation 480 nm and emission 590 nm and compared with a standard curve prepared with known concentration of Caelyx diluted in acidified isopropanol.

2.5.3. Evaluation of Caelyx-DiR accumulation by living image

Caelyx liposomes were labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindotricarbocyanine iodide (DiR) by incubation with 0.2 mol% of the lipophilic tracer DiR (Molecular Probes) for 15min at room temperature (Seynhaeve 2007). Mice were injected intravenously with pTNF/G3-HD-OEI polyplexes or pLuc/G3-HD-OEI polyplexes at c/p=1. 48 hours after polyplexes treatment, Caelyx-DiR was applied i.v. at a dose of 5mg/kg. From immediately after injection until 15 minutes after application, mice were imaged in the IVIS imager with aCCD camera using the ICG filter set (Xenogen Ivis), and the signal of fluorescence in tumor tissue was measured. At 24 hours after treatment, mice were imaged again. Mice were kept anaesthetised by inhalation of isoflurane (Forene, Abbott, Wiebaden-Delkenheim, Germany) by a gas manifold with a flow rate of 2% during live imaging. At 72 hours after treatment, mice were sacrificed, organs were resected and imaged in vitro.

2.5.4. Application of pTNF/G3-HD-OEI polyplexes in the subcutaneous murine neuroblastoma model

On day 0 (8 days after 1×10^6 Neuro2A tumor cells suspending in 100 µl PBS subcutaneous inoculation, when tumors reached 5-8 mm in size), day 3 and day 6 respectively, pTNF/G3-HD-OEI polyplexes or pLuc/G3-HD-OEI polyplexes at c/p=1 (prepared as above described, 50µg DNA/ mouse) were intravenously injected every third days into A/J mice (n=6) bearing subcutaneous Neuro2A tumors. The tumor size and body weight of the animals were monitored. Differences in tumor growth were statistically analyzed by T-TEST.

2.5.5. Application of pTNF/G3-HD-OEI polyplexes and Caelyx in the subcutaneous murine neuroblastoma model

At 10 days after neuroblastoma subcutaneous inoculated, A/J mice (n=12) were intravenously injected with pTNF /G3-HD-OEI polyplexes or pLuc/G3-HD-OEI polyplexes every forth days for three cycles, and every second days after polyplexes application, Caelyx was applied at a dose of 1.5mg/kg (30 µg diluted in 250 µl PBS per mouse). The tumor size and body weight were monitored every two days. Differences in tumor growth were statistically analyzed. In the retreated (or boost) model, an additional treatment cycle 6 days after last treatment was conducted with 7 out total 11 mice in the group, and the tumor size was monitored continuously.

2.5.6. Application of pTNF/G3-HD-OEI polyplexes and Caelyx in the metastatic murine neuroblastoma model

At day 6 after intravenous inoculation of 1×10^6 Neuro2A lenti Luc cells in 200 µl PBS, mice were intravenously injected with pTNF/G3-HD-OEI polyplexes or pdeTNF/G3-HD-OEI polyplexes (plasmid without promoter, used as control) every forth days for three times totally, and every second days after polyplexes application, Caelyx was applied at 1.5mg/kg (n=10). Bioluminescence imaging

(BLI) was carried out (ventral, dorsal and lateral side of mice) after intraperitoneal injection of luciferin. Mice were kept anaesthetised by inhalation of isoflurane (Forene, Abbott, Wiebaden-Delkenheim., Germany) by a gas manifold with a flow rate of 2% during live Bioluminescence imaging (BLI). Luciferase activity was quantified in the tumor area by BLI and expressed as photons/second/region of interest (ROI). At 6 days after last treatment, mice were sacrificed, and tumor metastases in the body cavity were observed and pictures taken by camera. Bowels (including heart, lung, liver, spleen, kidney, small and large intestine) were taken out and weighted, and the weight of tumors was calculated by deducting the average weight of visceral of mice (n=3+) without tumor cell inoculation but with the same treatment of polyplexes and Caelyx. The organs and tumor tissues of mice were either fixed in formalin for HE staining or stored in Tissue Freezing Medium at -80 °C for further cryosectioning. The experiment was performed twice independently.

2.5.7. Establishment of the LS 174t lenti Luc cell line in an intrasplenic liver metastasis model

The procedure has been carried out by Katarina Farklasova (veterinarian) and is described in detail in her PhD thesis. In brief, NMRI-nude mice (n = 34) were injected with $7{,}5x10^5$ LS174t lenti Luc cells into the spleen. For tumor injection the mice were anaesthetized by inhalation of isoflurane in oxygen (2.5% (v/v)) at a flow of 1 liter/min. Bepanthene® was put on the eyes to protect them. Rimadyl® (5mg/kg) was injected subcutaneously prior to tumor injection. The mouse was positioned on the right lateral side. The skin was first swabbed with ethanol, after it evaporated, the skin was swabbed with braunol®. The skin was raised and a vertical cut (ca. 0,5cm) throw the skin caudal to the costal arch was made. The muscle-layer and the peritoneum were raised and another vertical cut (ca. 0,5cm) was made to open the abdominal cavity. The lower part of the spleen was partially displaced out of the abdomen and the cell suspension was slowly injected using a 27G needle. Using a cotton swab, gentle pressure was

made on the injection site to prevent cell reflux throw the injection channel and bleeding. The spleen was placed back to the abdomen. The peritoneum and the muscle were sutured in one layer and the skin in another layer using Monosyn® 5/0. The mice were separately put into cages with gloves filled with hot water to keep them warm and to recover undisturbed from the other mice from surgery. 24 and 48 hours after tumor injection the mice were injected subcutaneously 5mg/kg Rimadyl® (Katarina Farkasova 's phD Thesis 2011).

2.5.8. Application of pTNF/ LPEI-PEG-GE11 polyplexes and Caelyx in the metastatic colon adenocarcinoma model

At 3 days after the inoculation of the cells, mice were intravenously injected with pTNF /LPEI-PEG-GE11I polyplexes or pdeTNF/LPEI-PEG-GE11 polyplexes (plasmid without promoter, used as control) every third day for three times totally, and every second days after polyplexes application, Caelyx was applied at 1.5mg/kg. Bioluminescence imaging (BLI) was carried out from abdominal side of mice after intraperitoneal injection of luciferin. Luciferase activity was quantified in the tumor area by BLI and expressed as photons/second/region of interest (ROI) (n=11). Mice were kept anaesthetised by inhalation of isoflurane (Forene, Abbott, Wiebaden-Delkenheim, Germany) by a gas manifold with a flow rate of 2% during live Bioluminescence imaging (BLI). At 12 days after cells inoculation, mice were sacrificed, and tumor metastases in the viscral were observed and pictures were taken by camera. The livers and spleens of mice were taken out and weighted, a liver lobe fixed in formalin for HE staining and the residual liver and spleen were frozen at -80°C. Frozen livers were grinded to powder with liquid nitrogen which were then lysed with1× cell lysis reagent (Promega, Mannheim, Germany). The concentration of total protein in supernatant of liver lysate was measured with the kit of Pierce BCA Protein Assay (Thermo Scientific) and the bioluminescence signal was measured in a tube luminometer (Lumat LB 9507, Berthold Technologies, Bad Wildbad, Germany) with an integration time of 30 seconds following a 2 seconds lag time

after the injection of luciferase assay reagent (LAR; 20mM glycylglycine, 1 mM MgCl2, 0,1mM EDTA, 3mM DTT (Sigma-Aldrich), 0,55mM ATP (Roche, Mannheim, Germany), 0,3mM CoA (Sigma-Aldrich), 0,5 mM Dluciferin (Promega), pH 8,5). And the results expressed as relative light units (RLU) per µg total protein.

2.6. Evaluation of TNF gene expression in tumors and livers by qPCR

The high pure RNA Tissue Kit (Roche, Switzerland) was used for the isolation of total RNA from tumor and liver. cDNA was obtained by using the high fidelity transcriptor cDNA synthesis kit. The qPCR assay was handled using light cycler 480 probes Master buffer in the Roche Light Cycler 480 qPCR machine (Basel, Switzerland). The expression of TNF and of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in tumor and liver was determined in parallel from the same reverse transcriptase reaction, each done in duplicate per sample. The primer used for TNF gene: forward primer: GCTTCCAGAACAGCAGACG; reverse primer: AGGCAGAACAGGGTGGTG; the primer for the GAPDH gene:

forword primer:AGCTTGTCATCAACGGGAAG; reverse primer:TTTGATGTTAGTGGGGTCTCG. The untreated sample was used as calibrator cDNA. Since the murine TNF cDNA sequence of pTNF was synthesized for optimal codon usage in mice and hence different to the wild-type TNF sequence is was possible to determine mRNA levels of transgenic TNF in tumor and liver by qPCR. By blasting the standard TNF with our codon optimal TNF sequences, the least similarity places were found out and designed the primers, then the primers were evaluated by normal PCR to prove that they can only amplify transgenic TNF in pTNF/G3-HD-OEI polyplexes treated samples, whereas wild type TNF is not amplified. The primer of transgenic TNF gene: forward primer: AAGCTCTAGCCAGAATAGCTCC; reverse primer: ACAGCCACTCCAGCTGCT;

Here we used liver cDNA as calibrator cDNA. At 48 hours after

pTNF/G3-HD-OEI polyplexes were intravenously injected into A/J mice bearing subcutaneous Neuro2A tumors, mice (n=3+ stddev) were sacrificed and tumor and liver tissue were collected. TNF expression in tumor tissue was determined at mRNA level by RT-qPCR and was normalized to the expression of the GAPDH housekeeping gene. All samples were done in duplicates. Relative transgenic TNF mRNA in tumor tissue of pTNF treated group was also determined at mRNA level by RT-qPCR and was normalized to the expression of TNF mRNA in liver tissue.

2.7. Immunohistological stain

2.7.1. Immunostaining with anti-CD31

One week after Neuro2A cell inoculation, mice were treated with pTNF/G3-HD-OEI polyplexes or pLuc/G3-HD-OEI polyplexes every third day for three times totally, at the third days after the last injection mice were sacrificed and tumors were resected. Tumor cryosections (5µm) on microslides were fixed in cold methanol for 5 min, and were blocked with 1% BSA for 15 minutes, with 10% goat serum for 15 minutes, and subsequently incubated with rat anti murine CD31 mAb (CD31: endothelial marker) (eBioscience San Diego, CA) for 2 hours, thereafter washed twice with 1%BSA/PBS, the slide was stained with Alexa Fluor 647 goat anti-rat IgG for 1hour and also washed twice with PBS. Then the slides were incubated with 0.5µg/ml DAPI for 10 min to stain nuclei with DAPI (blue), and then washed three times with water and mounted with Vectashield mounting media (Burlingame, USA) after drying under dark. The slides were evaluated with a Zeiss Axiovert 200 microscope (Carl Zeiss, Jena, Germany).

2.7.2. Immunostaining with anti-TNF

At 6 days after intravenous inoculation of Neuro2A lenti Luc cells, mice were treated with pTNF/G3-HD-OEI polyplexes or pdeTNF/G3-HD-OEI polyplexes every forth days for three times totally, and every second days after polyplexes

application Caelyx was applied at a dose of 1.5mg/kg. At 22 days after tumor cell inoculation, mice were sacrificed and dissected, the tumor tissue collected and embedded in Sakura Tissue Tek in Cryomolds and stored at -80°C. Tumor cryosections (5µm) on microslides were fixed in cold methanol for 5 min, and were blocked with 1% BSA for 15 minutes, with 10% goat serum for 15 minutes, and subsequently incubated with rabbit anti-mouse TNF- α antibody (abcam ab9739) for 2 hours and then washed twice with 1%BSA/PBS. The slides were incubated with Alexa Fluor 488 labeled goat-anti- rabbit secondary antibody (invitrogen) for 1 hour followed by two washing steps with PBS. Then the slides were incubated with 0.5µg/ml DAPI for 10 min to counterstained nuclei and then washed three times with water. After mounting with Vectashield mounting media (Burlingame, USA), the slides were evaluated under Zeiss Axiovert 200 microscope (Carl Zeiss, Jena, Germany) with 20x objective magnification.

2.7.3. Preparation cryosection slices

For the cryosection of tissue in the Caelyx treatment experiment, 48h after a single pTNF/ G3-HD-OEI polyplex in Neuroblastoma bearing mice, 5 mg/kg Caelyx were injected intravenously. 24 hours later, tumor and liver tissues were collected and the cryosections (5µm) on microslides were fixed in cold methanol for 5 min and mounted with Vectasheld mounting media after drying under dark and observed under Zeiss Axiovert 200 microscope (Carl Zeiss, Jena, Germany) with 20x objective magnification.

2.7.4. HE Staining

After sacrificing the mice, the organs were collected and fixed in formalin solution (4%) overnight. After two hours of washing in running water to remove formalin residues, the organs were stored in ethanol (70%). Then, the tissues were handled with tissue processor machine and slices (5µm of thickness) were cut with a LEICA RM2265 rotary microtome and transferred to glass-slides. For the staining, slides were de-waxed in two identical xylole

solutions (>98%, Roth) for 15min each. This step was followed by an alcohol dilution series (ethanol: 100%, 96%, 70%), washing the slides once in each dilution for 5min. After a short washing step with purified water, the slides were treated in Mayers- Haemalaun (Merck) for 1 min to stain the nuclei, and washed in running water again for 10 minutes. For cytoplasm staining, the slides were covered in eosin (2%, Chroma) for 8 min. Again, the slides were washed in running water for 3 min and then treated with the alcohol dilution series in reversed order (70% for 3min, 96% for 3min, 100% for 5min). At last, the slides were dewatered by using the xylole solutions twice, 10 min each. After evaporation of solvent residues, the slices were sealed with Eukitt (O.Kindler GmbH&Co) and a glass-cover. Then, slides were evaluated with a Zeiss Axiovert 200 microscope (Carl Zeiss, Jena, Germany) with 10x or 20x objective magnification. The obtained pictures were processed, using Adobe Photoshop CS5 for adjusting color and contrast adjustments and adding a scale bar and merging single pictures into bigger ones.

3. Results

3.1. Evaluation of plasmid pTNF transgene expression in vitro

We used a novel plasmid vector with CpG free backbone and an optimized promoter-enhancer combination (human CMV enhancer and EF1α promoter), since as mention in section **1.7.**, Stephen C Hyde *et al* observed that CpG-free plasmids with the hCMV/EF1α combination confer reduced inflammation and increase the magnitude and duration of gene expression in vivo (Hyde 2004). To evaluate the expression efficiency of the TNF coding plasmid pTNF, the L929 Bioassay was applied. L929, a murine aneuploid fibrosarcoma cell line, is used to assay TNF-alpha and TNF-beta, which lyse cells sensitized by pretreatment with actinomycin or cycloheximide. Treatment with TNF initiates apoptosis and subsequent cell death. We transfected Neuro2A cells with pTNF /G3-HD-OEI polyplexes or control polyplexes. In addition, polyplexes were formed with the plasmid pGSmuTNF (Kircheis R 2002), which contains a CMV driven expression cassette and a standard, CpG rich backbone. Collected the supernatant every 24 hours and changed for fresh medium. 24 hours after adding the condition medium from treated Neuro2A to L929 cell, the cytotoxicity caused by TNF was detected by MTT method. We found that the novel plasmid vector with CpG free backbone and an optimized promoter-enhancer combination led to sustained and high TNF transgene expression in vitro on Neuro2A murine neuroblastoma cells. Secreted TNF induced high cytotoxicity on L929 indicator cells and its expression in Neuro2A cells lasted for at least one week (**fig. 1 A**). As further evidence, an ELISA assay was also performed to detect the concentration of secreted TNF in conditioned medium and lysate of transfected cells. Clearly, the novel plasmid vector with CpG free backbone and an optimized promoter-enhancer combination led to sustained and high TNF transgene expression in vitro on Neuro2A murine neuroblastoma cells with up to 600 pg protein/mL/24h peaking between 48h and 72h after transfection in condition medium (**fig. 1 B**) and high amounts of TNF were also found in pTNF polyplex transfected Neuro2A cells (**fig. 1 C**). These results suggest that the

novel plasmid vector with CpG free backbone and an optimized promoter-enhancer combination is definitely powerful at inducing sustained and high transgene expression being far more efficient when compared to the CMV driven plasmid pGSmuTNF.

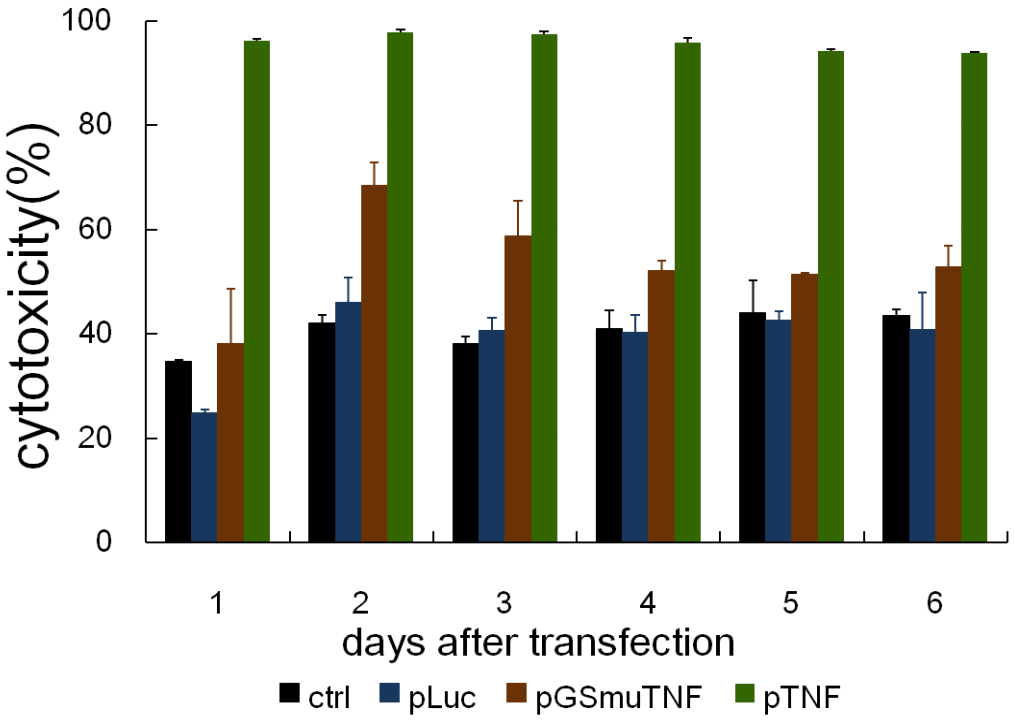


Fig. 1 A

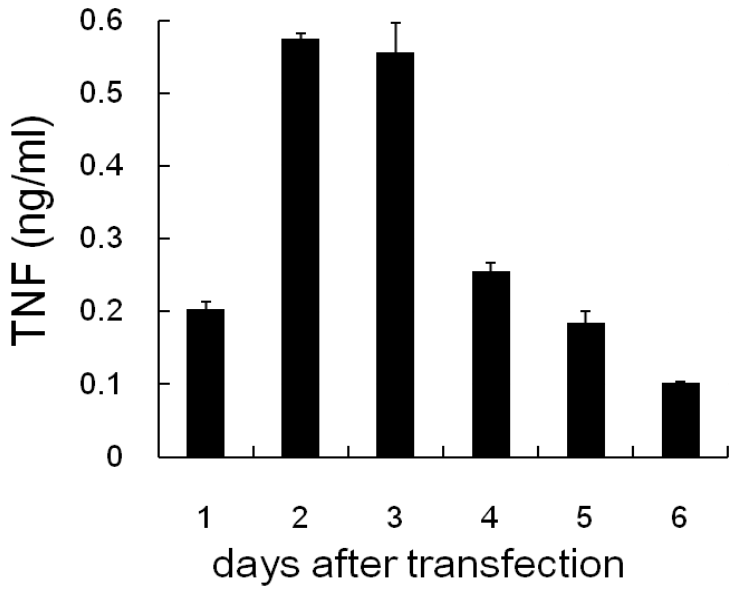


Fig. 1 B

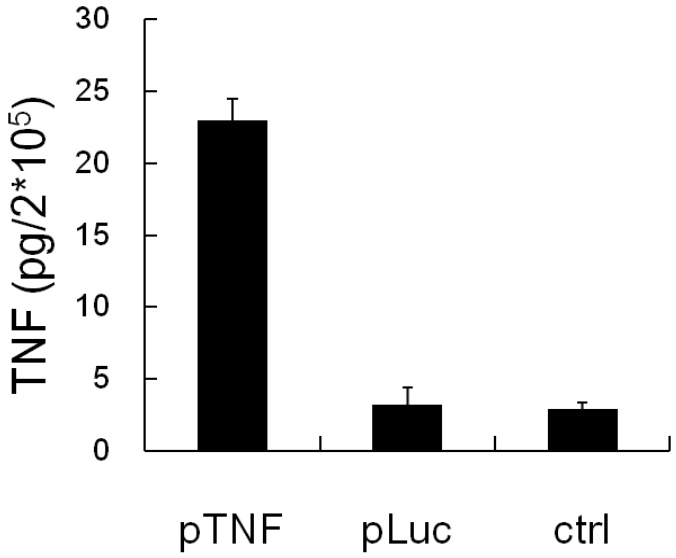


Fig. 1 C

Fig. 1: Evaluation plasmid pTNF transgene expression in vitro

A, Neuro2A cells were transfected with pTNF/G3-HD-OEI, pGSmuTNF/G3-HD-OEI or pLuc/G3-HD-OEI polyplexes respectively, and the supernatant were collected and changed freshly every 24h. Bioactivities of soluble TNF in the condition medium were detected by L929 indicator cell bioassay. **B**, **C**, the concentrations of TNF in both condition medium and lysate solution of transfected cells were measured by ELISA.

3.2. Evaluation the influence of pTNF plolyplexes on endothelial cell permeability in a transwell system

To find out the effect of pTNF polyplexes treatment on endothelial cell, a transwell experiment was performed. **Fig. 2 A** showed the schematic trans-well system. We found that secreted TNF in the conditioned medium from Neuro2A cells transfected with pTNF/ G3-HD-OEI polyplexes combined with actinomycin D increased endothelial cell permeability in a trans-well system, and was comparable with the effect induced by TNF protein at final concentration 1ng/ml (**fig. 2 B**), while there was no significant transfer of FITC- Dextran detected induced by the conditioned medium from Neuro2A cells transfected with pLuc/G3-HD-OEI polyplexes combined with actinomycin D. These results might due to the secreted TNF in the condition medium induced by pTNF polyplexes transfection leading to the apoptosis of PEC and/or enlargement of the cell gaps which facilitated the pass through of dextran-FITC. This is

consistent with other papers which declared that TNF induce the major destruction of the vascular bed (Nawroth 1986; Palladino 1987; Nawroth 1988; Clauss 1990; Gasparri 1999). However, when we transfected the cell lines Neuro2A, HuH7, U87MG, BT549, MBA-MD-231,MBA-MD-453 or L929 with pTNF/G3-HD-OEI or pLuc/G3-HD-OEI polyplexes and measured the cytotoxicity caused by secreted TNF directly, we found that TNF did not exert direct tumor cell toxicity on HUH7, Neuro2A, U87MG and MBA-MD-231 cells while moderate toxicity was found on MBA-MD-453 and BT549 cells (**fig. 2 C**). L929 murine fibroblasts were used as positive control, as significant reduction in cell viability is seen on L929 (the same as used in L929 bioassay in **fig. 1 A**).

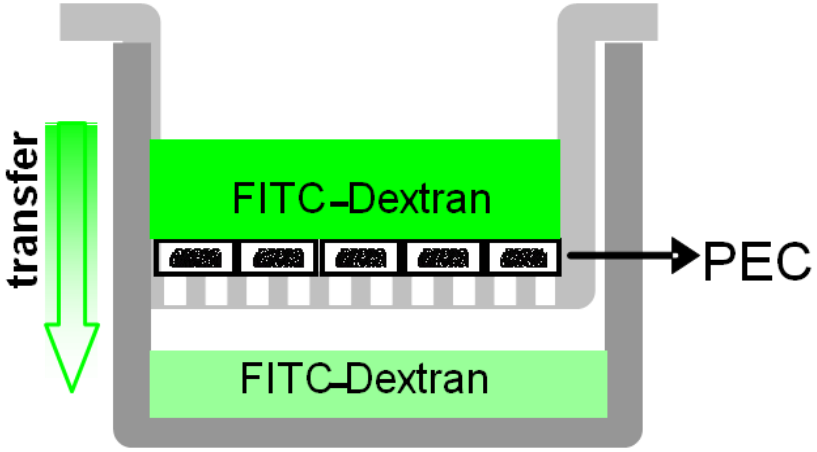


Fig. 2 A

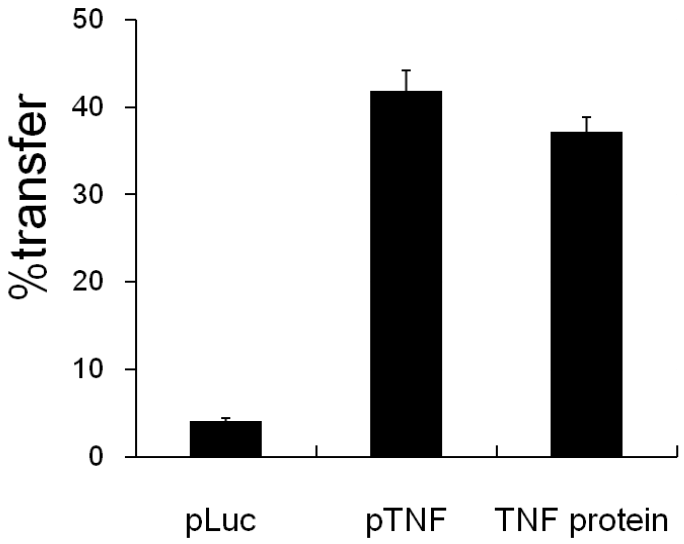


Fig. 2 B

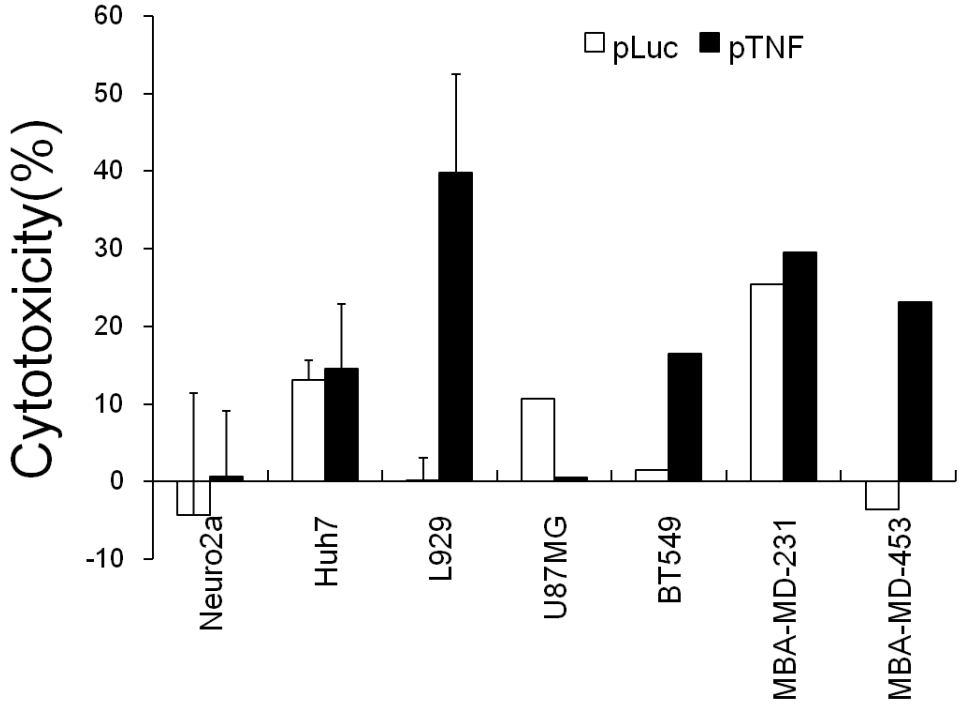


Fig. 2 C

Fig. 2: Evaluation the influerence of pTNF/G3-HD-OEI plolyplexes on endothelial cell permeability in a trans-well system

A, Schematic trans-well system. **B**, PEC were seeded at 20,000 per well in the inner chamber of the transwell (coated with collagen A), and grew for about 72 hours until confluence of cells. Then, the medium in the inner chamber was exchanged for medium containing TNF protein (1ng/ml) or conditioned medium from Neuro2A cells transfected with G3-HD-OEI/pTNF polyplexes or G3-HD-OEI/pLuc polyplexes (as control), together with 1µg/ml actinomycin D. FITC-dextran (MW=40,000, Sigma) was added to a final concentration of 1mg/ml in the inner chamber. 50 min later, the medium in both chambers was collected, the fluorescence of dextran measured in a Cary Eclipse fluorescence spectrometer (Lexington, USA) at Ex=490nm, Em=530nm and compared with a standard curve prepared with known concentration of FITC-dextran diluted in medium. The percent of transfer was calculated as amount of FITC-dextran in outer chamber divide amount of total dextran applied in the inner chamber. The percent of transfer was calculated as the percent of amount of FITC-dextran in outer chamber divided amount of total dextran applied. **C**, 24h after Neuro2A, HuH7, U87MG, BT549, MBA-MD-231, MBA-MD-453 or L929 cell lines were seeded, the cells were transfected with pTNF/G3-HD-OEI or pLuc/G3-HD-OEI polyplexes and 24h later the cytotoxicity was measured by MTT assay.

3.3. Evaluation TNF targeting expression plasmid pRGD-TNF or selective expression plasmid pTNF-miR143

As mention in section **1.2.4.**, the antitumor activity of TNF depends on indirect mechanisms associated with selective destruction of the tumor-associated

vascular bed, which provide the rationale for developing vasculature-targeting strategies aimed at increasing the therapeutic index of this cytokine. As mention in section **1.3.2**, several peptides such as CNGRC and CDCRGDCFC, containing Asn-Gly-Arg (NGR) or Arg-Gly-Asp (RGD) motifs, have been used for targeting chemotherapeutic drugs, proapoptotic peptides, and cytokines to tumors in experimental models (Arap 1998; Zarovni 2004). Thus, similar as Natasa Zarovni and some other scientists did (Zarovni Apr. 2004), here we try to employ the RGD motif in our pTNF plasmid to target transgenic TNF as a RGD-TNF fusion to the endothelium. To construct the pRGD-TNF plasmids, sequence encoding ACDCRGDCFCG was inserted by polymerase chain reaction (PCR) upstream of the mature TNF-coding region. To evaluate the expression efficiency of the pRGD-TNF plasmid, L929 Bioassay was performed. Compared with pTNF plasmid, the plasmid containing vasculature-targeting peptide RGD gene fused with mature TNF gene, pRGD-TNF, induced expression and secretion of RGD-TNF into the medium which led to higher cytotoxicity on L929 indicator cell (**fig. 3 A**). This could be because the transgenic RGD peptide targeted the intergrinα5 on the surface of L929 cell, which further promoted internalization and subsequently induced higher cytotoxicity on L929 indicator cells. In order to investigate the therapeutic potential of pRGD-TNF and compare with pTNF plasmid in vivo, we applied the pRGD-TNF/ G3-HD-OEI or pTNF/G3-HD-OEI polyplexes in the murine neuroblastoma model and measured the tumor size continuously. We found that three rounds pRGD-TNF polyplexes intravenous application led to inhibition of tumor growth in mice; however, the inhibition effect was only moderately enhanced when compared to pTNF polyplex treatment (**fig. 3 B**).

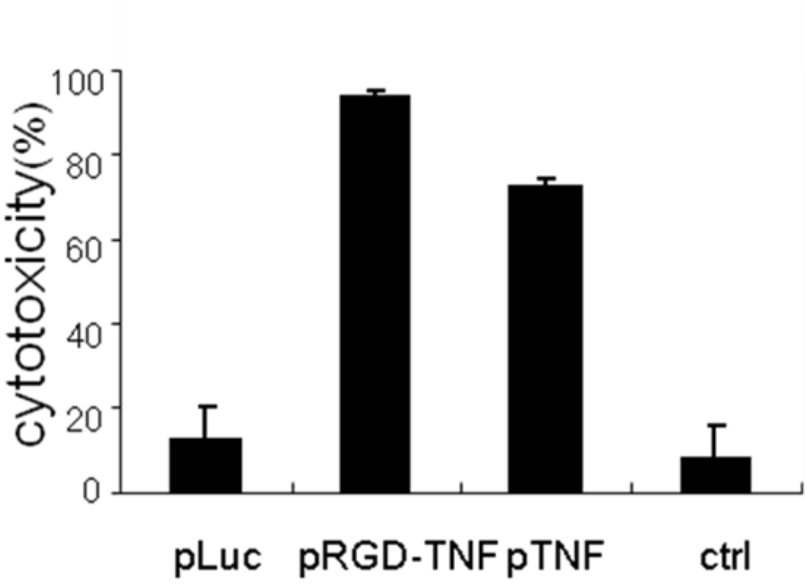


Fig. 3 A

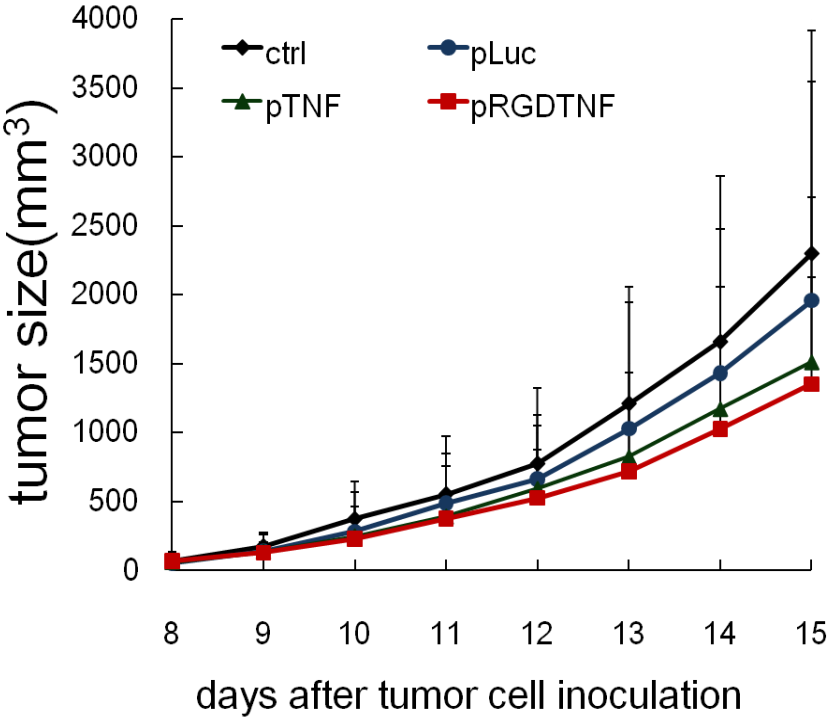


Fig. 3 B

Fig. 3 A, B: Evaluation of TNF targeting expression plasmid pRGD-TNF

A, 24 hours after Neuro2A cells were seeded, the cells were transfected with pTNF/G3-HD-OEI, pRGD-TNF/G3-HD-OEI or pLuc/G3-HD-OEI polyplexes and 24h later, the condition medium were collected. Bioactivities of soluble TNF in the condition medium were detected by L929 indicator cell bioassay. **B**, Eight days after Neuro 2A cell inoculation, mice were treated with pTNF/G3-HD-OEI, pRGD-TNF/G3-HD-OEI or pLuc/G3-HD-OEI polyplexes every third day, and the tumor size was measured coutinuously (n= 6).

As mention **in section 1.3.3.**, in view of the natural function of microRNA and the specific abundance of miR-143 in different tissues, here pTNF-mir143 was constructed by inserting miR-143 targeting sequence into plasmid pTNF. To detect the influence of miR-143 on the TNF transgene expression in different cell line, we transfected PSMC (Primary porcine smooth muscle cells, high in miR-143, investigated by Florian Kopp and Maria Schnödt, unpublished results) and PC3 cells (human prostate carcinoma, low in miR-143, investigated by Florian Kopp and Maria Schnödt, unpublished results) with pTNF-miR143/G3-HD-OEI and pTNF /G3-HD-OEI polyplexes as control. Secreted TNF in condition medium were detected by L929 bioassay. The results of the bioassay proved that transgene expression of TNF induced by the plasmid containing miR-143 targeting sequence (pTNF-miR-143) is significantly down- regulated in PSMC while no influence is found in PC3 cells (**fig. 3 C**). However, when we transfected another cancer cell line, Neuro2A (murine neuroblastoma), with pTNF-miR143 /G3-HD-OEI polyplexes, as the result of bioassay shown, the expression of TNF induced by the pTNF-miR143 /G3-HD-OEI polyplexes was also decreased (**fig. 3 D**), which might suggest that not all tumor cell line exhibit a low miR-143 level.

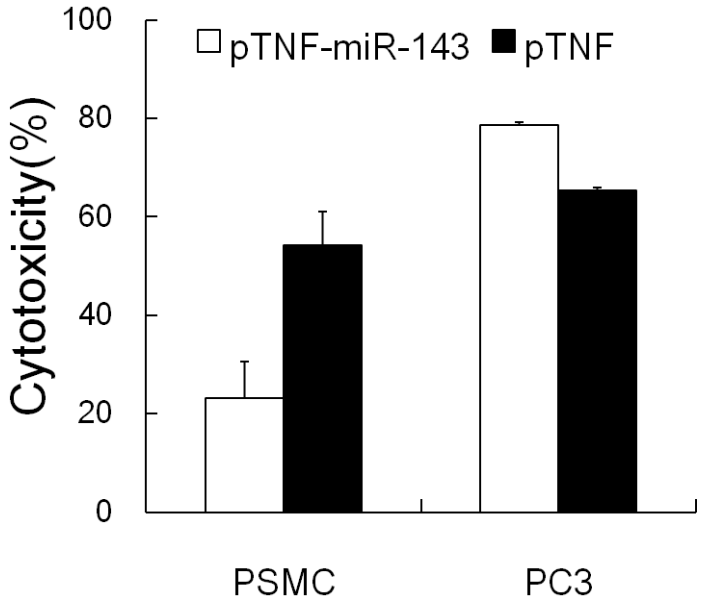


Fig. 3 C

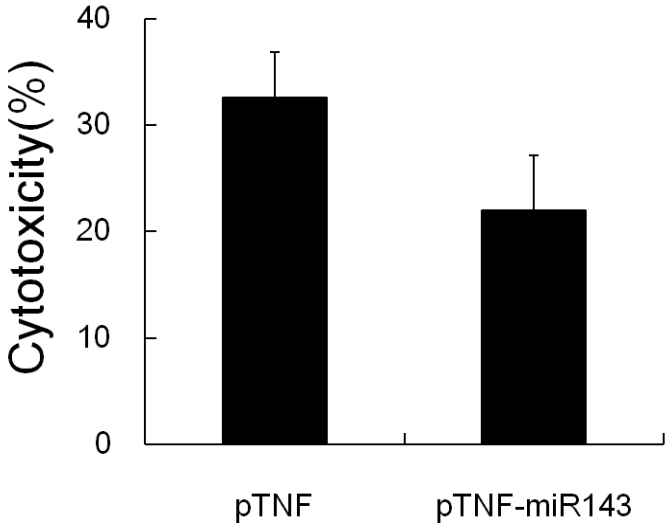


Fig. 3 D

Fig. 3 C,D: Evaluation TNF selective expression plasmid pTNF-miR143
24 hours after PSMC or PC3 **(C)** and Neuro2A cells (**D**) were seeded, the cells were transfected with pTNF/G3-HD-OEI or pTNF-mir143/G3-HD-OEI polyplexes and 24h later the condition medium was collected. Bioactivities of soluble TNF in the condition medium were detected by L929 indicator cell bioassay.

3.4. Evaluation of G3-HD-OEI as gene carrier of luciferase reporter gene in vivo after systemic application

As mention in section **1.4.3.**, several grafted polypropylenimine dendrimers were synthesized by Dr. Verena Russ in our lab through modifying either polypropylenimine (PPI) dendrimer generation 2 (G2) or generation 3 (G3) via 1.6-hexandioldiacrylate with oligoamines, like branched oligoethylenimine 800Da (OEI) or PPI dendrimer G2 (Russ Dec 2008). In vitro results showed that transfection levels obtained with OEI-grafted dendrimers were the highest, being similar or even higher as compared to standard polyethylenimines (linear and branched). Here, these polymers were evaluated for transfecting subcutaneous Neuro2A tumors after repeated systemic polyplex application with pLuc (luciferase encoding plasmid) containing polyplexes. After tail vein injections of G3-HD-OEI/pLuc polyplexes into mice bearing subcutaneous Neuro2A tumors, BLI imaging was performed every 24h and showed that the tumor targeting transgenic expression of luciferase could be detected for at

least one week in G3-HD-OEI/pLuc polyplexes treated mice (**fig. 4 A**). As quantification results of the photon strength also showed, transgenic activity of polyplexes in tumor tissue remained high for at least one week (**fig. 4 B**). When organs of mice were resected and homogenized, bioluminescence was measured in the supernatant of organs lysate. The same trends were found, that the transgenic expression in tumor tissue is significant higher than in other organs (**fig. 4 C**). This again shows that G3-HD-OEI, a non viral, biodegradable gene carrier (G3-HD-OEI) exhibits intrinsic tumor affinity.

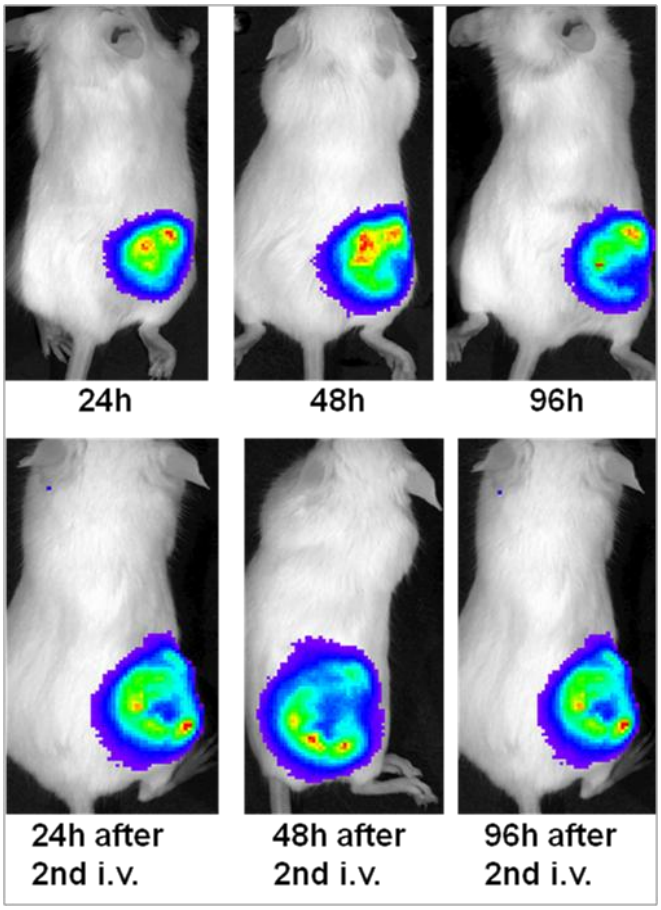


Fig. 4 A

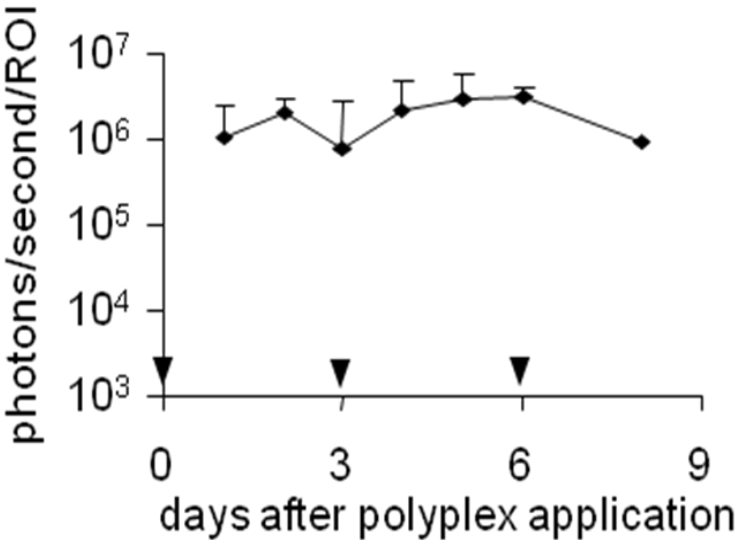


Fig. 4 B

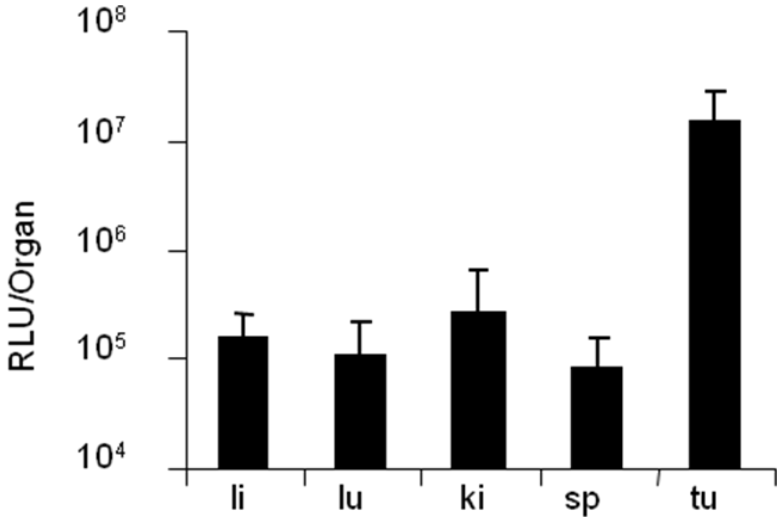


Fig. 4 C

Fig. 4: Evaluation of G3-HD-OEI as gene carrier of luciferase reporter gene in vivo after systemic application

pLuc/G3-HD-OEI polyplexes were intravenously injected every third days into A/J mice bearing subcutaneous Neuro2A tumors. **A**, Bioluminescence imaging (BLI) was carried out in the IVIS imager with a CCD camera (Xenogen Ivis) after intraperitoneal injection of luciferin (6mg/ mouse). **B**, Luciferase activity was quantified in the tumor area by BLI and expressed as photons/second/region of interest (ROI) (n=3 + stddev), arrowheads indicate polyplex applications. **C**, The luciferase activity in different organs was quantified. li, abbrevation for liver, lu for lung, ki for kidney, sp for spleen, tu for tumor .

3.5. Evaluation of TNF gene expression in tumors and livers by qPCR

To detect the transgene expression of pTNF plasmid in vivo, the transgenic

TNF expression induced by pTNF/G3-HD-OEI polyplexes in vivo at mRNA level was determined by qPCR and normalized to the expression of the GAPDH housekeeping gene. The relative total TNF mRNA level (both, endogeneous and transgenic TNF mRNA) in tumor and liver of mice from pTNF/G3-HD-OEI polyplexes treated group were significant higher than in pLuc/G3-HD-OEI polyplexes treated mice and untreated mice (**fig. 5 A**), and the TNF mRNA level in pLuc/G3-HD-OEI polyplexes treated mice was higher than in untreated mice (**fig. 5 A**). A higher level of TNF mRNA in tumor than in liver was found which indicates again that G3-HD-OEI polypelxes exhibits intrinsic tumor affinity, as shown in **fig. 4**. Utilizing properly designed primers, it was possible to distinguish between exogeneous and endogeneous TNF mRNA. By blasting the standard TNF with our codon optimal TNF sequences, the least similarity places were found out and designed the primers which were then evaluated by normal PCR to prove that they can only amplify transgenic TNF in pTNF/G3-HD-OEI polyplexes treated samples, whereas wild type TNF is not amplified. In the pTNF/G3-HD-OEI polyplexes treated group, the relative transgenic (or exogenous) TNF mRNA in tumor tissue is about 6 times higher than in the liver tissue (**fig. 5 B**). This might verify of success transgene expression of pTNF plasmid, as pTNF/G3-HD-OEI polyplexes application leads to efficient TNF transgene expression, at least, at the RNA level.

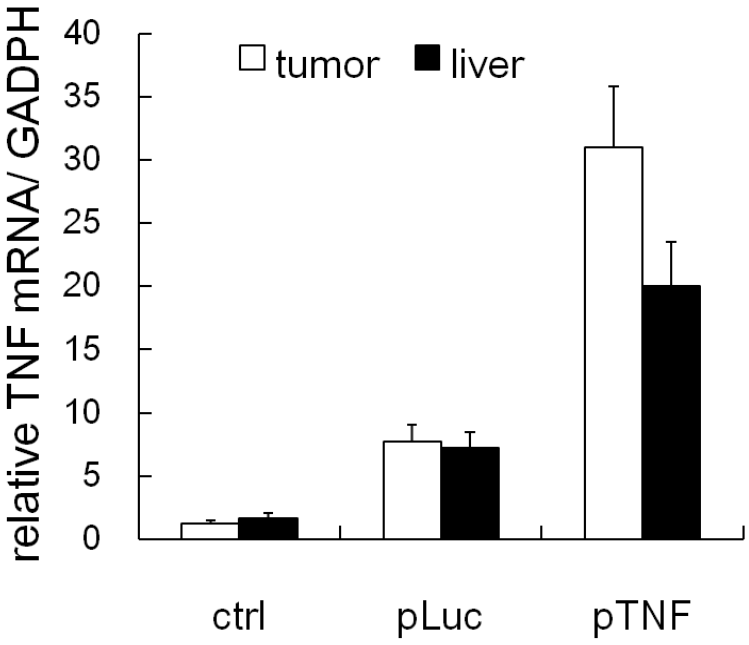


Fig. 5 A

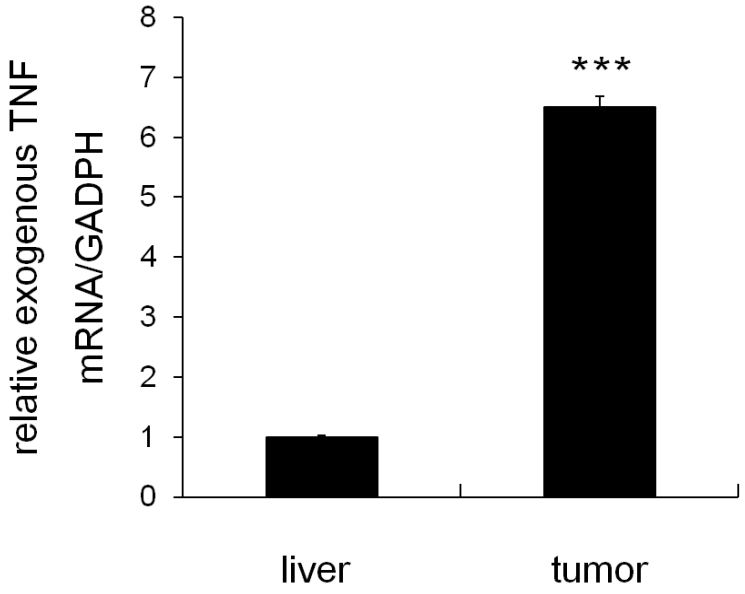


Fig. 5 B

Fig. 5: Detection of pTNF/G3-HD-OEI polyplexes transgene expression in vivo by qPCR

At 48 hours after pTNF/G3-HD-OEI polyplexes were intravenously injected into A/J mice bearing subcutaneous Neuro2A tumors, mice (n=3+ stddev) were sacrificed and tumor and liver tissue were collected. TNF expression in tumor tissue was determined at mRNA level by RT-qPCR and was normalized to the expression of the GAPDH housekeeping gene. All samples were done in duplicates. **A**, Relative TNF mRNA in tumor tissue and liver tissue; **B**, Relative transgenic TNF mRNA in tumor tissue of pTNF treated group was also determined

at mRNA level by RT-qPCR and was normalized to the expression of TNF mRNA in liver tissue.

3.6. Evaluation the influence of pTNF/G3-HD-OEI polyplexes on endothelial cell density in tumors

To evaluate the influence of TNF on endothelial cell in vivo, eight days after subcutaneous Neuro2A cell inoculation, mice were treated with pTNF/G3-HD-OEI or pLuc /G3-HD-OEI polyplexes every third day for three times totally, and at 17 days after Neuro2A tumor cell inoculation mice were sacrificed, tumor tissue resected and cryosection were prepared. The tumor cryosections were stained with a fluorescently labeled antibody directed against murine endothelial cell marker anti CD31 (also known as PECAM-1, Platelet Endothelial Cell Adhesion Molecule-1, a membrane protein constitutively expresses on the surface of endothelial cells). The staining results show the decreased CD31 expression in the tumor tissues from pTNF/G3-HD-OEI polyplexes treated mice (**fig. 6**) and hence a decreased vessel density in tumor microenviroument caused by the transgenic TNF after pTNF/G3-HD-OEI polyplexes application.

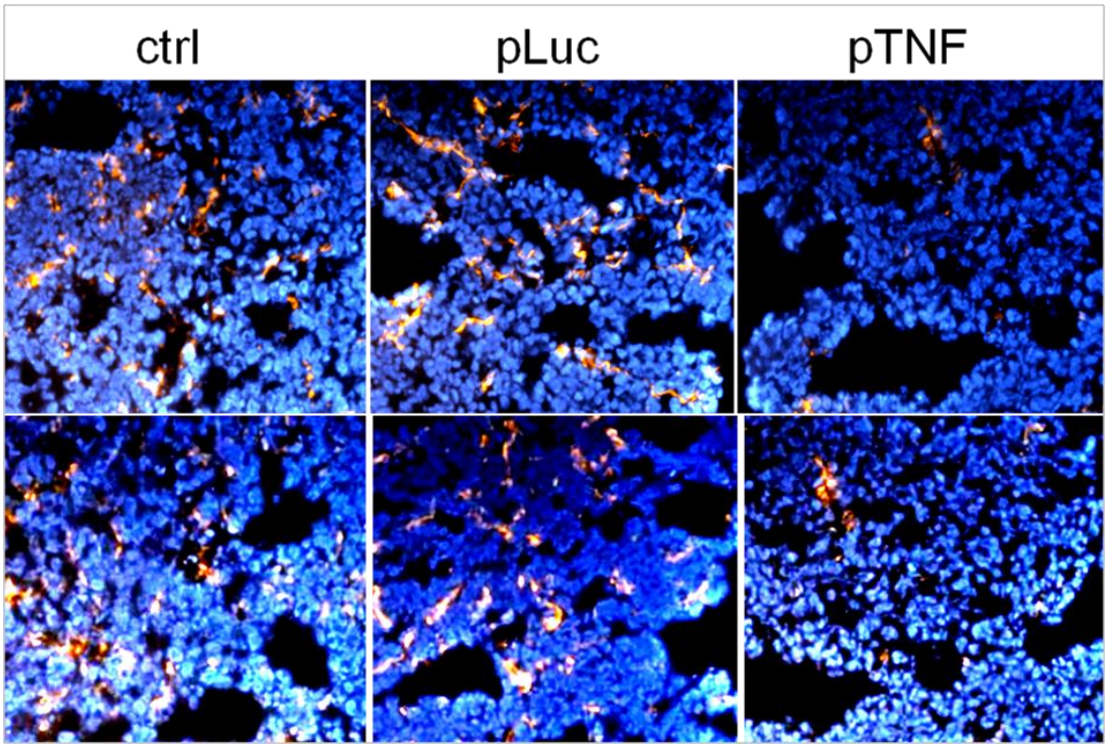


Fig. 6

Fig. 6: Evaluation the influence of pTNF/G3-HD-OEI polyplexes on endothelial cell density in tumors

Eight days after subcutaneous Neuro2A cell inoculation, mice were treated with pTNF/G3-HD-OEI or pLuc /G3-HD-OEI polyplexes every third day for three times totally. At 17 days after setting, Neuro2A tumors were resected, tumor cryosections prepared and stained with a fluorescently labeled antibody directed against murine endothelial cell marker anti CD31 (orange), nuclei were counterstained with DAPI (blue). Slices were observed under Zeiss Axiovert 200 microscope with 10× objective magnification.

3.7. Assessment of Caelyx® uptake and in tumor and tissue distribution and evaluation of Caelyx®-DiR accumulation by living image

To assess how pTNF polyplexes treatment affect the accumulation of liposomal doxorubicine Caelyx® in tumor tissue, A/J mice bearing subcutaneous, syngeneic Neuro2A tumors were treated with pTNF polyplexes based on the biodegradable polymer G3-HD-OEI, and HuH7 hepatoma xenografts in SCID mice received pTNF polyplexes based on linear polyethylenimine carrying a peptide (GE11) selectively binding to the epidermal growth factor receptor, which is highly overexpressed in HuH7. After pretreating mice with pTNF polyplexes, Caelyx® was applied intravenously. Tumor and liver tissues were collected 24h thereafter and cryosections were prepared and observed under microscope. From the results of cryosection slices, we conclude that pTNF polyplexes application enhances accumulation of liposomal doxorubicine Caelyx in tumor tissue and led to nuclear localization of doxorubicine in tumor tissue in Neuroblastoma using G3-HD-OEI as gene carrier (**fig. 7 A**). The quantification results of Caelyx® concentration also showed that pTNF polyplexes pretreatment significantly enhanced the accumulation of Caelyx® in tumor tissue in Neuroblastoma using G3-HD-OEI as gene carrier, while the same trends were found in HuH7 hepatoma xenografts model using LPEI-PEG-GE11 (**fig. 7 B**) or G2-HD-OEI (**fig. 7 B**) as gene carrier. The enhancement was more significant when LPEI-PEG-GE11 was employed as gene carrier. This might due to the fact that LPEI-PEG-GE11 is based on linear polyethylenimine carrying a peptide (GE11) selectively binding to the epidermal growth factor receptor (EGFR), which is over-expression in HUH7 cells, thus

leading to pronounced endocytosis of polyplexes and subsequently high transgene expression efficiency. With the help of BLI, significantly enhanced tumor accumulation of Caelyx® fluorescently labeled with the lipid DiR emitting in the near infrared at 15 minutes (**fig. 7 C**) or 24 hours (**fig. 7 C**) after injection was observed. The fluorescence signals were quantified and results are showed in **fig. 7 D**. Further, when the organs were collected and imaged, significant enhancement of Caelyx®-DiR in tumor tissue and a certain decrease in liver tissue in pTNF/ LPEI-PEG-GE11 polyplexes treated mice were found (**fig. 7 E**). All these results suggest a TNF mediated increase of tumor blood vessel permeability augmenting the accumulation of liposomal chemotherapeutics.

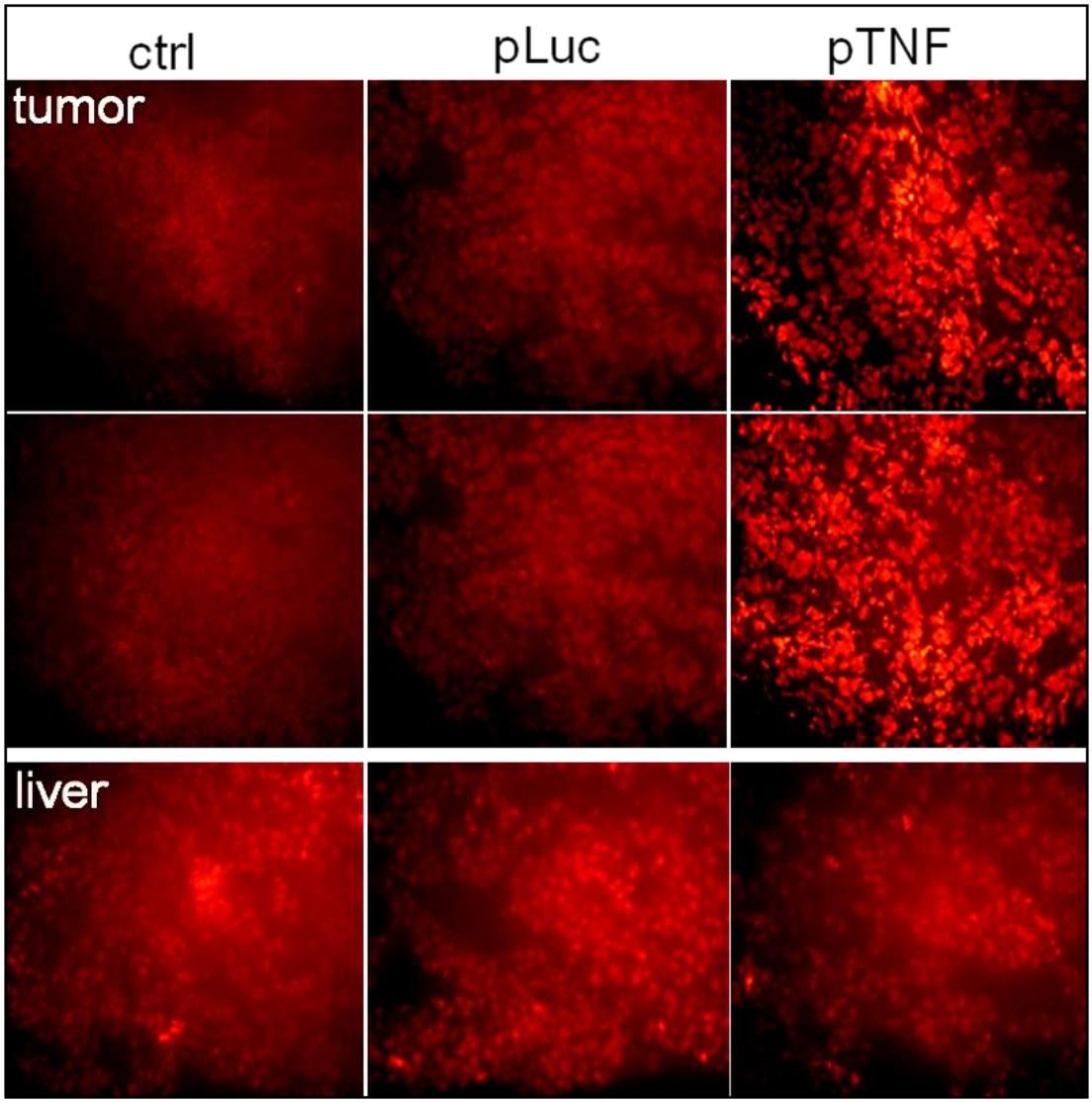


Fig. 7 A

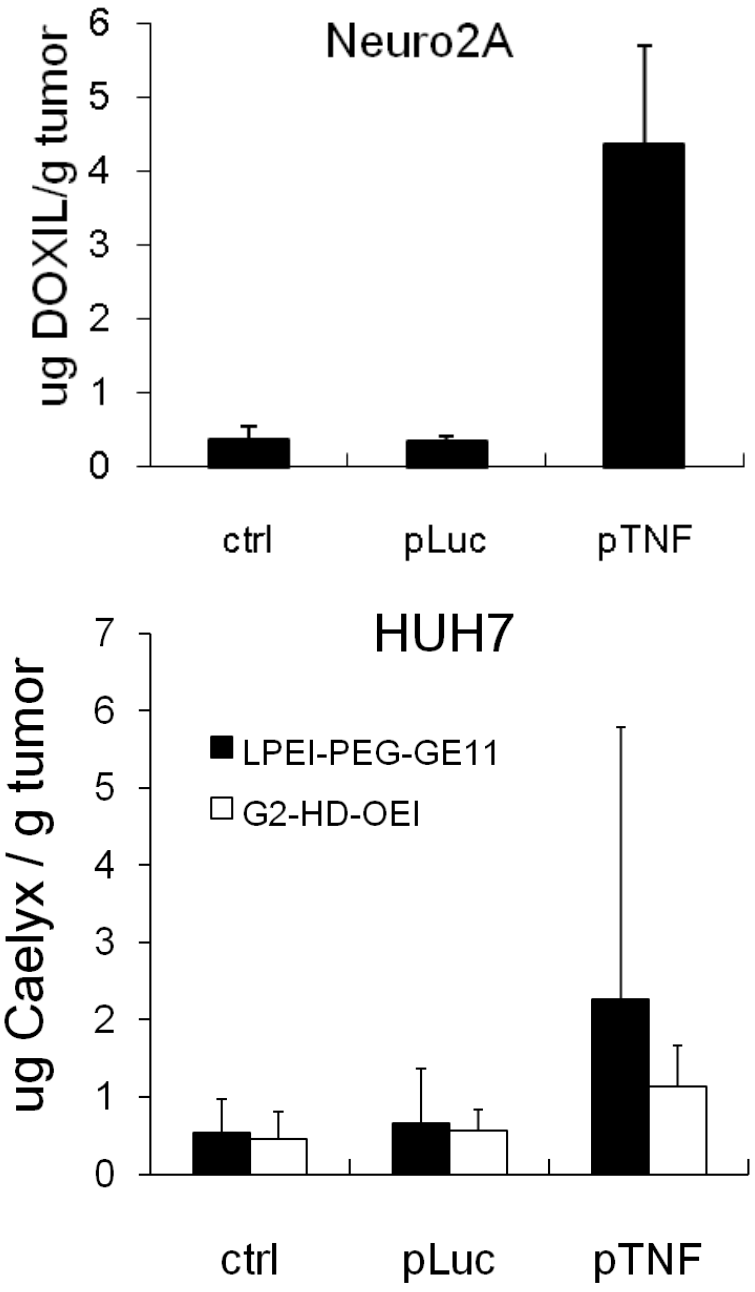

Neuro2A
ug DOXIL/g tumor
0
1
2
3
4
5
6
ctrl
pLuc
pTNF
HUH7
ug Caelyx / g tumor
LPEI-PEG-GE11
G2-HD-OEI
0
1
2
3
4
5
6
7
ctrl
pLuc
pTNF

Fig. 7 B

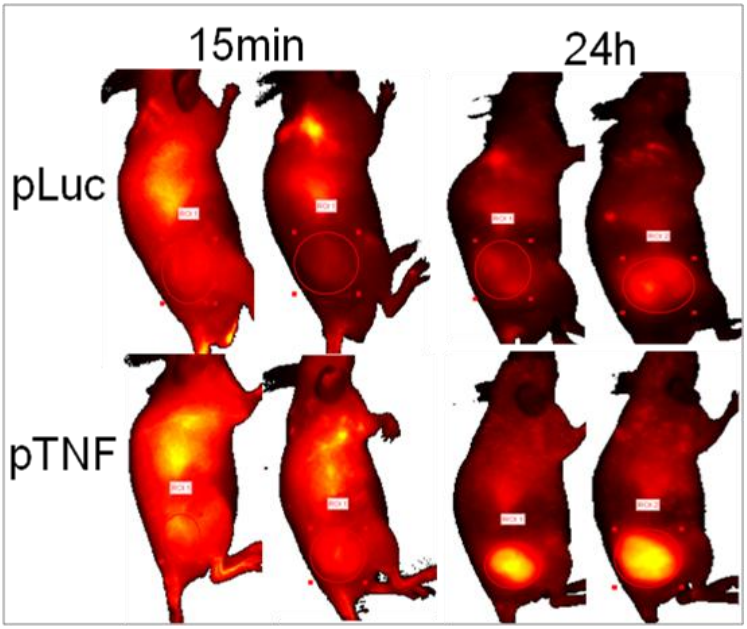

15min
24h
pLuc
pTNF

Fig. 7 C

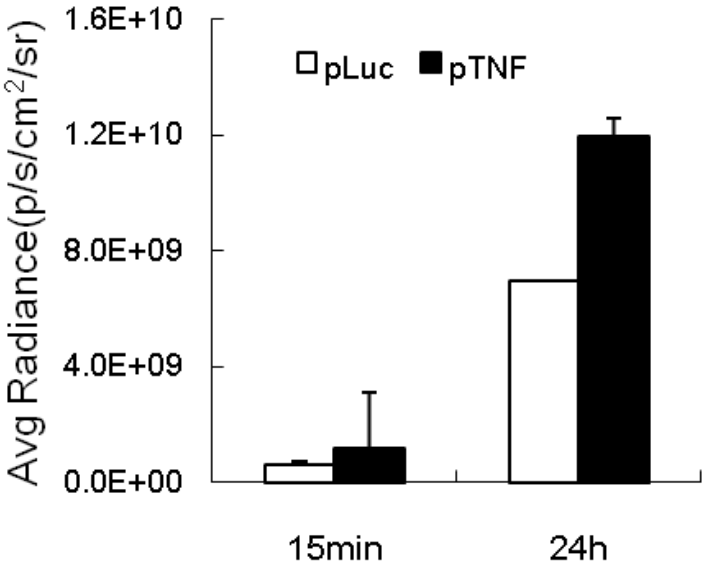

1.6E+10
1.2E+10
8.0E+09
4.0E+09
0.0E+00
Avg Radiance(p/s/cm2/sr)
pLuc
pTNF
15min
24h

Fig. 7 D

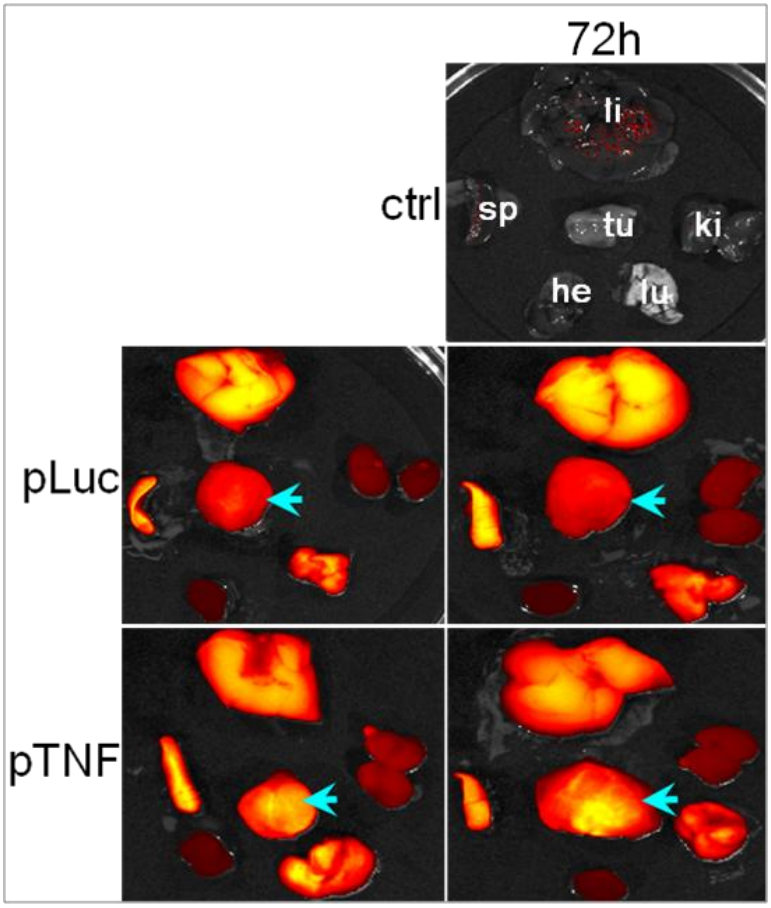


Fig. 7 E

Fig. 7: Assessment of Caelyx® uptake and in tumor and tissue distribution and evalution of Caelyx®-DiR accumulation by living image

48h after a single intravenous injection of pTNF/G3-HD-OEI polyplexes into Neuro2A (Neuroblastoma) bearing mice (**A**), or pTNF/ GE11-PEG-PEI polyplexes into HUH7 (human hepatocellular carcinoma) bearing mice (**B**), 5 mg/kg Caelyx® were injected intravenously. 24h later, tumors and liver tissues of mice were collected. **A,** Cryosections slices of tumors from Neuroblastoma model were fixed in methanol and observed under Zeiss Axiovert 200 microscope with 20 × objective magnification. **B**, Tumors from both models were homogenized in acidified isopropanol and doxorubicine quantified in a spectrofluorometer using a standard curve with defined Caelyx concentration. **C,** Immediately till 15 minutes or 24 hours after Caelyx®-DiR injection, mice were imaged in the IVIS imager with a CCD camera using the ICG filter set (Xenogen Ivis), and the signal of fluorescence in tumor tissue was measured. **D**, Average Radiance (p/s/cm^2/sr) in tumors (15 minutes and 24 hours after injection) was quantified. **E**, 72h after Caelyx-DiR injection, mice were sacrificed and tumors and organs were collected and imaged in the IVIS imager with a CCD camera using the ICG filter set (Xenogen Ivis).

3.8. Application of pTNF /G3-HD-OEI polyplexes in the subcutaneous murine neuroblastoma model Neuro2a

Since many papers (Kircheis R 2002; Kursa 2003; Koppu 2010) indicated the

direct effect of TNF in inhibition of tumor growth, here, in order to see the therapeutic effect of pTNF/G3-HD-OEI polyplexes treatment in the murine neuroblastoma model, on the ninth day after Neuro2A cell inoculation, mice were treated with pTNF/G3-HD-OEI or pLuc/G3-HD-OEI polyplexes every third day, the tumor size and the body weight were measured continuously. From the results, we found that pTNF polyplexes treatment reduced tumor growth in murine neuroblastoma model when compared with Luciferase polyplexes treated group (**fig. 8 A**), however, obviously, pTNF polyplex treatment alone is not enough to inhibit the tumor growth for a long time. There is no significant change on body weight between these two groups (**fig. 8 B**), which indicates the undetectable side effect caused by pTNF polyplexes application. All experiments were repeated twice and exhibited the same tendency.

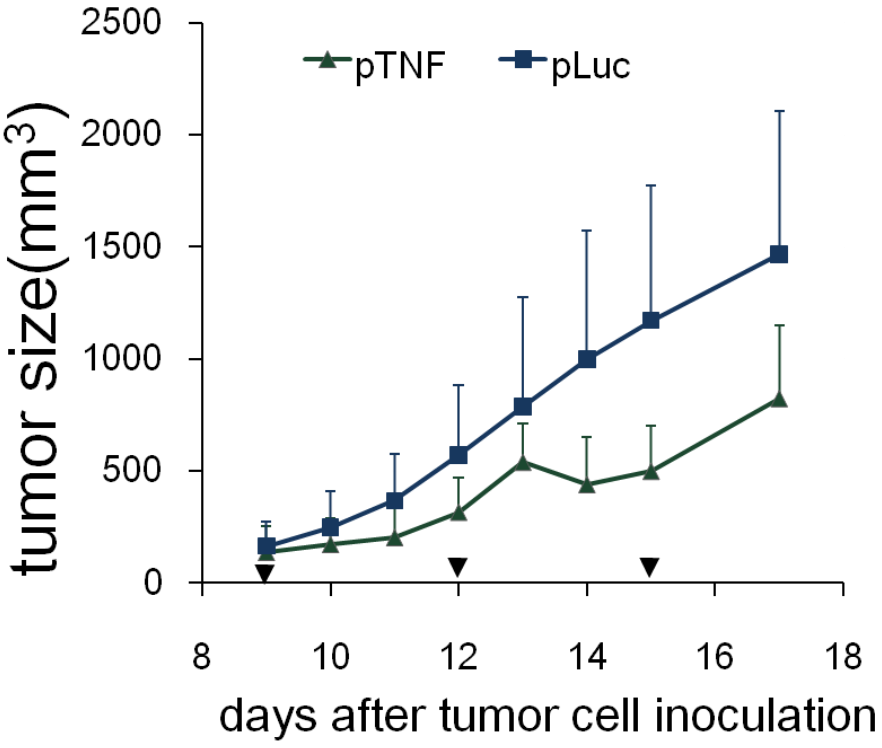


Fig. 8 A

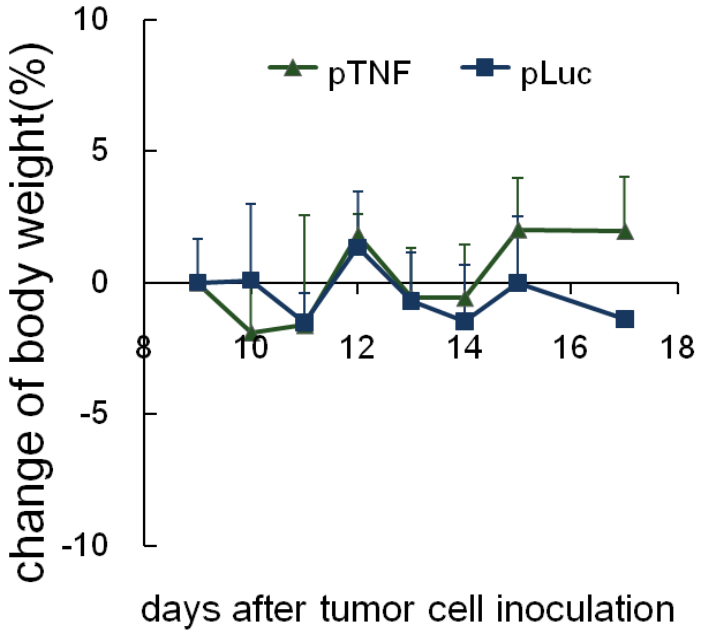


Fig. 8 B

Fig. 8: Application of pTNF polyplexes reduces tumor growth in the murine neuroblastoma model

A, Eight days after Neuro 2A cells inoculation, mice were treated with pTNF/G3-HD-OEI or pLuc /G3-HD-OEI polyplexes every third day, and the tumor size was measured continuously, arrowheads showed the days poplyplexes applied; **B**, The body weight of mice were measured continuously.

3.9. Application of pTNF/G3-HD-OEI polyplexes and Caelyx® in the subcutaneous murine neuroblastoma model

Here, in order to evaluate the combined therapeutic effect of pTNF/G3-HD-OEI polyplexes and chemotherapy and the influence of pTNF polyplexes pretreatment on the therapeutic effect of chemotherapy in the murine neuroblastoma model, three rounds of systemic pTNF polyplexes application and subsequent Caelyx® treatment were performed in A/J mice carrying subcutaneous Neuro2A tumors . As the results shown, pTNF polyplexes application and subsequent Caelyx® treatment led to tumor growth inhibition lasting until eight days after the last treatment (**fig. 9 A**). The pictures of experimental mice showed vivid evidence that the tumor growth of mice treated with pTNF polyplexes and subsequent Caelyx® were significantly inhibited as

compared with mice pLuc polyplexes/Caelyx® treated, only Caelyx® treated or totally untreated (**fig. 9 B**). Almost all pTNF polyplexes/Caelyx® treated mice developed hemorrhagic tumor necroses. Necrosis was most pronounced in the center of the tumor, whereas hemorrhagic tumor necrosis was virtually not found in control animals and only rarely found in Caelyx® or pLuc polyplexes/Caelyx® treated animals (**fig. 9 A, B**, picture of representative mice shown). However, there was only in very few cases a complete eradication of the tumor in this model. This is consistent with other papers applying TNF as treatment in the murine neuroblastoma model (Kircheis R 2002). Moreover, in the retreated (or boost) model, an additional treatment cycle 6 days after last treatment was conducted with 7 out total 11 mice in the group, and the tumor size were monitored continuously. Re-growth of treated tumors could be inhibited by a second treatment cycle indicating that no resistance towards combined pTNF polyplexes /Caelyx® treatment occurred (**fig. 9 C**). Immunological staining of the tumor cryosection with a fluorescently labeled antibody directed against murine endothelial cell marker anti CD31 showed the significant decrease of CD31 expression in the tumors of re-treated mice as compared with non retreated mice (**fig. 9 D**), which indicates a decrease vessel density caused by pTNF polyplexes /Caelyx® retreatment, or to say, the transgenic TNF destroyed the endothelium. When CD31 fluorescence was quantified by image analysis using ImageJ software, less positive pixels per field in pictures from pTNF polyplexes /Caelyx® retreated mice were observed (**fig. 9 E)**. All these results suggest that TNF pretreatment and subsequent Caelyx® chemotherapy do reduce the growth of tumor cells in vivo in murine neuroblastoma model, which could because that pTNF polyplexes pretreatment increase of tumor blood vessel permeability and augment the accumulation of liposomal chemotherapeutics.

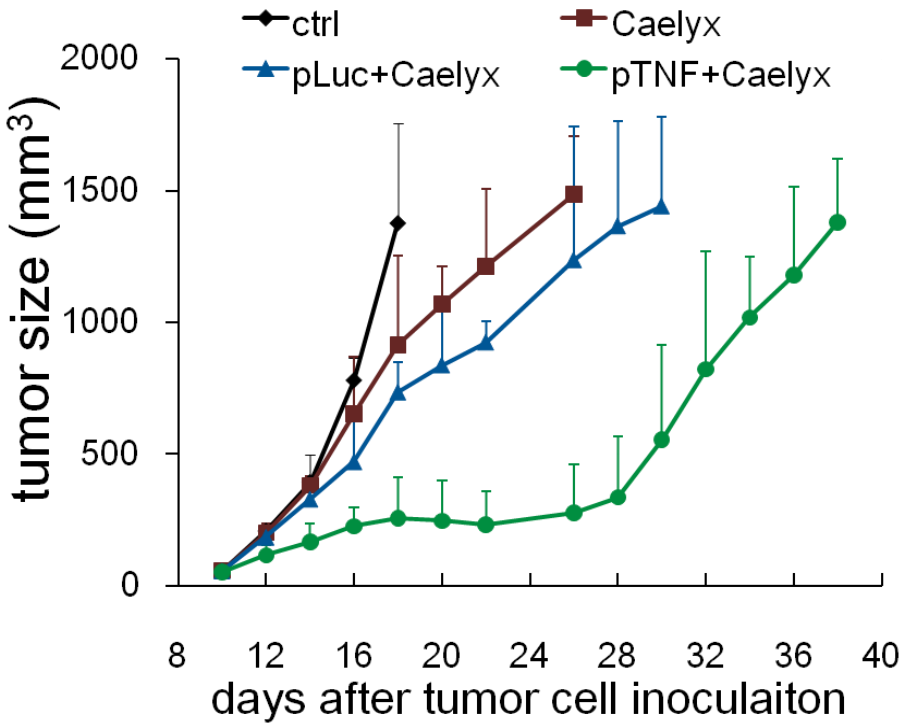

ctrl
Caelyx
pLuc+Caelyx
pTNF+Caelyx
tumor size (mm3)
2000
1500
1000
500
0
8
12
16
20
24
28
32
36
40
days after tumor cell inoculaiton

Fig. 9 A

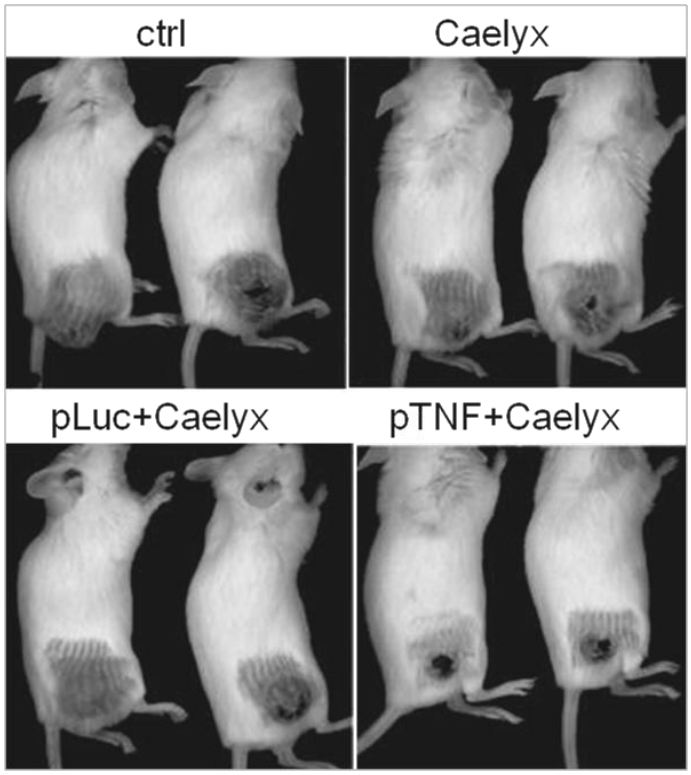

ctrl
Caelyx
pLuc+Caelyx
pTNF+Caelyx

Fig. 9 B

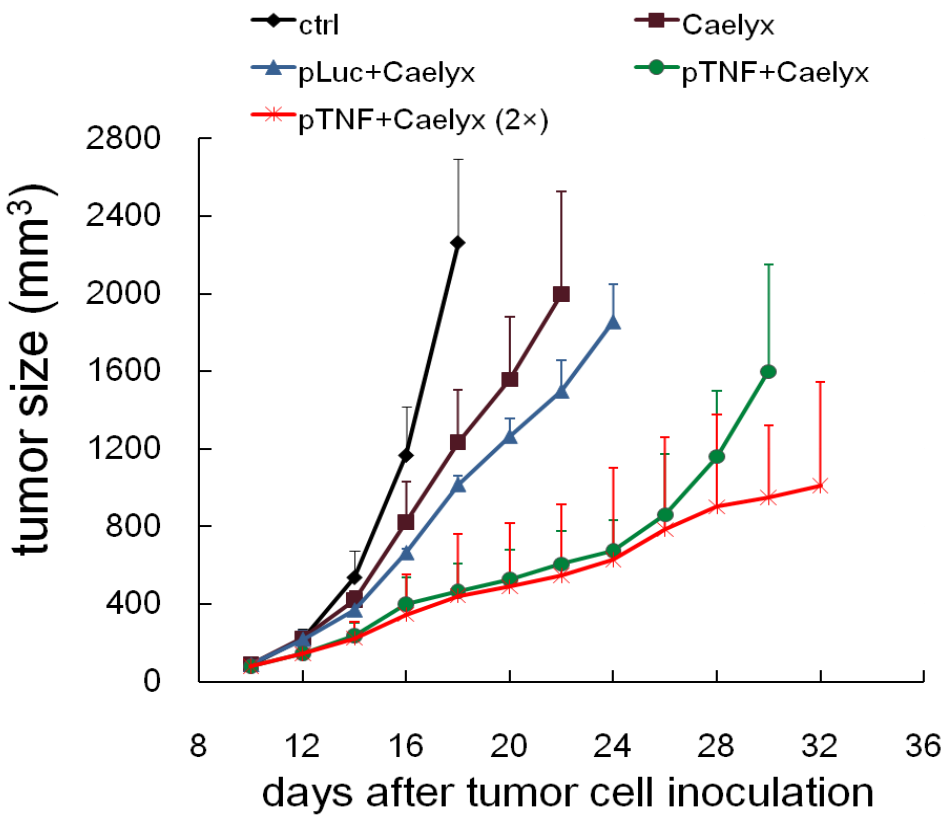

ctrl
Caelyx
pLuc+Caelyx
pTNF+Caelyx
pTNF+Caelyx (2×)
tumor size (mm3)
2800
2400
2000
1600
1200
800
400
0
8
12
16
20
24
28
32
36
days after tumor cell inoculation

Fig. 9 C

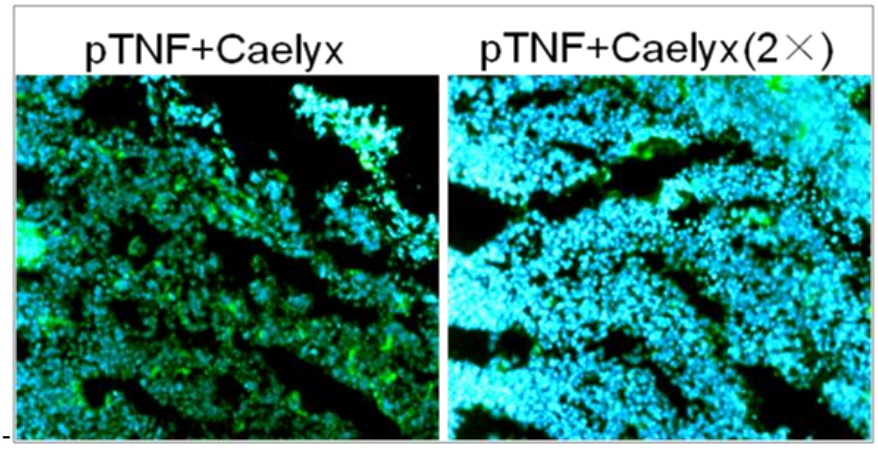

pTNF+Caelyx
pTNF+Caelyx(2×)

Fig. 9 D

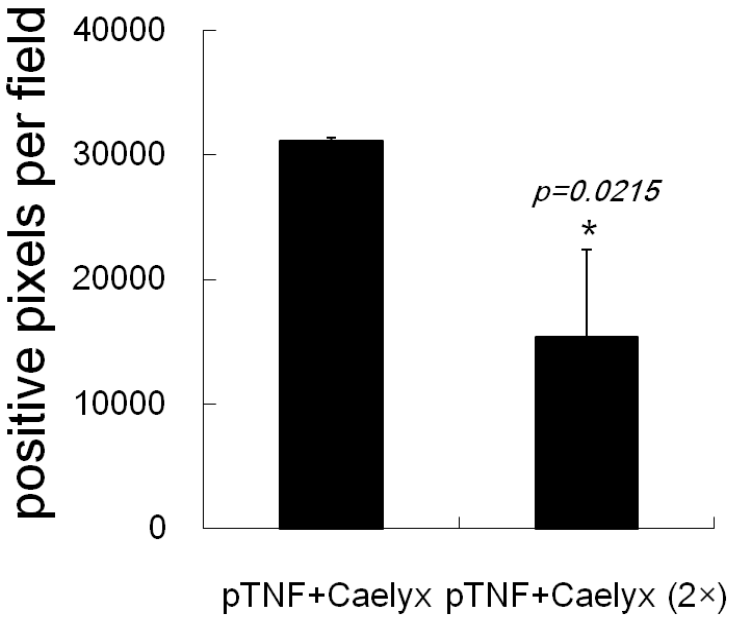


Fig. 9 E

Fig. 9: Application of pTNF/G3-HD-OEI polyplexes and Caelyx® in subcutaneous murine neuroblastoma model

A, 10 days after subcutaneous inoculation of Neuro2A cells,mice were treated with intravenously applied pTNF/G3-HD-OEI or pLuc/G3-HD-OEI polyplexes every 4 days for three times respectively, and every 2 days after polyplex application Caelyx® was applied at a dose of 1.5mg/kg. The tumor size was measured continuously. **B**, The pictures of mice in different groups were taken at the day of last treatment. **C**, In the retreated (or boost) model, the same treatment as in **A**, only that 6 days after last treatment, an additional treatment cycle was conducted with 7 out total 11 mice in pTNF polyplexes /Caelyx® treated group, and the tumor size were monitored continuously. **D**, At 32 days after tumor cell inoculation, mice were sacrificed, and Neuro2A tumors were resected and tumor cryosections were stained with a fluorescently labeled antibody directed against murine endothelial cell marker anti CD31 (green), nuclei were counterstained with DAPI (blue). Slices were observed under Zeiss Axiovert 200 microscope (Carl Zeiss, Jena, Germany) with 10x objective magnification, and pictures were taken. **E**, CD31 fluorescence was quantified by image analysis, using ImageJ software. The parameter total number of positive pixels per field was analyzed for each image.

3.10. Application of pTNF/G3-HD-OEI polyplexes and Caelyx® in the metastatic murine neuroblastoma model

As we have found that pTNF polyplexes application synergizes with Caelyx® chemotherapy leading to the inhibition of tumor growth in subcutaneous murine neuroblastoma model, here we tried to find out the therapeutic effect of pTNF polyplexes/ Caelyx® treatment towards metastatic murine neuroblastoma

model. At 6 days after intravenous Neuro2A lenti Luc cells inoculation, mice were treated with pTNF /G3-HD-OEI or pdeTNF/G3-HD-OEI polyplexes every forth days for three cycles, and every second days after polyplexes application Caelyx® was applied at a dose of 1.5mg/kg. Bioluminescence imaging (BLI) was carried out after intraperitoneal injection of luciferin and luciferase activity was quantified in the tumor area (from back, flank and abdomen side) by BLI (**fig. 10 A**) and expressed as photons/second/region of interest (ROI) (n=10). Similar as in the subcutaneous murine neuroblastoma model, in the metastatic murine neuroblastoma model three rounds of systemic pTNF polyplexes application and subsequent Caelyx® treatment also led to tumor growth inhibition **(fig. 10 A)**. Quantifying bioluminescence imaging (BLI) results as a measure for total tumor burden in **fig. 10 B** showed the significantly lower BLI signal of the tumor cells in pTNF polyplex/ Caelyx treated mice than in the pdeTNF polyplexes/ Caelyx® treated mice or untreated mice (pdeTNF, plasmid without promoter, used as the control plasmid of pTNF). At 6 days after the last treatment, mice were sacrificed, and tumor metastases in the body cavity were evaluated and pictures taken by camera. As the photos of mice show, there are clearly less and smaller visible tumor metastases within the organs in pTNF polyplexes/Caelyx treated mice, while there are obviously more tumor metastases were found in pdeTNF polyplexes/ Caelyx® treated mice or untreated ones (**fig. 10 C**). Almost all pTNF polyplexes/Caelyx® treated mice showed significantly inhibition of tumor growth and metastasis, while it was virtually not found in untreated animals and only rarely found in pdeTNF polyplex/ Caelyx® treated animals (**fig.10 A, B**, **C**, representative mice are shown). To find out more direct evidence for reduction of tumor, bowels (including heart, lung, liver, spleen, kidney, small and large intestine) were taken out and weighted, and the weight of tumors was calculated by deducting the average weight of visceral of mice (n=3+) without tumor cell inoculation but with the same treatment of polyplexes/Caelyx®. Compared with the control group, pTNF polyplex/ Caelyx treatment significantly decreased the tumor

weight in mice, while the tumor weight from pdeTNF polyplexes/ Caelyx® treated mice is also lower than the untreated group (**fig. 10 D**). This tendency among the three groups is consistent with the above described results for Bioluminescence imaging (BLI) and photos. Moreover, to observe the tissue lesions of mice, the collected organs were fixed in formalin and HE staining was performed. As it can be seen from the pictures of tissues slices, almost no tumors were found in kidney lung and spleen tissues. The pictures in **fig. 10 E-1** show the healthy, tumor free kidney, lung and spleen tissues. However, many metastatic tumors were found in the liver tissues. Hepatocytes and tumor cells can easily be distinguished by their color: Tumor cells are darker colored and slightly bluish. Morphological characteristics also show difference: while hepatocytes tend to have an angular shape, tumor cells seem to be round, no clear membrane boundary between cells, containing larger nuclei and therefore containing less stroma (**fig. 10 E-2**). There are obvious many metastatic tumors in livers from pdeTNF polyplexes/Caelyx® treated mice or untreated mice, while there are almost no tumor metastases in liver in the pTNF polyplex/ Caelyx® treated mice, and the livers appeared quite healthy and almost similar as the background group (mice without tumor cells inoculation) (**fig. 10 E-2**). In conclusion, all these results from Bioluminescence, figures of body cavities, tumor weight and the HE staining of tissue sections suggest that pTNF polyplexes pretreatment and Caelyx® chemotherapy does reduces the growth and metastasis of tumor cells in vivo in the metastatic murine neuroblastoma model, and that combining liposomal chemotherapeutic with TNF gene therapy leads to a synergistic effect on tumor reduction in both subcutaneous and metastatic murine neuroblastoma model.

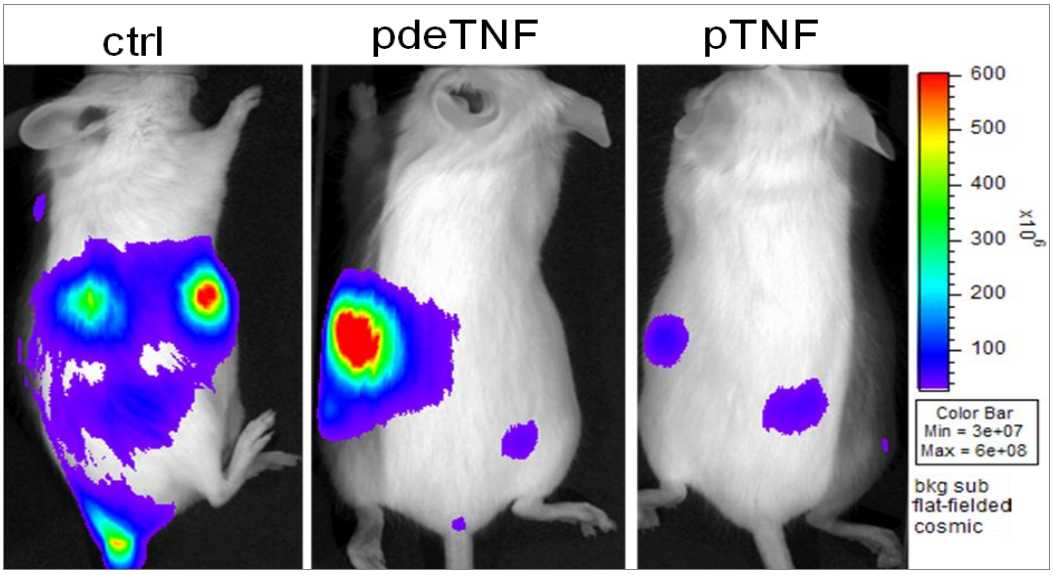


Fig. 10 A

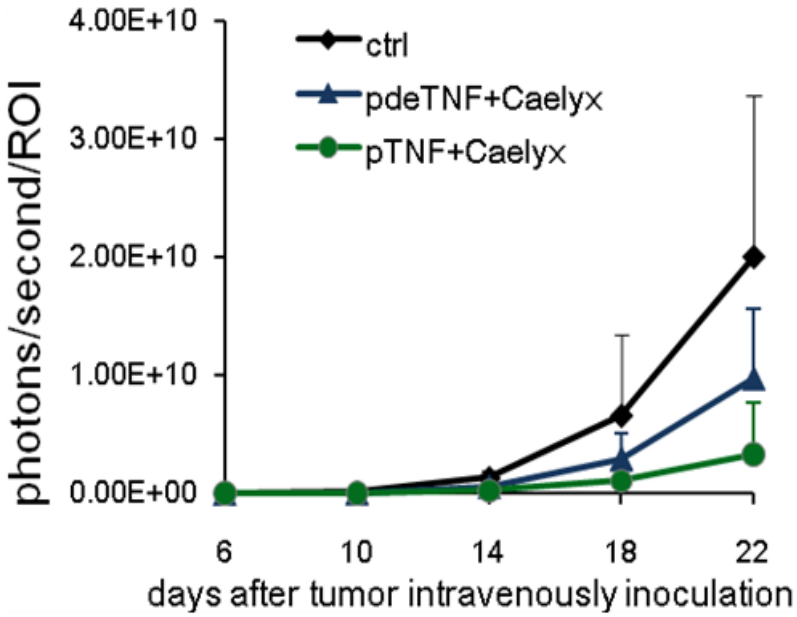


Fig. 10 B

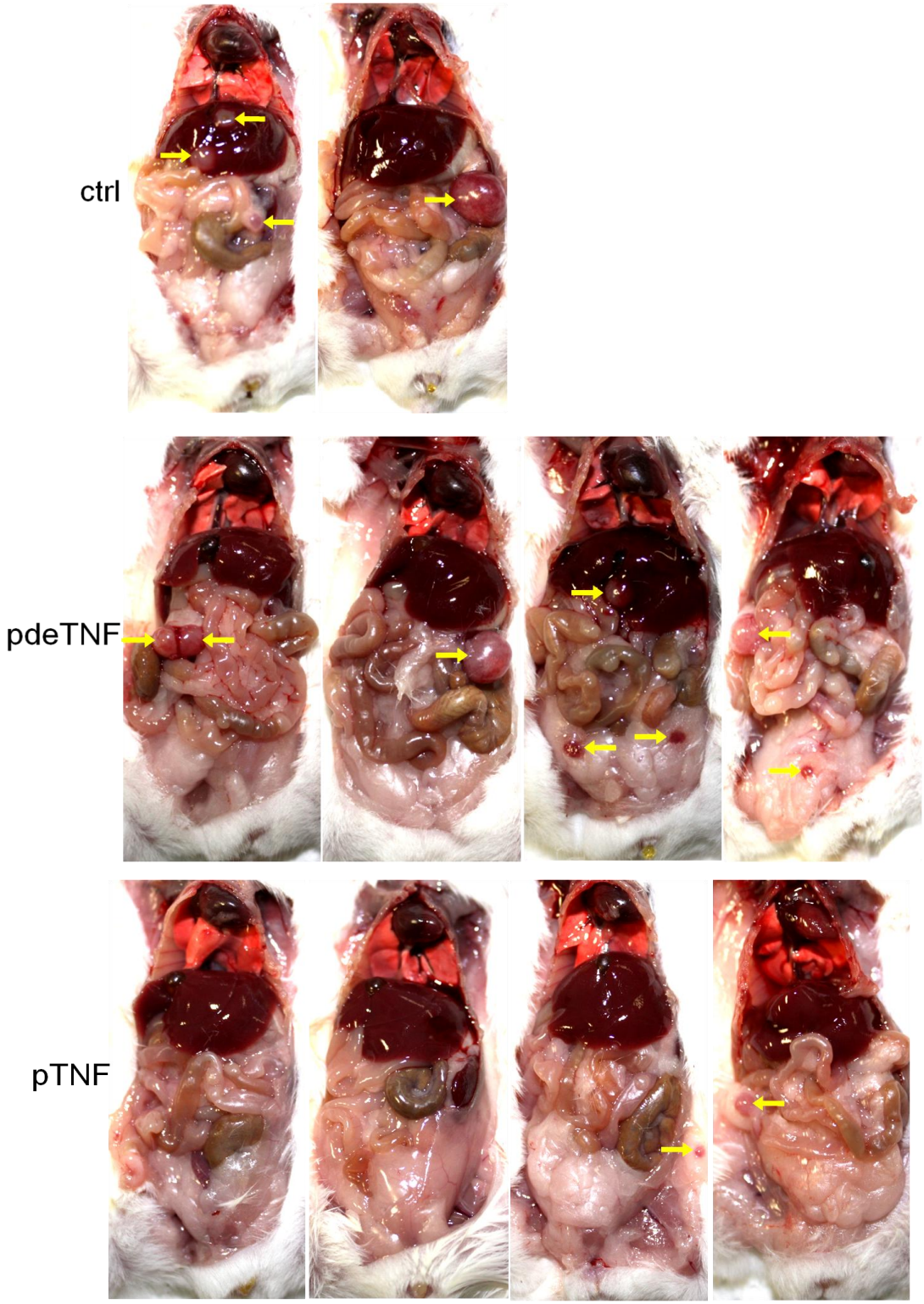


Fig. 10 C

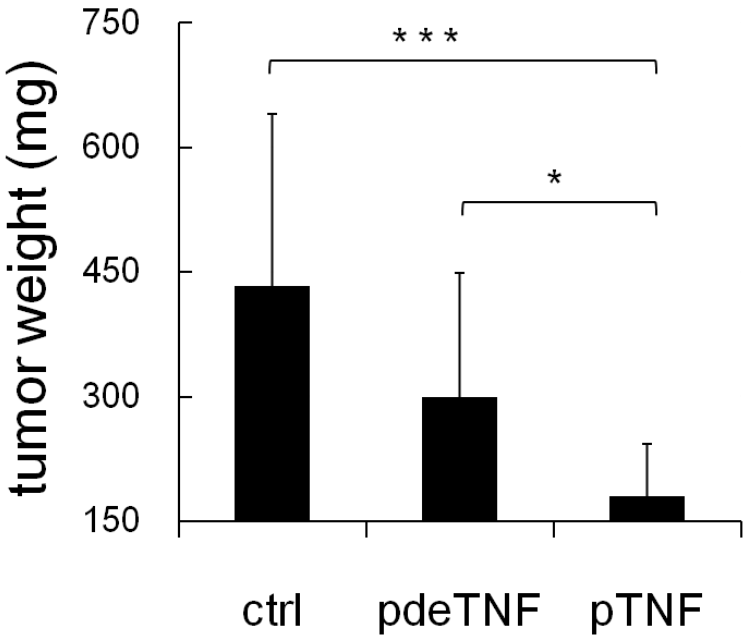
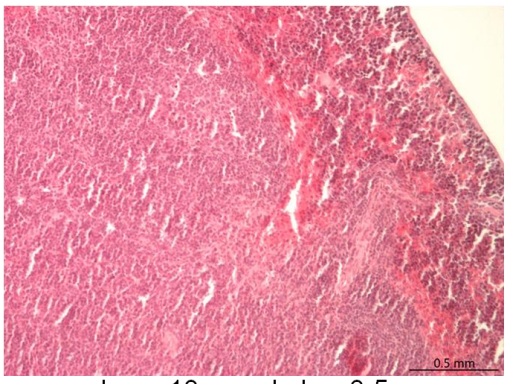


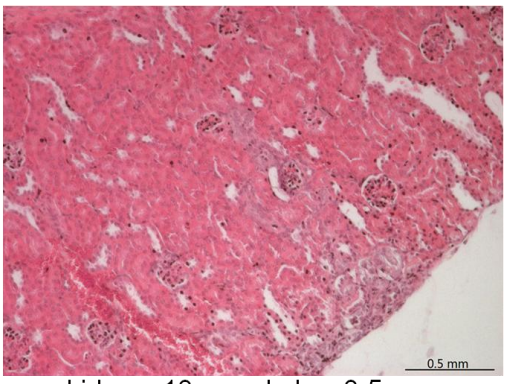
Fig. 10 D

Figures of HE Staining

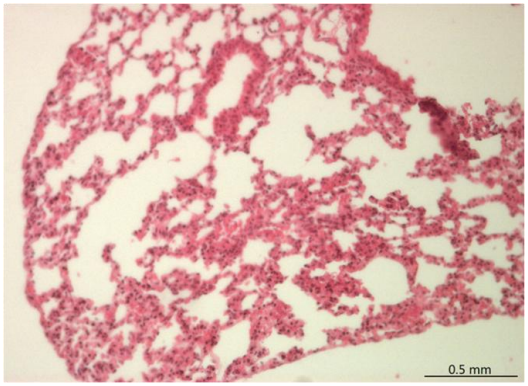
Ⅰ Healthy, tumor free spleen kidney and lung



spleen, 10x, scale bar 0,5mm



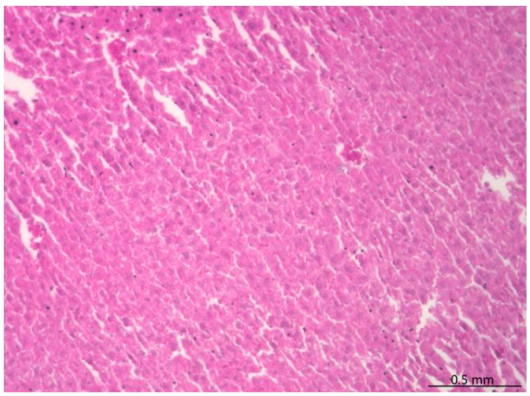
kidney, 10x, scale bar 0,5mm



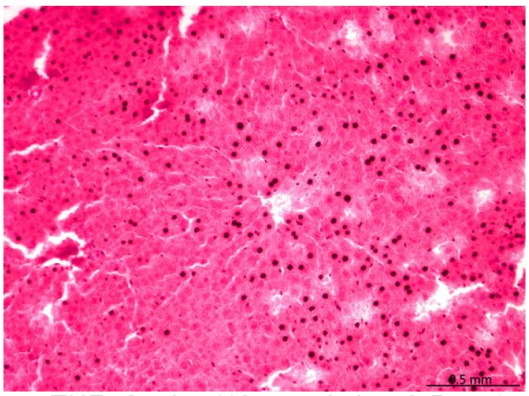
lung, 10x, scale bar 0,5mm

Fig. 10 E -1

Ⅱ Metastatic tumors in liver



Background (10×, scale bar 0.5 mm)

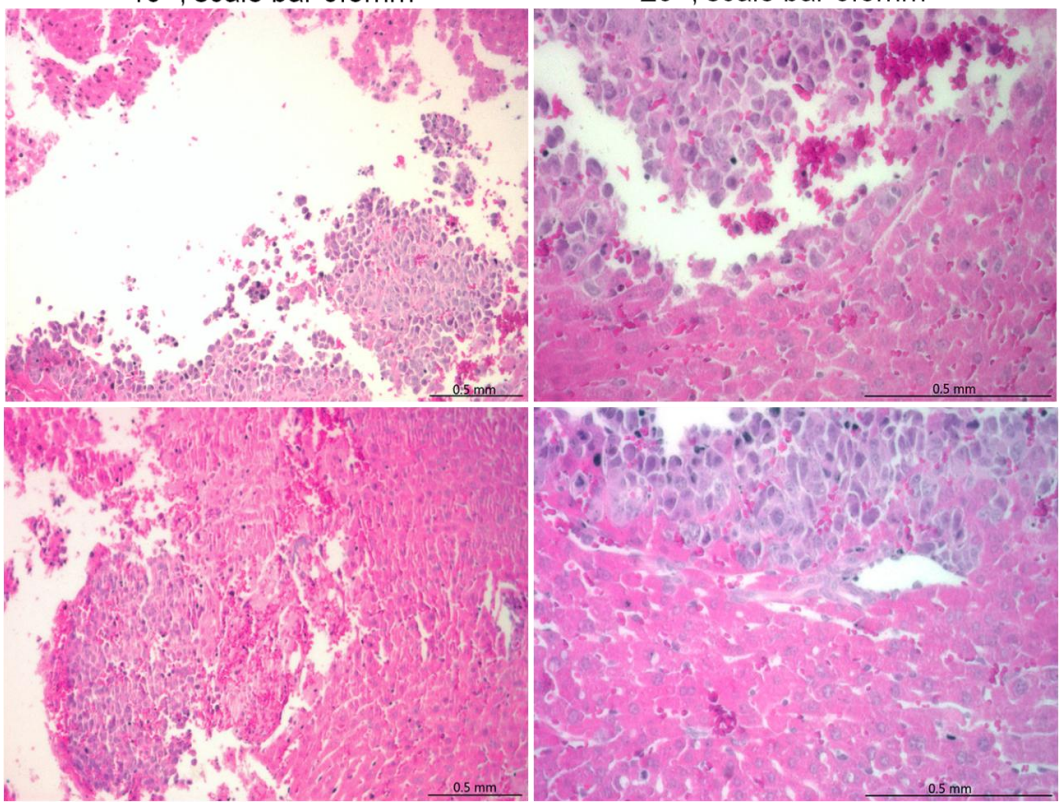


pTNF+Caelyx (10×, scale bar 0.5 mm)

pdeTNF+Caelyx®

10×, scale bar 0.5mm

20×, scale bar 0.5mm



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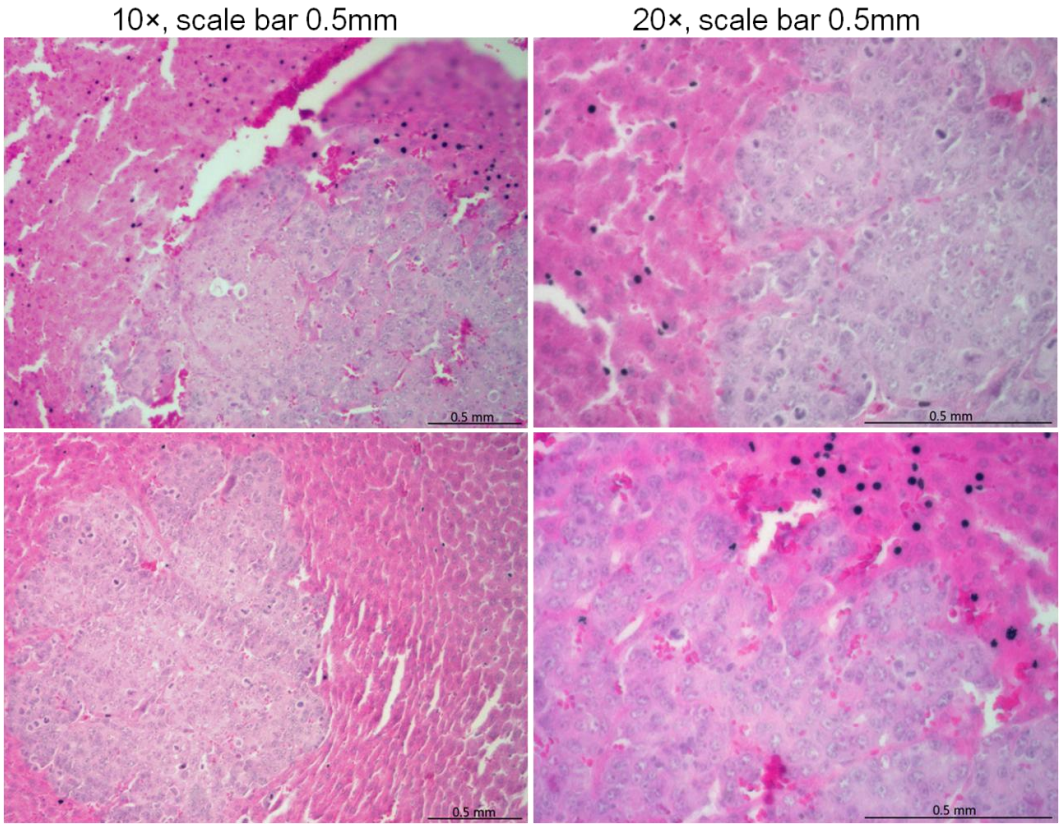

Fig. 10 E-2,

Fig. 10: Application of pTNF/G3-HD-OEI polyplexes and Caelyx® in the metastatic murine neuroblastoma model

At 6 days after intravenous inoculation Neuro2A lenti Luc cells, mice were treated with pTNF/G3-HD-OEI or pdeTNF/G3-HD-OEI polyplexes every 4 days for three times, and every 2days after polyplexes application, Caelyx® was applied at a dose of 1.5mg/kg. A set mice was left totally untreated as a control. **A**, Bioluminescence imaging (BLI) was carried out after intraperitoneal injection of luciferin and luciferase activity was quantified in the tumor area (from back, flank and abdomen side) by BLI and expressed as photons/second/region of interest (ROI) (n=10) ; **B**, Quantification results of the photon signal, expressed as photons/second/region of interest (ROI) ; **C**, At 6 days after last treatment, mice were sacrificed, and tumor metastases in the body cavity were observed and pictures taken by camera; **D**, Bowels (including heart, lung, liver, spleen, kidney, small and large intestine) were taken out and weighted, and the weight of tumors was calculated by deducting the average weight of visceral of mice (n=3+) without tumor cell inoculation but with the same treatment of polyplexes/Caelyx®. **E**, After the mice were sacrificed, organs were collected and fixed in formalin and the HE staining of organs were done, and slices were observed under Zeiss Axiovert 200 microscope. The obtained pictures were processed, using Adobe Photoshop CS5 for adjusting color and contrast adjustments and adding a scale bar. **Fig. 10 E-1** shows the pictures of healthy, tumor free spleen kidney and lung, with 10×objective magnification, scale bar 0,5mm; **fig. 10 E-2** shows the pictures of livers, with 10× or 20×objective magnification, scale bar 0,5mm, tumor cells in livers from pdeTNF or ctrl group are darker colored and slightly bluish, seem to be round and no clear

membrane boundary between cells, thus can been distinguished from hepatocytes easily.

To find further evidence of the transgene expression of pTNF polyplexes in vivo in metastatic murine neuroblastoma model, especially at protein level, we employed an immunostaining method. At 6 days after last treatment, mice were sacrificed and dissected; the tumor tissues collected and cryosection were prepared. Microslices of tumors were immunostained with rabbit anti murine TNF as first antibody and Alexa Fluor 488 labeled goat-anti- rabbit secondary antibody and the stained sections observed under a microscope. Pictures from both pdeTNF and pTNF polyplexes treated groups showed fluorescence which might be due to the fact that TNF could be produced by either the host or malignant cells originating from inflammatory processes, an effect naturally occuring in the body (Balkwill 2009). Considering natural occurrence of TNF seems fairly considerable. Nevertheless, there was a notable difference: not only the intensity of the fluorescence was much stronger in the pTNF polyplexes treated group, but also the source of the signal was located closer to the cells than in control group, which might verify successful transgene expression of pTNF plasmid. Hence, pTNF/G3-HD-OEI polyplex application indeed led to efficient TNF transgene expression in Neuro2A metastases. As shown in the power panels of **fig. 10. F**, possibile dysfunctional staining was excluded by the use of a control group, which did not show fluorescence - indicating the fluorescent second antibody (Alexa Fluor 488 labeled goat-anti-rabbit) has only specific binding ability to the first antibody (rabbit anti-mouse TNF).

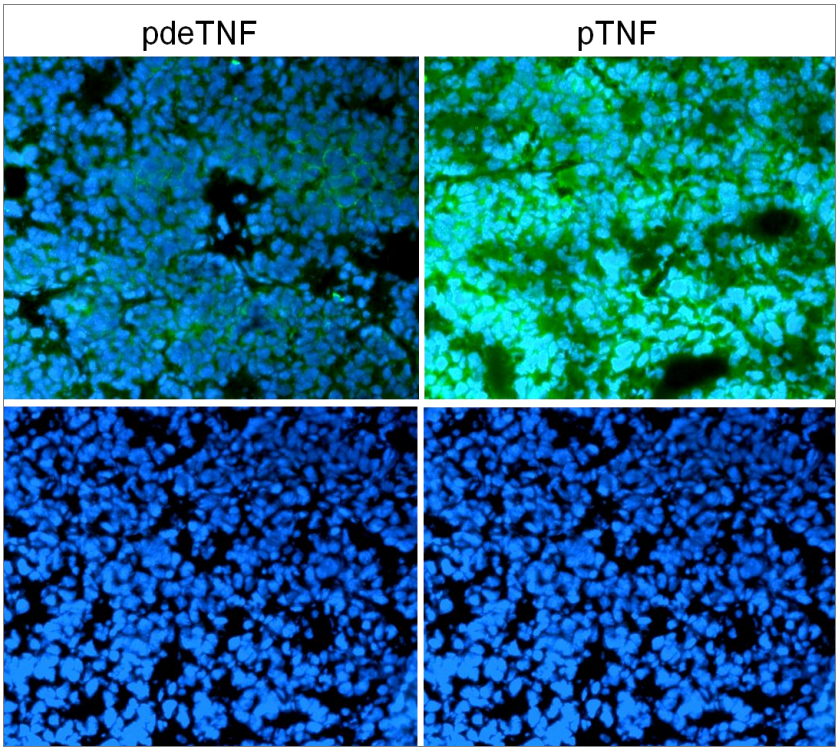

Fig. 10 F: Detection of pTNF polyplexes transgene expression in vivo by immunostaining

Mice were treated as mention in **fig.10**. Then, at 6 days after last treatment, mice (n=3) were sacrificed and dissected, tumor tissues were collected and cryosectioned. The microslice of tumor were immnostained with anti-TNF first antibody and Alexa Fluor 488 labeled goat-anti- rabbit second antibody and the stained slice were observed under Zeiss Axiovert 200 microscope with 20× objective magnification. The figures in the down line showed the control for unspecific second antibody attachment. Green: TNF, blue: cell nuclei (DAPI).

3.11. Application of pTNF /LPEI-PEG-GE11 polyplexes and Caelyx® in the metastatic colon adenocarcinoma model

As we have found in subcutaneous and metastatic murine neuroblastoma models that pTNF polyplexes application synergizes with the Caelyx® chemotherapy, we also investigated how pTNF and Caelyx® treatment will affect cancer models other than murine neuroblastoma, such as metastatic human colon adenocarcoma. Here we tried to find out a possible therapeutic effect of pTNF polyplexes and Caelyx® on intrasplenically injected LS174T colon adenocarcinoma liver metastases in nude mice. At 3 days after the LS 174T lenti Luc cells were intrasplenically inoculated, mice were treated with pTNF/LPEI-PEG-GE11 or pdeTNF/ LPEI-PEG-GE11 polyplexes every third days for three cycles, and every second days after polyplexes application,

Caelyx was applied at a dose of 1.5mg/kg. Bioluminescence imaging (BLI) was carried out after intraperitoneal injection of luciferin and luciferase activity quantified in the tumor area (from abdominal side) by BLI (**fig.11 A**) and expressed as photons/second/region of interest (ROI) (n=10). Similar as in the subcutaneous and metastatic murine neuroblastoma model, three rounds of systemic pTNF polyplex application and subsequent Caelyx® treatment also led to tumor growth inhibition of LS174T lenti Luc tumors: bioluminescence imaging (BLI) results in **fig. 11 A** and the quantification results of BLI in **fig. 11 B** led to significantly lower BLI signal of the tumor cells when compared to then control groups. There was also certain therapeutic effect of Caelyx and control polyplexes, as reduced tumor growth was also observed in pdeTNF and Caelyx® treated mice when compared with untreated mice. At 1 day after the last treatment, mice were sacrificed and dissected to monitor the tumor metastases in the visceral of mice (particularly the supposed metastasis organ liver and the tumor inoculation site spleen). As the photos of livers demonstrate, there are significantly less tumor metastasis on the surface of livers from pTNF polyplexes/ Caelyx® treated mice, while obviously more tumor metastasis were observed on the surface of livers from mice either pdeTNF polyplexes/ Caelyx® treated or untreated (**fig. 11 C**). There is one liver from an untreated mouse, where almost the whole liver is covered with tumors with almost no healthy liver tissue left.The growth of tumor cells in the spleens from pdeTNF polyplex/ Caelyx® treated or untreated mice was so fast that half of the spleen surface is covered with tumor tissue and the spleens are swollen or seriously expanded. In contrast, there are far less tumors on the surface of spleens of pTNF polyplexes/ Caelyx® treated mice. To give a quantitative measure for tumor burden, we collected and weighted liver and spleen. Compared with the control group, pTNF polyplex/ Caelyx® treatment led to significantly decreased spleen and liver weight. Also the weight of organs from pdeTNF polyplex/Caelyx® treated mice is lower when compared to totally untreated and tumor bearing mice (**fig. 11 D**), which indicates the therapeutic effect of Caelyx® in this cancer

model. Then, the concentration of total protein and the bioluminescence signal in supernatant of liver lysates were measured and expressed as relative light units (RLU) per µg total protein. Significantly lower Bioluminescence signal strength in the liver lysate which means significantly less tumor cells in the livers are found in pTNF poplyplexes/ Caelyx® treated mice than pdeTNF polyplexes/ Caelyx® treated or untreated mice (**fig. 11E**). However, no lower Bioluminescence signal strength was found in livers from pdeTNF polyplexes/ Caelyx® treated mice than untreated mice (**fig. 11 E**). Moreover, to observe the tissue lesions of mice, the supposed sites of metastasis, liver, and the tumor cell inoculated organ spleen were collected, fixed in formalin and the HE staining of organs performed. Hepatocytes and tumor cells in liver sections can be easily distinguished by their color, as tumor cells appear darker colored and slightly bluish. Morphological characteristics also show differences: while hepatocytes tend to have an angular shape, the tumor cells seem to be round, no clear cell membrane boundary between cells is visible, and some of them even containing multiple nuclei and therefore also less stroma (**fig. 11 F-1**). As seen in the pictures of stained liver slices, there are obviously multiple sites of tumors metastases in the liver from pdeTNF polyplex / Caelyx® treated or untreated mice, and in almost all mice had more than half of the liver is invaded with tumors. Many tumors do not show signs of necrosis as it can be seen from the merged big tumors on the left side of **fig. 11 F-1,** and only some of the very big tumors developed necrosis in the middle of the tumor lesion. The tumors metastases in liver from the pTNF polyplexes / Caelyx® treated mice are significantly reduced, as much less tumors were found: only one obvious tumor was found within four different livers, which already developed a central necrosis albeit relatively small in size (**fig. 11 F-1**). These results of HE staining also demonstrate that pTNF polyplexes / Caelyx® treatment slowed the growth of cancer cells or prevented the metastasis of the tumor cells in liver. As seen on the pictures of stained spleens, it was obvious that all spleens from three groups carry tumors; almost the whole spleens where invaded with tumor cells

and no tumor free spleens were observed in mice either pdeTNF polyplexes/ Caelyx® treated or untreated. Fewer tumors were found in mice pTNF polyplexes/ Caelyx® treated, and a higher fraction of tumors developed necrosis (**fig. 11 F-2**). However, even the spleens where no tumors were visible on the surface, were also seriously tumor infected (**fig. 11 F-2**). In conclusion, all these results from bioluminescence, macroscopic observations and weight of the tumor infected organs liver and spleen, and the HE staining of tissue section suggest that a combination of pTNF polyplexes pretreatment and then Caelyx® chemotherapy does reduce the growth and metastasis of tumor cells in vivo in the intrasplenic liver metastatic colon adenocarcinoma model, and that combining liposomal chemotherapeutic with TNF gene therapy leads to a synergistic effect on tumor reduction in this cancer model.

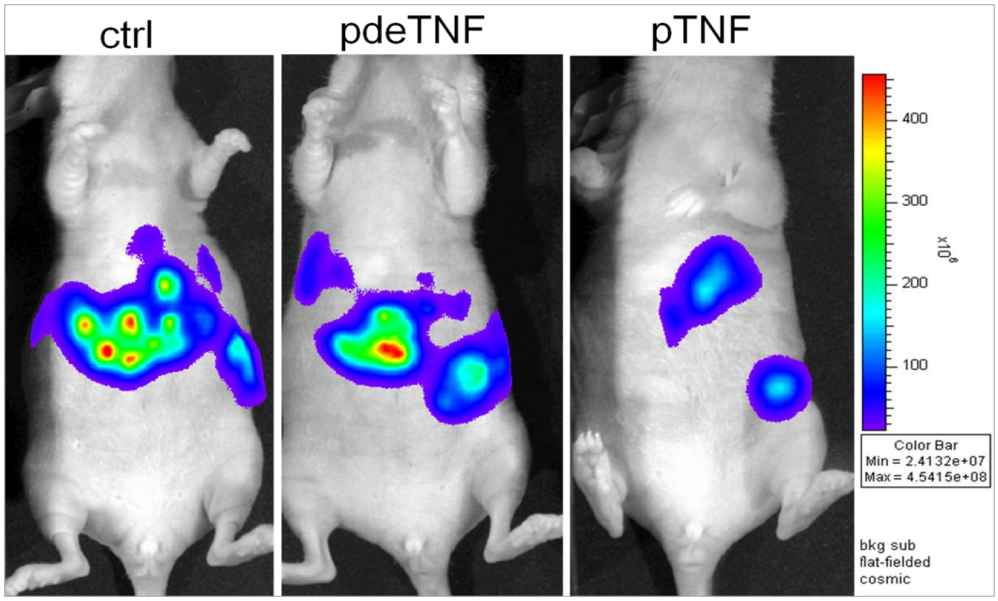

Fig. 11 A

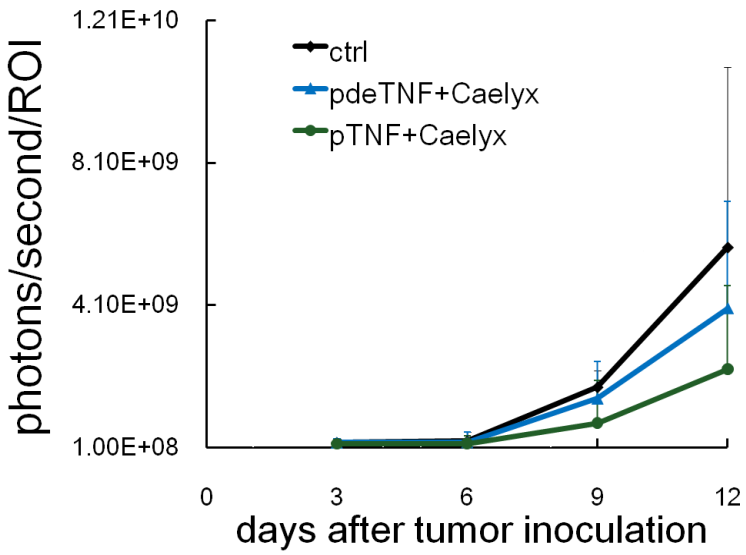

Fig. 11 B

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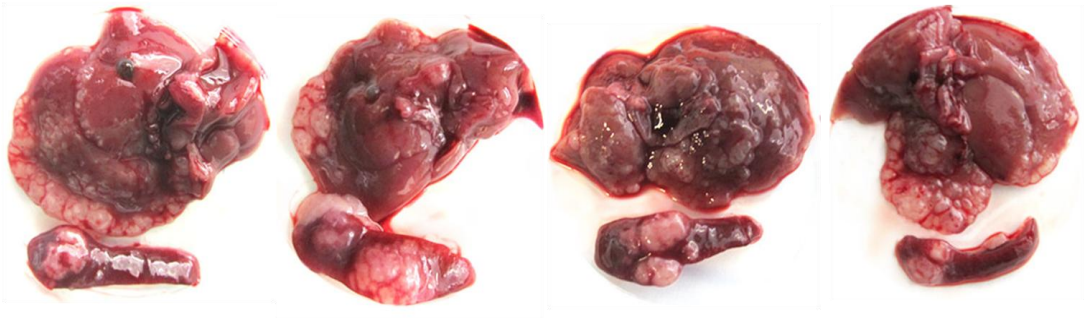
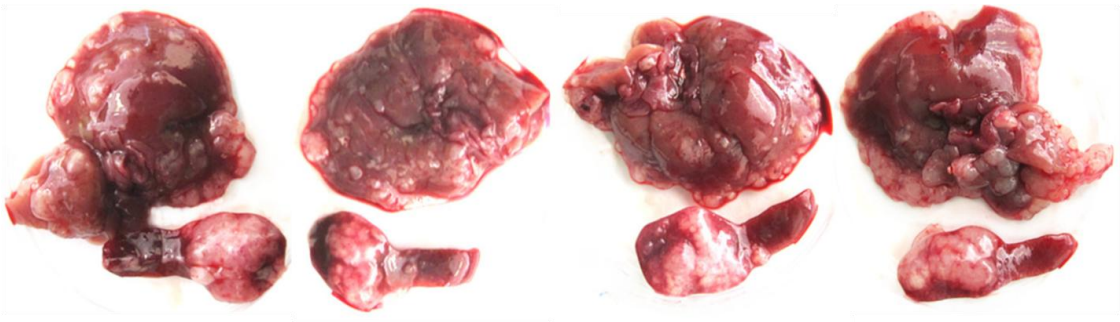


pdeTNF+ Caelyx®



pTNF+ Caelyx®

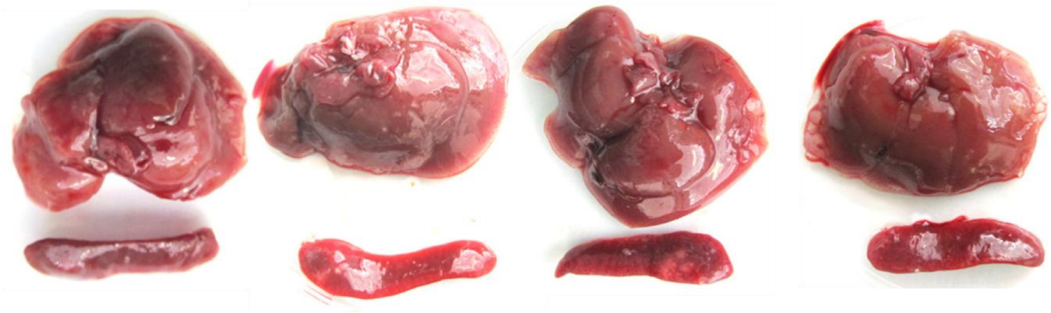
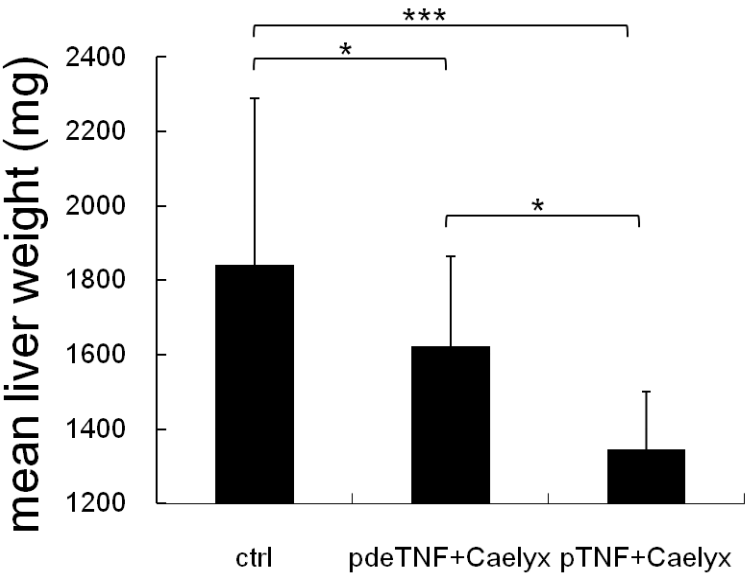
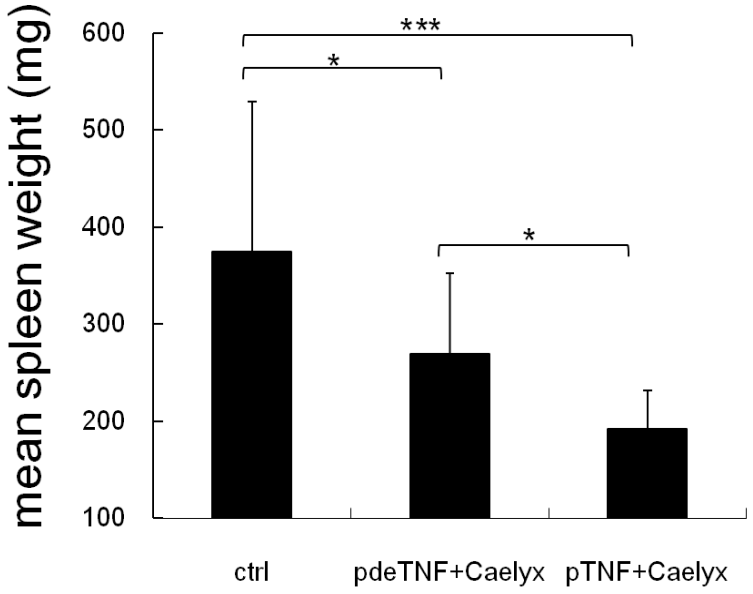


Fig. 11 C


***
*
*
mean liver weight (mg)
2400
2200
2000
1800
1600
1400
1200
ctrl
pdeTNF+Caelyx
pTNF+Caelyx


***
*
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mean spleen weight (mg)
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500
400
300
200
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pdeTNF+Caelyx
pTNF+Caelyx

Fig. 11 D

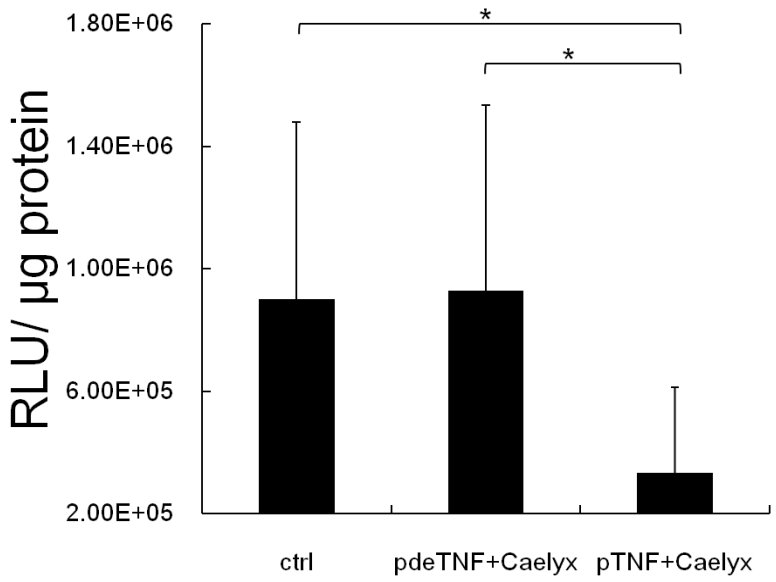
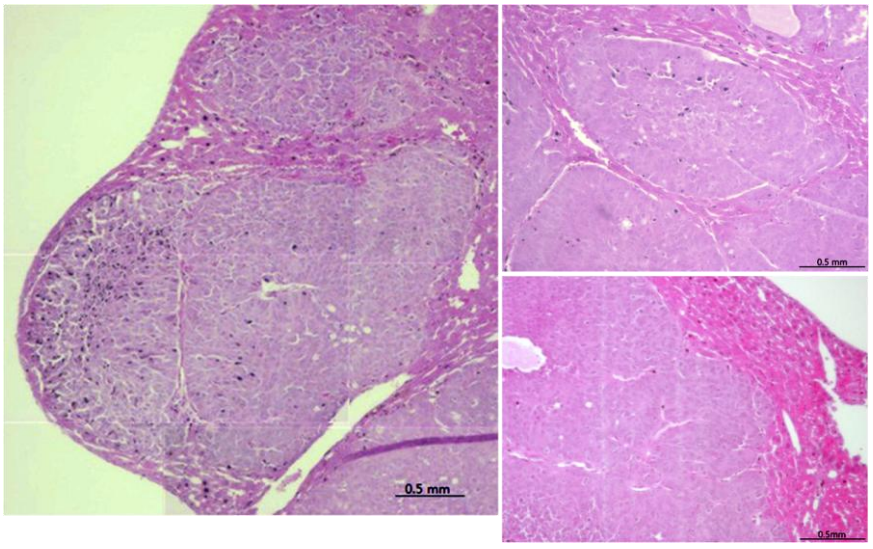


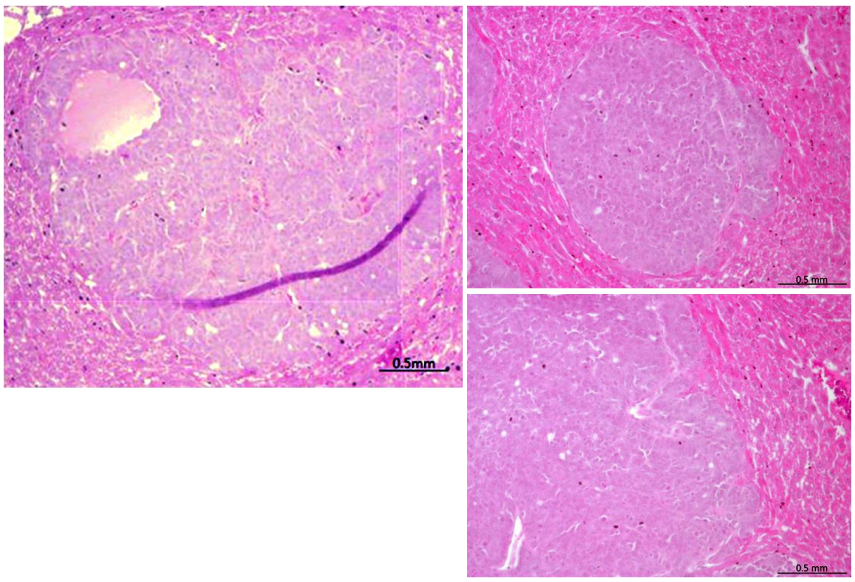
Fig. 11 E

Figures of HE staining

Ⅰ Metastatic tumors in liver

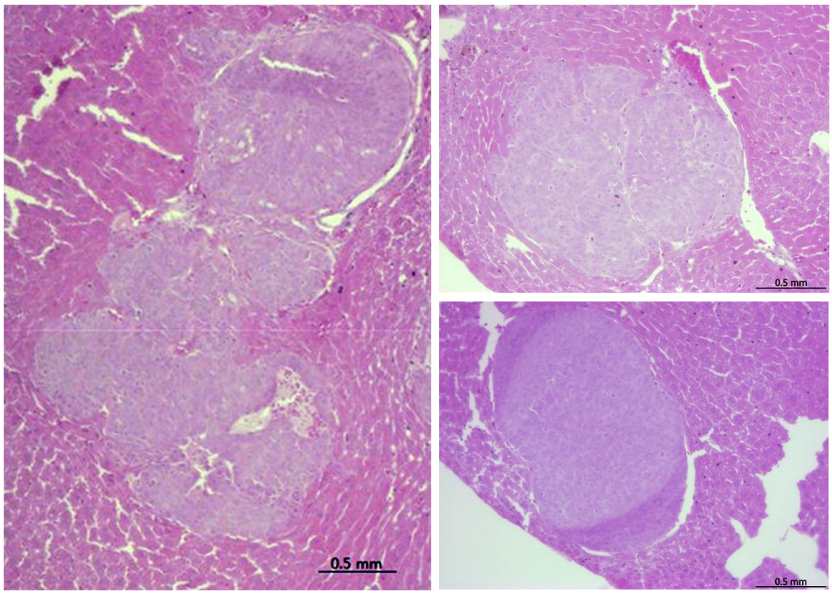
ctrl

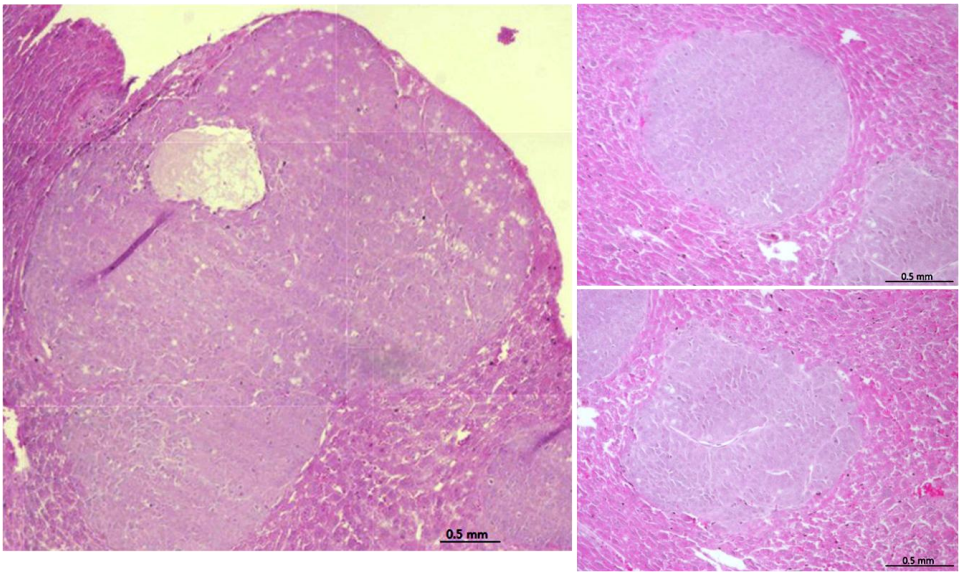




Left: merged big tumors from several single pictures; Right: single pictures; Pictures were observed with 10× objective magnification, scale bar 0,5mm

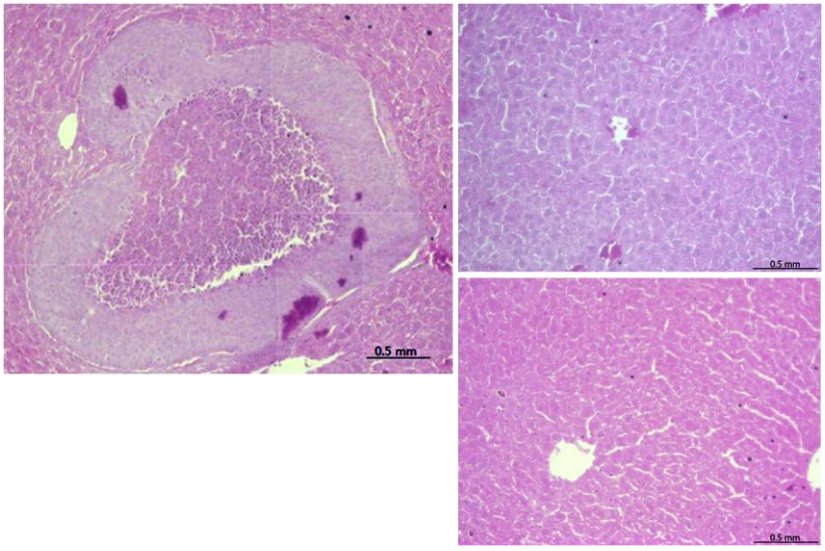
pdeTNF+Caelyx®





Left: merged big tumors from several single pictures; Right: single pictures; Pictures were observed with 10× objective magnification, scale bar 0,5mm

pTNF+Caelyx®

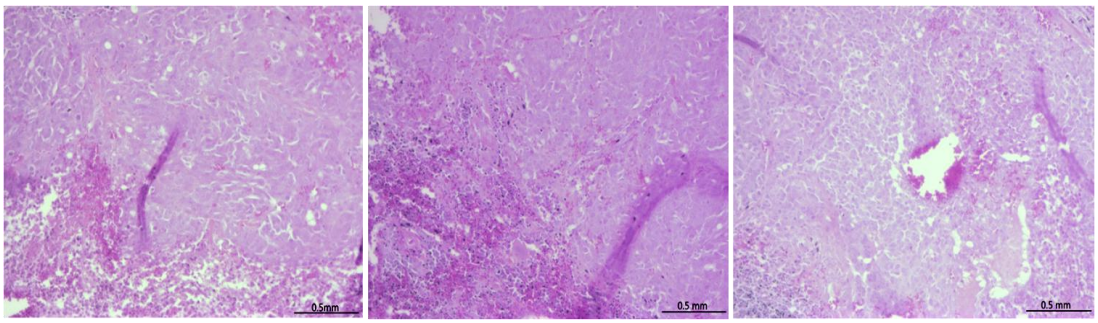


Left: merged big tumors from several single pictures; Right: single pictures; Pictures were observed with 10× objective magnification, scale bar 0,5mm

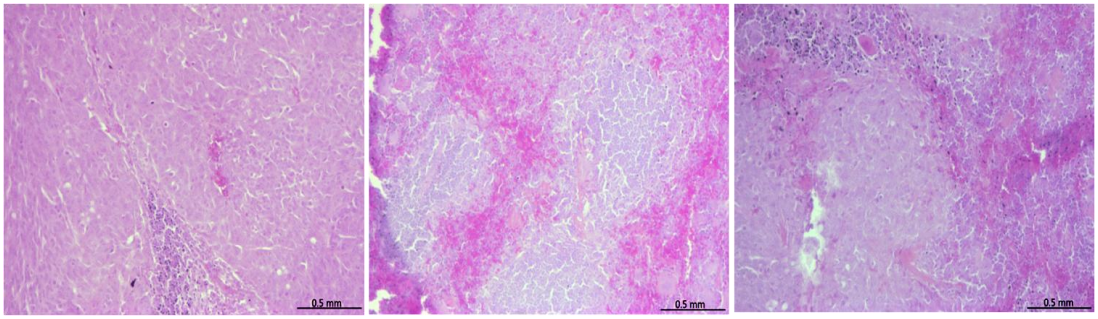
Fig. 11 F-1

Ⅱ Tumors in spleen

ctrl



pdeTNF+Caelyx®



pTNF+Caelyx®

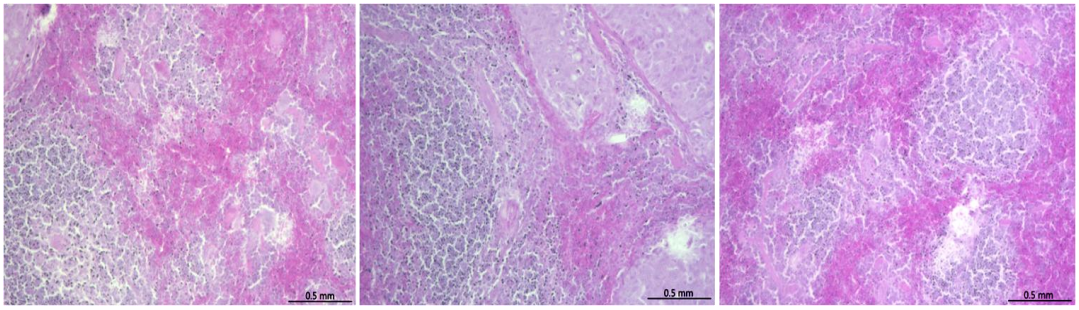


Fig. 11 F-2

Fig. 11: Application of pTNF/LPEI-PEG-GE11 polyplexes and Caelyx® in the metastatic colon adenocarcinoma model

At 3 days after LS 174T lenti Luc cells where intrasplenic inoculated, mice were intravenously injected with pTNF / LPEI-PEG-GE11 or pdeTNF/ LPEI-PEG-GE11 polyplexes every 3 days for three times, and every 2 days after polyplexes application, Caelyx® was applied at a dose of 1.5mg/kg. As a further control, tumor inoculated mice were left untreated. **A**, Bioluminescence imaging (BLI) was carried out after intraperitoneal injection of luciferin and luciferase activity was quantified in the tumor area (from back, flank and abdominal side) by BLI and expressed as photons/second/region of interest (ROI) (n=10); **B**, Quantification results from **fig. 11 A** expressed as photons/second/region of interest (ROI) ; **C**, At 1 day after last teatment, mice were sacrificed and dissected to observe the metastasis of tumor cells in the visceral of mice (particularly liver and spleen), and the pictures of livers and spleens were taken (n = 11) ; **D**, Collected livers and spleens were weighted and differences statistically analyzed (n=11, T-Test, * $p<0.05$, ** $p<0.01$, ***

p<0.001) **E,** Frozen livers were grinded to powders with liquid nitrogen which were then lysed with protein lysis buffer, the concentration of total protein and bioluminescence signal in supernatant of liver lysates were measured and expressed as relative light units (RLU) per µg total protein. **F**, After the mice were sacrificed and dissected, livers and spleens were collected and fixed in formalin. HE staining was done, and slices were observed under Zeiss Axiovert 200 microscope. The obtained pictures were processed, using Adobe Photoshop CS5 for adjusting color and contrast adjustments and adding a scale bar and merging single pictures into bigger ones (as seen at the left of the pictures of livers). **Fig. 11 F-1** shows the pictures of livers, with 10×objective magnification, scale bar 0,5mm; Left: merged big tumors from several single pictures. Tumor cells in livers from in pictures are darker colored and slightly bluish, seem to be round and no clear membrane boundary between cells, thus can been distinguished from hepatocytes easily. **Fig. 11 F-2** shows the pictures of spleens, with 10×objective magnification, scale bar 0,5mm.

4. Discussion

4.1. Novel plasmid pTNF with human CMV enhancer and CpG free backbone induces persistent transgene expression in vitro

It was shown that CpG-dinucleotides in the plasmid DNA have a strong negative effect on the duration of transgene expression (Yew 2000; Yew 2002; Hodges 2004; Hyde 2008; Mitsui 2009). In the plasmid, the hCMV enhancer is more efficient driving transcription in human cells than the murine analogue (Isomura 2003). Our results suggest that the novel plasmid vector with CpG free backbone and an optimized promoter-enhancer combination (with hCMV as enhancer and EF1 as promoter) is definitely powerful at inducing sustained and high transgene expression and could be used in vitro (**fig. 1 A, B,C**) and in vivo for sustained gene therapy. These results are consistent with several papers showing that with a CpG-free pDNA expression vector sustained *in vivo* transgene expression can be achieved (Navarro 2010), without inflammatory reactions in the lung (Hyde 2008).

4.2. Secreted TNF increases endothelial cell permeability in a transwell system, but exerts only moderate toxicity towards tumor cells.

Our results of the transwell assay (**fig. 2 B**) show that secreted transgenic TNF induced by pTNF polyplex transfection increased endothelial cell permeability, which might due to TNF mediated apoptosis of some endothelial cells and then an enhancement of leakage. This is consistent with other papers (Nawroth 1986; Palladino 1987; Nawroth 1988; Clauss 1990; Gasparri 1999; Lienard 1999). Meanwhile, we found TNF did not exert direct tumor cell toxicity on HUH7, Neuro2A, U87MG and MBA-MD-231 cell lines, while exerting a moderate toxicity on MBA-MD-453 and BT549 cell lines (**fig. 2 C**). The different sensitivity of the cell lines to TNF could be due to the abundance of the TNF receptor. All these cell lines are known to express low levels of TNF-R1. Tumor cell lines including gliomas (Duan 2001; Carroll-Anzinger 2006) and hepatoma (Plümpe 2000) are resistant against apoptosis initiated by treatment with TNF-α,

but can be sensitized through costimulation with metabolic inhibitors (inhibitors of protein or RNA synthesis), such as actinomycin D, CDDP, cyclohexamide or mitomycin C. Even TNF sensitive cell lines such as L929 always respond more sensitive to the TNF treatment when applied together with inhibitors of protein or RNA synthesis. As concluded by some papers, TNFR 2 (TNF-R75), must be responsible for the strongly enhanced systemic toxicity of murine TNF (Ostade 1993), while some other papers suggested that although only sensitive cell lines express transcripts for p75 TNF receptor 2, changes in TNF receptors were not found to contribute to the susceptibility for TNF (Duan 2001). Many papers suggested that the toxicity of TNF towards malignant cells might dependent on the activation of TNFR-1 and Fas-mediating the apoptosis pathway. TNF-R1-mediated cytotoxicity can be selectively inhibited under conditions in which Fas-mediated cell death is not affected. Activation of both receptors results in synergistic signaling of apoptosis (Wong 1994). It was reported that in tissue culture studies, purified or recombinant TNF was selectively toxic for malignant cells, as were TNF-containing supernatants from activated macrophages (Colotta 1984). However, many of these data were generated in the presence of metabolic inhibitors such as actinomycin D, cyclohexamide or mitomycin C (Colotta 1984; Sugarman 1985; Dealtry 1987) or in combination with interferon-γ (IFN γ) (Williamson 1983; Fransen 1986). It might be that the antitumor activity of TNF depends on indirect mechanisms associated with selective destruction of the tumor-associated vascular bed and TNF-induced alteration of endothelial barrier function and of immune mechanisms rather than having toxic effects directly on tumor cells.

4.3. The plasmid pRGD-TNF leads to targeting TNF expression and pTNF-mir143 leads to selective TNF expression

Similar as Natasa Zarovni and some other scientists did (Zarovni 2004), here, we employed the sequence of RGD motif (which can been recognized, bound and internalized by αvβ3 integrins, belonging to a family of cell-surface proteins,

with high expression on endothelium) in our pTNF plasmid to target TNF transgene expression to endothelium. Integrins are dimeric adhesion receptors that mediate cellular attachment to the extracellular matrix (ECM) or to adjacent cells. Integrin-mediated cell–matrix interactions regulate often divergent biological events including cell adhesion, migration, proliferation, differentiation and survival (Giancotti 1997). The αvβ3 integrin plays a fundamental role during the angiogenesis process by inhibiting endothelial cell apoptosis (Scatena 1998). 3-type integrins (αvβ3 and αIIbβ3) are expressed in multiple cell types including invasive tumor cells, osteoclasts, activated endothelial and smooth muscle cells, platelets, megakaryocytes and macrophages (Sloan 2006). Endothelial enhanced the expression of αvβ3 integrin in the tumor vasculature (Sloan 2006). Besides, after TNF treatment ,endothelial cells may increase the activation and ligation of αvβ3 while decreasing the activation and ligation of α5β1-integrins to facilitate cell migration (GAO 2002). Compared with pTNF plasmid, the plasmid containing vasculature-targeting peptide RGD gene fusing with mature TNF gene, pRGD-TNF, induced higher TNF biological activity into the medium which led to higher cytotoxicity on L929 indicator cells than pTNF (**fig. 3 A**).This could because that RGD motif attached on L929 mouse fibroblasts through αvβ3 integrin (expressed by L929 cells)-mediated interactions (Katagiri 1996; Karakecili 2007) and then leads to higher endocytosis of TNF and subsequently TNF-mediated cytotoxicity. When we applied the pRGD-TNF/G3-HD-OEI polyplexes in vivo in the subcutaneous murine neuroblastoma model, we found that three rounds of intravenous pRGD-TNF polyplex application led to inhibition of tumor growth in mice (**fig. 3 B**); however, the inhibition effect was only moderatly enhanced compared to pTNF polyplexes treatment and the effect is not as significant as some papers (Zarovni 2004) declared. This might either suggests that RGD-TNF expressed directly in tumor cells is not reaching the endothelias cells in sufficient amounts or that the targeting TNF expression might also not enough for the tumor inhibition without chemotherapy treatment; even it is most expressed in

vasculature around the tumor (or tumor microenvironment). This will be investigated in future research projects.

As mention in section **1.3.3.**, in view of the natural function of microRNA and the specific abundance of miR-143 in different tissues, here we construct pTNF-mir143 by inserting miR-143 targeting sequence into plasmid pTNF. We found the plasmid containing miR-143 targeting sequence (pTNF-miR143) do lead to lower TNF expression in PSMC (primary porcine smooth muscle cells, high level of miR-143, Florian Kopp, unpublished results) while no influence towards PC3 tumor cell line (low in miR-143, Florian Kopp, unpublished results) (**fig. 3 C**), which might indicate that when applied in vivo, pTNF-miR143 plasmid might selectively TNF transgenic expression in tumor cells, while reducing the transgene expression in in other tissues besides tumor tissue and lead to lower related side effect. However, the miR-143 targeting sequence containing plasmid also decreased the expression of TNF in murine neuroblastoma (Neuro2A) cell line (**fig. 3 D**), which suggests that not all tumor cell line have a low level of miR-143 , and the application of miR-143 targeting sequence in selectively transgene expression also dependent on tumor types.

4.4. pTNF/G3-HD-OEI poplyplexes induces transgenic TNF gene expression in vivo

Here, the transgenic TNF expression induced by pTNF polyplexes in vivo was determined by qPCR at mRNA level. The relative total TNF mRNA level in both tumor and liver of mice from pTNF polyplex treated mice is significantly higher than in pLuc polyplex treated and untreated mice (**fig. 5 A**), which verifies that pTNF polyplex application leads to efficient expression of transgenic TNF in vivo, at least at mRNA level. Moreover, the transgenic TNF could also stimulate the endogenous TNF expression. TNF binding to TNFR1 induces a range of inflammatory mediators and growth factors through activation of the AP1 transcription factors or IκB kinases (IKKs) that, in turn, activate nuclear

factor-κB (NF-κB), which then leads to inflammation and more TNF expression (Balkwill 2009). Meanwhile, the TNF mRNA level in pLuc polyplexes treated mice is also higher than in completely untreated mice.This could be due to inflammation caused by polyplex treatment inducing the initiation of the intracellular signal pathway leading to expression of inflammatory factors such as TNF and IL-6. To have direct evidence of the protein expression of TNF induced by pTNF polyplex application in vivo in the metastatic murine neuroblastoma model, we employed an immunostaining method. Pictures from both pdeTNF and pTNF treated groups show fluorescent staining specific for TNF. It is likely that the diffuse fluorescence found in all anti-TNF stained specimens originates from inflammatory processes intrinsically occuring in the body (either produced by the host or by malignant cells (Balkwill 2009)), while the distinct, strong fluorescence shown in **fig. 10 F** indicates higher TNF- α levels in vicinity to the tumor cells. Considering the affected cells were producing TNF- α at higher dose, this leads to the verification of positive treatment with the plasmid.

4.5. Application of pTNF polyplexes decreases endothelial cell density in tumor

Several evidences suggest that the antitumor activity of TNF depends on indirect mechanisms associated with selective destruction of the tumor-associated vascular bed. Our results of trans-well experiments show, that secreted TNF increases endothelial cell permeability, which might be due to the decreased endothelial cell density caused by pTNF/G3-HD-OEI polyplexes treatment. This is consistent with staining results in vivo showing the decreased CD31 expression in the tumor tissues from pTNF/G3-HD-OEI polyplexes treated mice (**fig. 6**) proving the decreased vessel density in tumor microenviroument caused by the transgenic TNF after pTNF/G3-HD-OEI polyplex application.

4.6. Application of pTNF polyplexes enhances accumulation of liposomal doxorubicine Caelyx® in tumor tissue and leads to nuclear localization of doxorubicine, while no significant effect was found on PEG-QD

As mention in section **1.2.4.**, it is reported in several publications that low doses of TNF Increase tumor blood vessel permeability, thus augmenting tissue concentrations of hemotherapeutic drugs (Nawroth 1986; Palladino 1987; Nawroth 1988; Clauss 1990; Gasparri 1999; Lienard 1999), as the observed synergy between TNF and chemotherapy in locoregional treatment of patients with advanced tumors of the limb (Lienard 1999). We found that pTNF polyplex application enhances accumulation of liposomal doxorubicine Caelyx® in tumor tissue, in both Neuro2A murine neuroblastoma model using G3-HD-OEI as gene carrier and in HUH7 hepatoma xenografts model using GE11-PEG-PEI or G2-HD-OEI as gene carrier (**fig. 7 B)**, and it led to nuclear localization of doxorubicine (**fig. 7 A**). Moreover, significantly enhanced tumor accumulation of Caelyx® fluorescently labeled with the lipid DiR emitting in the near infrared at 15 minutes or 24 hours after injection was observed by in vivo imaging (**fig. 7 C),** and the quantification results of the signal of fluorescence (**fig. 7 D)** exhibited the same tendency. While more convincing results were observed in the results of organs image (**fig. 7 E)**, as there was the significant enhancement of Caelyx®-DiR in tumor tissue and a slight decrease in liver tissue in the TNF polyplex pretreated group. All these results suggest a TNF mediated increase of tumor blood vessel permeability augmenting the accumulation of liposomal chemotherapeutics. However, when we used PEG-QD (coating Quantum dots with PEG, with the size of about 80nm, zeta-potential of -0.42) fluorescence signal in living image instead of Caelyx®-DiR, there is no significant enhancement of the accumulation of Caelyx® in tumor tissue was found. This could be explained by a possibly too short circulation time of PEG-QD (with only a few hours half life in vivo, even when Quantum dots coated with PEG (Andreas Offenhäusser 2009)) in comparison to Caelyx®, which reportedly circulates for >24h in the blood stream (Seynhaeve 2007). It was reported,

twelve hours after injection, 51 ±25% of the liposomal doxorubicine was still present in the blood circulation, while even after 72 h, still 15 ± 2% of the liposomes were measured in the circulation (Seynhaeve 2007).

4.7. Application of pTNF polyplexes reduces tumor growth in the subcutaneous murine Neuroblastoma model

Neuroblastoma is the most common extra cranial solid cancer in childhood and the most common cancer in infancy. It is one of the few human malignancies known to demonstrate spontaneous regression from an undifferentiated state to a completely benign cellular appearance. Their unpredictability and high rate of metastasis combined with their malignant character make them though a challenge for therapy. Consequently, different efforts were taken in treating this tumor type. Meanwhile, some of those features, such as the easy establishment of the mice model in A/J and so on all favor the use of neuroblastoma for research use. Since many papers declared the direct effect of TNF in inhibition of tumor growth, we evaluated the therapeutic effect of pTNF/G3-HD-OEI polyplexes treatment in the Neuro2a murine neuroblastoma model and showed that application of pTNF/G3-HD-OEI polyplexes reduces tumor growth in mice.

The mechanisms how TNF functions in tumor therapy might be complicated. As mentioned in section **1.2.4.**, both isoforms of TNF (membrane bound and soluble TNF) bind two distinct receptors that are ubiquitous, TNF-R1, and TNF-R2 (Dembic Z 1990). It is an apoptotic pathway initiated by the clustering of death domain containing proteins upon the combination of TNF with the related receptor (Tartaglia LA 1993; Grell M 1994). Many papers (Kircheis R 2002; Kursa 2003; Koppu 2010) declared the direct effect of TNF in inhibition of tumor growth, our results of murine neuroblastoma model (**fig. 8 A**) also showed that pTNF polyplexes reduces tumor growth in the murine neuroblastoma model when compared with luciferase polyplexes treated group, while there is no significant change in body weight (**fig. 8 B**) between these two

groups, suggesting no significant side effect caused by pTNF polyplex treatment. However, as late as 17 days aftertumor setting, the tumor already grew quite big and mice had to be sacrificed, which also suggest that TNF alone is not enough in significantly delaying tumor growth. The therapeutic effect is not as good as compared with results declared by Swati Koppu *et al.* (a rapid and sustained tumor regression over one month, with longterm survival of 100% of the animals (90% complete response, 10% partial response))(Koppu S. 2010), this might due to the dose dependency principal, Swati Koppu *et al.* treated mice daily (50µg DNA) for ten days , while we only treated mice three times for every three days. However, the results could be also consistent with publications, which declare that TNF is only in combination with metabolic inhibitors that its cytotoxic potential is unmasked, and the antitumor activity of TNF depends mainly on indirect mechanisms associated with selective destruction of the tumor-associated vascular bed and TNF-induced alteration of endothelial barrier function, and of immune mechanisms rather than having toxic effects directly on tumor cells (Balkwill 2009).

4.8. The combined therapeutic treatment of pTNF polyplexes and Caelyx® inhibits tumor growth in the subcutaneous murine neuroblastoma model

Our results found that in Neuro2A murine neuroblastoma model, three rounds of systemic pTNF polyplexes application and subsequent Caelyx® treatment led to tumor growth inhibition lasting until eight days after the last treatment (**fig. 9 A**). The pictures of mice after last treatment (**fig. 9 B**) also showed that the tumor growth of mice treated with pTNF polyplexes and Caelyx® were significantly growth inhibited when compared with mice treated with pLuc polyplexes and Caelyx® or completely untreated. Besides, re-growth of treated tumors could be inhibited by a second treatment cycle indicating that no resistance towards combined pTNF polyplexes/ Caelyx treatment occurred (**fig. 9 C**). Immunological staining of the tumor cryosection with a fluorescently

labeled antibody directed against the murine endothelial cell marker CD31 showed the significant decrease of CD31 expression in the tumors of re-treated mice when compared with non retreated mice (**fig. 9 D**), which indicates a decrease in vessel density caused by the transgenic TNF. All these results suggest that a TNF pretreatment and then Caelyx® chemotherapy does reduce the growth of tumor cells in vivo in mice, which could because that TNF pretreatment increases of tumor blood vessel permeability and augments the accumulation of liposomal chemotherapeutics. These results are consistent with many papers which declare that TNF alone was ineffective in their setting but synergized with melphalan chemotherapy in a rat osteosarcoma ILP model, with mild hyperthermia optimizing the anti-tumor effect (de Wilt 1999). A combination of TNF and doxorubicin had comparable effects in rat sarcoma models (van der Veen 2000). Previous results observed in our lab that enhanced therapeutic effects was reached in a murine B16F10 melanoma model by the combination of tumor-targeted TNF gene therapy with liposomal doxorubicin DOXIL® (Wagner E. 2004). It appears that low doses of TNF increase tumor blood vessel permeability (Seynhaeve 2007), thus augmenting tissue concentrations of chemotherapy and destroying the tumor vasculature.

4.9. The combined therapeutic treatment of pTNF polyplexes and Caelyx® inhibits the tumor growth in the metastatic murine neuroblastoma model

While some tumors are benign which do not invade or metastasize and can be removed from the body without further spreading, other tumors cells display uncontrolled growth, invasion that intrudes upon and destroys adjacent tissues, metastasis, or spreading to other locations in the body via lymph or blood, called malignant tumors. While the benign tumors usually can be removed by surgery, the malignant and metastatic tumors are often uncurable, metastasis causes most cancer deaths, yet this process remains the most poorly understood component of cancer pathogenesis. Chaffer *et al* suggested that

metastasis can be portrayed as a two-phase process: the first phase involves the physical translocation of a cancer cell to a distant organ, whereas the second encompasses the ability of the cancer cell to develop into a metastatic lesion at that distant site , which named colonization (Christine L.Chaffer 2011). Neuroblastoma is one of the few human malignancies, their unpredictability and high rate of metastasis combined with their malignant character make them though challenge for therapy.

Here we evaluated the effect of combined treatment of pTNF polyplexes and Caelyx® in the Neuro2A metastatic murine neuroblastoma, and found similar effect on inhibition of the tumor growth as in the subcutaneous murine neuroblastoma model. The similar therapeutic effect found in metastatic model might be due to the idea shared by Sugino et al.(Sugino 2002): the metastasis doesn't origin from a "cancer stem cell", but an aggregation of tumor cells, surrounded by epithelia, therefore preserving its organisation. It is possible that some cell aggregates were formed after inoculation and showed embolus behavior – with or without the ability to pass the vessel walls. The low doses of TNF increase tumor blood vessel permeability (Seynhaeve 2007), thus augment tissue concentrations of chemotherapy in the metastatic tumors which are located in the vincity of a main blood vessel and then enrich in microvessels, subsequently lead to the enhanced therapeutic effect caused by chemotherapy. As Bioluminescence imaging (BLI) results (**fig. 10 A)** and its quantification results (**fig. 10 B**) show, signals of luminescense of the tumor cells are significantly lower in mice pTNF polyplexes/ Caelyx® treated than mice pdeTNF polyplexes / Caelyx® treated or untreated mice. The photos of mice body cavity at 6 days after tumor cell inoculation (**fig. 10 C**) showed that mice treated with pTNF polyplexes/ Caelyx® do have significantly less metastatic tumors and the total tumor burden in mice was significantly decreased (**fig. 10 D**) when compaed to the pdeTNF/Caelyx® treated or untreated mice. As figures of HE staining showed, in all groups almost no tumors were found in kidney, lung or spleen (**fig. 10 E-1**). However, many metastatic tumors were

found in the liver (**fig. 10 E-2**). This is consistent with the first published description of this metastatic Neuro2A model in A/J mice (Amirkhosravi A. 1997). The high metastatic tumor cells in liver in this tumor model was surprising, since in a tail vein model of metastasis, the organ of first encounter is the lung and more than 90% of injected cells are initially trapped in the lungs (Amirkhosravi A. 1997). The exact reason might be complicated, and one possible explanation suggested by Amirkhosravi *et al*. is like this: it is likely that spread occurred beyond the lung stems from cells released from lung metastases into the arterial circulation. The liver provides more favorable conditions for the growth of this neuroblastoma cell line than the lung, which may be also due to availability of appropriate growth factors in the liver or an appropriate match of cell receptors and binding ligands. Moreover, other researchers also provided many other reasons. The formation of metastases in certain favored target organs may be influenced by structural differences in the capillaries of various tissues. In certain tumor types, the layout of the circulation may be the major determinant of metastatic tropism. Organ-specific homing may also constitute an active process, where tissue and cancer cell–specific features determine metastatic dissemination. The expression by metastasizing cancer cells of specific proteins (for example, integrins) seems to play a key role in this process (Christine L.Chaffer 2011). Thus, the preference of liver metastases in metaststic murine neuroblastoma model could also due to both the layout of the circulation and the organ-specific homing. We found many tumor metastases in livers of pdeTNF polyplexes/ Caelyx® treated or untreated mice, while there were almost none found in livers of the pTNF polyplexes/Caelyx® treated mice, which are almost as similar healthy as the background group (mice without tumor cells inoculation) (**fig. 10 E-2**). Here, all these results we got from Bioluminescence, figures of mice body cavity, tumor weight and the HE staining of tissue section suggest that a TNF pretreatment and then Caelyx® chemotherapy do reduce the growth and metastasis of tumor cells in vivo in the metastatic murine neuroblastoma model, and that combining

liposomal chemotherapeutic with TNF gene therapy leads to a synergistic effect on tumor reduction in this cancer model, All these results in the Neuro2A murine neuroblastoma model come to the conclusion that low doses of TNF increase tumor blood vessel permeability (Seynhaeve 2007), thus augmenting tissue concentrations of chemotherapy and destroying the tumor vasculature in both subcutaneous and metastatic murine neuroblastoma model.

4.10. The combined therapeutic treatment of pTNF polyplexes and Caelyx® inhibits the tumor growth in metastatic colon adenocacinoma model

Almost all colon cancers are primary adenocarcinoma, which has its origin in the cells of glandular structures in the inner layer of the colon. The malignancy then spreads first into the wall of the colon and potentially into the lymphatic system and other organs. Colon cancer is the third most common cancer in both men and women in North America and Western Europe. Colon adenocarcinoma can be treated, with 50 percent of patients surviving for at least five years, however, colon cancer survival is directly related to detection and the type of colon cancer involved. Surgery remains the primary treatment, while chemotherapy and/or radiotherapy are used to reduce the likelihood of metastasis developing, shrink tumor size, or slow tumor growth (André T. 2006; F.Giuliani 2010; Lombardi 2010). Despite the divergent treatment exist, the complete cure is rare and for cancer diagnosed at late stages (Cancer that metastasizes to distant sites (stage IV)) is usually not curable. Thus, adenocarcinoma is one of the most studied cancers by researchers.

Here, we evaluated the effect of combined therapeutic treatment of pTNF polyplexes and Caelyx® in the intrasplenic liver metastatic colon adenocarcinoma model, and found the similar effect of inhibition of the tumor growth as in subcutaneous and metastatic murine Neuroblastoma. A possible reason for the therapeutic effect might be similar as mentioned in **4.10.** As Bioluminescence imaging (BLI) results (**fig. 11 A)** and its quantification (**fig. 11**

B) shows, luminescence signals from tumors nodules are significantly lower in mice pTNF polyplexes/ Caelyx® treated than in mice pdeTNF poplyplexes/ Caelyx® treated or untreated. The photos of the mice body cavity (particularly the supposed metastasis organ liver and tumor cell inoculated organ spleen) at one day after last treatment showed that mice treated with pTNF/LPEI-PEG-GE11 polyplexes and Caelyx® do have significantly less metastatic tumors and reduced tumor growth (**fig. 11 C**); as well as the significantly lower weight of livers and spleens of mice (**fig. 11 D**). The reasons of the strong preference for generating liver metastases in this colon adenocarcinoma model could mainly due to the layout of the circulation (Christine L.Chaffer 2011): The disseminating colorectal cacinoma (CRC) cells may be intrinsically poorly adapted for survival in the liver microenvironment. However, because of the portal circulation, which drains from the mesentery directly into the liver, abundant carcinoma cells may be dumped over extended periods of time into the liver microvasculature; on rare occasion, an otherwise low-probability event may then generate a liver metastasis. In these various cases, homing to a particular organ can be considered to be a passive process that is determined by circulation patterns and the physical properties of the vasculature rather than by particular biological properties of the disseminating cancer cell (Christine L.Chaffer 2011).

As our results showed, more than 70% of pTNF polyplexes/ Caelyx® treated mice showed significantly inhibition of tumor growth in spleen and metastasis in liver, while this was only rarely found in pdeTNF polyplexes/ Caelyx treated animals when compared to untreated animals (**fig.11 D**). Significantly lower Bioluminescence signal strength in the liver lysate which means significantly less tumor cells are found in the livers of pTNF poplyplexes/ Caelyx® treated mice than pdeTNF polyplexes/ Caelyx® treated or untreated mice (**fig. 11E**). There was no complete tumor eradication in this model: only one mouse found in the pTNF poplyplexes/ Caelyx® treated group seems to have almost no tumors as detected by the BLI and visual inspection of the organs by eyes.

However when checking the spleen by HE staining, also tumors were found. As figures of HE staining showed, there were obviously large numbers of tumors metastases visible in the supposed metastatic organ liver and the tumor cells inoculated organ spleen in pdeTNF polyplexes/ Caelyx® treated or untreated mice, while the tumor metastases in livers from the pTNF polyplexes/ Caelyx® treated mice were significantly reduced, and the only one obvious tumor lesion found in one slice already developed necrosis with a relative small tumor size , which might be because the pTNF polyplexes and subsequently Caelyx® treatment induced the necrosis of tumor lesions at a relative early stage (**fig. 11 F-1**). However, all spleens from three groups were seriously tumor infected (**fig. 11 F-2**), although when observed by eyes, some mice from pTNF poplyplexes /Caelyx® treatment have only small tumors on the surface of spleen (**fig. 11 C**). These results might suggest that our treatment can not totally inhibit the tumor growth in mice in this metastatic intrasplenic liver metastatic colon adenocarcoma model.

In conclusion, all these results from Bioluminescence, figures and weight of the tumor infected organs liver and spleen, and the HE staining of tissue section suggest that a pTNF polyplexes pretreatment and then Caelyx® chemotherapy do reduce the growth and metastasis of tumor cells in vivo in intrasplenic liver metastatic colon adenocarcinoma model, as well as in subcutaneous and metastatic murine neuroblastoma model, and that combining liposomal chemotherapeutic with TNF gene therapy leads to a synergistic effect on tumor reduction in vivo. However, how TNF gene therapy will help the chemotherapy in other cancers is still unknown and needs to be verified.

5. Summary

Above a minimal size a tumor lesions relies on a functional blood supply, which gives access to oxygen and nutrients. This is achieved by the tumor cell's secretion of various pro-angiogenic factors. Albeit being well vascularized, solid tumor often exhibit a disorganized vessel structure and an increase interstitial pressure precluding efficient systemic treatment with chemotherapeutic drugs. Hypoxic conditions due to incomplete vasculatization can even turn cancer cells more aggressive and increase their metastatic potential (Vaupel 2008; Hoeckel 1996). In order to obtain an effective drug concentration in the tumor tissue, elevated doses have to be applied leading to unwanted side effects in non target organs. Hence, improving the accessibility of tumors for small and macromolecular chemotherapeutic drugs is hence a major aim in tumor therapy. One attempt to improve accumulation of anticancer drugs is to enhance the permeability of tumor blood vessels. This is especially of importance when applying macromolecular drugs, like liposomes or other particulate drug carriers, or even recombinant viruses used as gene carriers or virotherapy.

An important endogenous mediator of inflammatory processes and endothelial permeability is the cytokine tumor necrosis factor alpha (TNFα). Tumor necrosis factor (TNF), as a major inflammatory cytokine involved in systemic inflammation, it can induce rapid haemorrhagic necrosis in experimental cancers. It was believed that recombinant human tumor necrosis factor (TNF) has pleiotropic properties; it has strong effects on angiogenic vessels in tumors, and the ability to cause apoptosis of tumor-associated endothelial cells which leads to the complete destruction of the tumor vasculature. In the study of ten Hagen's group, it was found that that applying low-dose tumor necrosis factor (TNF) by systemic injections with PEGylated long circulating liposomes augmented the tumor accumulation of these (Seynhaeve 2007). In addition, in the isolated limb perfusion, in which TNF is coadministered with a chemotherapeutic agent for the treatment of patients with limb threatening tumors, addition of TNF results in increased accumulation of drug inside the

tumor accompanied by improved response rates (van der Veen 2000; Hoving 2006). In 1992, surgeons Ferdy Lejeune, Alexander Eggermont and their colleagues performed a local approach of TNF therapy, isolated limb perfusion (ILP), to deliver high doses of TNF in combination with IFNγ and melphalan locoregionally to patients with cancers of the extremities. This caused specific destruction of tumor vasculature, haemorrhagic necrosis and complete tumor disappearance in patients with advanced soft tissue sarcomas or melanoma (Lienard 1992; Grunhagen 2006). Also, a combination of TNF and doxorubicin treatment leads to comparable therapeutically effects in rat sarcoma model (van der Veen 2000). TNF treatment lead to an increase in endothelial permeability which subsequently induces the improvement of chemotherapy penetration within the tumor tissue; in addition, killing of angiogenic endothelial cells results in tumor vessel destruction (Seynhaeve 2007).

However, the clinical use of TNF as an anti-cancer drug is limited to local treatments because of its dose-limiting systemic toxicity (Curnis Nov.2000). Systemic application of recombinant TNF is hampered by its high systemic toxicity dictating the need to target TNF activity selectively to the tumor. Here we demonstrate localized antitumor activity of TNF after systemic injection of a non viral, biodegradable gene carrier (G3-HD-OEI) exhibiting intrinsic tumor affinity in vivo in murine neuroblastoma model (Russ Dec 2008). We also used a novel TNF encoding plasmid with CpG free backbone and hCMV enhancer and EF1α promoter, since Stephen C Hyde *et al* observed that CpG-free plasmids with the hCMV/EF1α combination confer reduced inflammation and increase the magnitude and duration of gene expression in vivo (Hyde 2004). A combination of TNF and doxorubicin had comparable effects in rat sarcoma models (van der Veen 2000). It appears that low doses of TNF increase tumor blood vessel permeability, thus augmenting tissue concentrations of chemotherapy (Seynhaeve 2007) and destroying the tumor vasculature (Colotta 1984).

Here we found that when applying TNF encoding plasmid in a similar way,

inhibition of tumor growth was seen concomitant with decreased vessel density (CD31 expression). We could also show that systemic TNF gene delivery promoted tumor accumulation of liposomally encapsulated doxorubicine (Caelyx) in subcutaneous Neuro2A murine neuroblastoma and HUH7 human hepatocellular carcinoma. A/J mice bearing subcutaneous, syngeneic Neuro2A tumors were treated with polyplexes based on a biodegradable polymer (G3-HD-OEI), HUH7 hepatoma xenografts in SCID mice received TNF polyplexes based on linear polyethylenimine carrying a peptide (GE11) selectively binding to the epidermal growth factor receptor, which is highly overexpressed in HUH7. When pretreating mice with pTNF polyplexes, improved tumor accumulation of Caelyx® was observed in both tumor models as compared to control polyplexes plus Caelyx®. With the help of live bioluminescense imaging, enhanced tumor accumulation of Caelyx® fluorescently labeled with the lipid DiR emitting in the near infrared was observed. This suggests a TNF mediated increase of tumor blood vessel permeability augmenting the accumulation of liposomal chemotherapeutics. In the Neuro2A model, three rounds of systemic pTNF polyplexes application and subsequent Caelyx® treatment led to tumor growth inhibition lasting until eight days after the last treatment. Re-growth of treated tumors could be inhibited by a second treatment cycle indicating that no resistance towards combined pTNF polyplexes/ Caelyx® treatment occurred. Histological staining of cryosection showed significant decrease of CD31 expression in the re-treated group, which indicates a decreased vessel density caused by pTNF polyplexes/ Caelyx® treatment. Similar as in the subcutaneous model, in the murine metastatic neuroblastoma model, three rounds of systemic pTNF polyplexes application and subsequent Caelyx® treatment also led to tumor growth inhibition, as the bioluminescense signal signal of the luciferase marked tumor cells and the the total tumor burden are significantly lower than in pdeTNF polyplexes / Caelyx® (pdeTNF: control plsmid) treated or untreated mice. The photos of the body cavities also showed that mice treated with pTNF polyplexes and Caelyx® do

have significantly less tumor metastases and the tumor growth was significantly reduced. Results of HE staining of livers from mice also showed that there are almost no tumor metastases remaining in pTNF polyplexes/ Caelyx® treated mice, while there are many tumor metastases were found in livers from pdeTNF polyplexes/Caelyx® treated and untreated mice. Moreover, when we applied the combined therapeutic treatment of pTNF polyplexes and Caelyx® in a intrasplenic liver metastatic colon adenocarcinoma model, a similar effect of inhibition of tumor growth as in murine neuroblastoma model was found. Both, the bioluminescense signal of lciferase marked tumors and the tumor weightis significantly lower in pTNF polyplexes/ Caelyx® treated mice than in pdeTNF polyplexes /Caelyx® treated or untreated mice. The photos of the body cavities (particularly the supposed metastasis organ liver and tumor cell inoculated organ spleen) showed that mice treated with pTNF polyplexes and Caelyx® do have significantly less metastatic tumor cells and exhibit a reduced tumor growth. Also, the weight of livers and spleens of mice were significantly decreased when compared to pdeTNF polyplexes/ Caelyx® treated or untreated mice. Consistently, significantly lower Bioluminescence signal strength in the liver lysate which means significantly less tumor cells in the livers are found in pTNF poplyplexes/Caelyx® treated mice than pdeTNF polyplexes/ Caelyx® treated or untreated mice. More than 70% of pTNF polyplexes/ Caelyx® treated mice showed significantly inhibition of tumor growth in spleen and metastasis in liver, while it was only rarely found in pdeTNF polyplexes/ Caelyx® treated mice. HE staining results showed that there were obviously large numbers of tumor metastases in livers from pdeTNF polyplexes/ Caelyx treated or untreated mice, while the tumor metastases in livers of pTNF polyplexes/ Caelyx® treated mice were significantly reduced, only a single tumor was found in one section, which already developed necrosis although being rather small in size. Spleens from three groups were seriously tumor infected, however, in pTNF poplyplex / Caelyx® treated mice, more tumor necrosis was observed.

In conclusion, pTNF polyplexes pretreatment and subsequent Caelyx® chemotherapy does reduce the growth and metastasis of tumor cells in vivo in intrasplenic liver metastatic colon adenocarcinoma model, as well as in subcutaneous and metastatic murine neuroblastoma model, and that combining liposomal chemotherapeutic with TNF gene therapy leads to a synergistic effect on tumor reduction in vivo.

6. Appendix

6.1. Abbreviations

BLI, Bioluminescence imaging

BSA, bovine serum albumin

bPEI, branched PEI

c/p-ratio, weight ratio of conjugate to plasmid

CMV, Cytomegalovirus

CpG, cytosine-guanine dinucleotide

DMEM, Dulbecco´s modified Eagle´s medium

DMSO, dimethyl sulfoxide

DNA, deoxyribonucleic acid

DiR, 1,1’-dioctadecyl-3,3,3’,3’-tetramethylindotricarbocyanine iodide

DAPI: 4',6-diamidino-2-phenylindole

EGFP: Enhanced Green Fluorescent Protein

E. coli, Escherichia coli

EC, endothelial cell

EF-1, elongation factor-1α

eFLuc ,enhanced firefly luciferase

ELISA, Enzyme-linked immunosorbent assay

EGF, epidermal growth factor

EGFP, enhanced green fluorescent protein

FITC, fluoresceinisothiocyanate

FCS, fetal calf serum

Fig. ,figure

Ex, excitation

Em, emission

GAPDH, Glyceraldehyde-3-phosphate dehydrogenase

HBG, HEPES-buffered glucose

hCMV, mCMV human, murine cytomegalovirus

HEPES, N-(2-hydroxyethyl) piperazine-N’-(2-ethansulfonic acid)

HD, hexandioldiacrylate

HE stain, Hematoxylin Eosin stian

ILP, isolated limb perfusion

hCMV, human CMV

i.p., intraperitoneal

i.v., intravenous

IFN, interferon

IL, interleukin

LPS, lipopolysaccharide

LPEI, linear PEI

miR, microRNA

mCMV, murine CMV

mRNA, messenger RNA

MyD88, myeloid differentiation primary-response protein 88

N/P-ratio, molar ratio of PEI nitrogen to DNA phosphate

NFκB, nuclear factor 'kappa-light-chain-enhancer' of activated B-cells

NK, natural killer cell

Ori, origin of replication

OEI, oligoethylenimine

PEI, polyethylenimine

p/s, photons per second

PBS, phosphate-buffered saline

PCR, polymerase chain reaction

PEG, polyethylene glycol

pDNA, plasmid DNA

PPI, polypropylenimine

polyA, polyadenylation signal

PSMC, porcine smooth muscle cell

QPCR, real-time PCR

RLU, relative light units

ROI, region of interest

RNA, ribonucleic acid

SCID, severe combined immunodeficiency

TNF-α, tumor necrosis factor α

UTR, untranslated region

w/w, weight to weight ratio

Zeo, zeosine resistance gene

6.2. Publications

6.2.1. Poster presentations

1. **Su, Baowei,** Russ, V, Carlsen, J, Wagner, E, Ogris, M. (2009) Non-viral tumor gene therapy with novel plasmids expressing the cytotoxic cytokine TNF-alpha. Combined Meeting of the 17th European-Society-of-Gene-and-Cell-Therapy/16thGerman-Society-for-Gen e-Therapy/ 4thGerman-Society-for-Stem-Cell-Research NOV 21-25, 2009 Hannover, GERMANY. Human Gene Therapy 20: 1421-1421.
2. **Su, Baowei,** Wagner, E, Ogris, M. (2010) TNF Alpha Gene Therapy Synergizes with Liposomal Doxorubicin in the Treatment of Experimental Neuroblastoma and Hepatoma in Mice. 7th Annual Meeting of German-Society-for-Gene-Therapy (DG-GT e.V.) OCT 07-09, 2010 LMU Campus Grosshadern, Munich, GERMANY Human Gene Therapy 21: 1204-1205.

6.2.2. Publications

1. **Su, Baowei,** Cengizeroglu, A, Farkasova, K, Anton, M, Haase, R, Wagner, E, Ogris, M (2011) Systemic TNF alpha gene therapy synergizes with liposomal doxorubicine in the treatment of metastatic murine neuroblastoma and human colon carcinoma. In preparation
2. Haase, R, Magnusson, T, **Su, Baowei,** Wagner, E, Lipps, H, Haas, J, Baiker, A, Ogris, M (2011) Generation of a tumour- and tissue-specific episomal non-viral vector system. In preparation

7. Reference

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9. Curriculum vitae

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