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GROWTH FACTOR GENE THERAPY IN DIABETIC PIGS: TRANSGENIC KERATINOCYTE TRANSPLANTATION TO FULL-THICKNESS WOUNDS

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Introduction: Healing of impaired and diabetic wounds represents a difficult challenge with the currently available treatment. 25% of patients with diabetes will suffer from a wound problem in their lifetime. Several studies have shown reduced expression of growth factors in chronic diabetic wounds. Our laboratory showed that Insulin-like growth factor levels are up to 40-fold decreased in the wound fluid in diabetic porcine full-thickness wounds. The potential topical application of growth factors, however, is limited by short half life and expensive production. Gene therapy to over-express growth factors might be the needed therapeutic alternative for treatment of impaired wounds.

Methods: Diabetes was induced in female Yorkshire pigs by Streptozotocin injection (150 mg/kg BW). Two weeks later 18 to 24 full thickness wounds were created on the dorsum and enclosed in polyurethane chambers. Autologous Keratinocytes were harvested using a dermatome and transfected ex vivo with plasmid DNA encoding EGF, PDGF-BB or IGF-1. Wounds were randomly divided into three groups and received suspensions of either growth factor transfected Keratinocytes, untransfected Keratinocytes or normal saline treatment. Serum and wound fluid glucose concentrations were monitored on a daily basis and growth factor concentration in the wound fluid was quantified by ELISA. Wound contraction was measured by photoplanimetry. Wound biopsies were taken on day 12 and reepithelialization was measured to determine wound healing state.

Results: Our new wound healing model showed reliably impaired wound healing in all diabetic pigs compared to non-diabetic pigs. Serum glucose was significantly increased (>350 mg/dl) in all study groups. Transgenic cells showed significant growth factor overexpression in vivo up to 150 fold. IGF-1 and EGF significantly increased wound healing in diabetic pigs ($p=0.0037$), whereas PDGF-BB showed no significant changes.

Conclusion: Transgenic Keratinocytes served as an efficient vehicle for growth factor delivery into porcine diabetic full-thickness wounds. IGF-1 and EGF gene therapy in diabetic impaired wounds are to improving wound healing significantly. Further studies are needed and ongoing to determine the synergistic effects of combined growth factor gene therapy in diabetic wound healing.

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Graft versus Leukemia with little Graft versus host disease? A clinical phase I/II study using transfusion of transduced donor T-cells for donor leukocyte transfusion

Running title: Fate of HSV-Tk- T-cells after allo-HSCT

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This work was supported in part by: Deutsche José-Carreras-Leukämie-Stiftung Grant No DJCLS-R98/11 (to Arnold Ganser) and European study group (Bonini et al.) Suicide Gene transfer in Stem Cell Transplantation (No: QLRT-2000-01265)

Abstract:

Seven patients with acute myeloid leukemia (AML) and 2 patients with chronic myelogenous leukemia (CML) were transplanted from their HLA-identical siblings with CD34-enriched stem cells (HSCT) without further immunosuppression. In order to induce graft versus leukemia (GVL) and to investigate the possibility of controlling graft-versus-host disease (GvHD), the standard transplantation protocol was adapted to include transfusion of gene-modified donor T-cells after HSCT. Donor-T-cells were transduced with the replication-deficient retrovirus SFCMM-3, which expresses the herpes simplex thymidine kinase (HSV-Tk) as a suicide gene and the truncated low affinity nerve growth factor receptor (Δ LNGFR) for selection purposes. After transfusion, SFCMM-3 transduced T cells were detectable in all patients by PCR and FACS-analyses immediately after transfusion and during the follow up period (range: 1.1-3.8 years). One of 9 patients developed acute GvHD of the skin, grade 1, 56 days after the transfusion of the transduced cells. Loss of bcr-abl gene expression was achieved in patient UPN914, after an expansion of transduced cells. Donor chimerism was stabilized after transfusion of the transduced cells in all patients treated. To date, all patients are alive, well and, with the exception of the 2 relapses, in complete clinical remission.

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Improvement of innate properties of DNA vaccines and bridging innate and adaptive immune responses by sequence modifications of the plasmid backbone

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Introduction: The main limitation of pDNA based vaccines is a low efficiency requiring high amounts of pDNA to induce strong cellular and humoral immune responses. Herein, we tested the influence of vector backbone sequence modifications, mainly CpG-content, on the innate immune system of mice and humans.

Methods and results: Various vectors backgrounds (pΔS [50,6% CpG], pΔS110⁻ [47,5% CpG]) derived from pcDNA5/FRT (Ref [100% CpG]) were tested. In comparison to pΔS, pΔS110⁻ has a 110-nucleotides long deletion in the pUCori. In contrast to Ref and pΔS110⁻, pΔS-DNA induces the secretion of high amounts of IFN γ and IL-6 after *in vitro* stimulation of naive mouse splenocytes. Analyses of cytokines patterns showed strong Th1- but not Th2-polarisation by pΔS-DNA.

Elimination of five CpGs within the 110-region in pΔS results in weak stimulation of proinflammatory cytokines indicating a strong influence of CpGs on induced immune responses. This hypothesis was confirmed by stimulation of splenocytes from wildtype and TLR9^{-/-} mice. Whereas pΔS-DNA stimulates splenocytes from wildtype mice to produce high amounts of IFN γ and TNF α , the effect was totally aborted in TLR9^{-/-} mice.

Additionally, human *in vitro* stimulations of human plasmacytoid dendritic cells with pΔS-DNA resulted in high amounts of type I interferon (IFN α) whereas no effect was detectable using pΔS110⁻ vector.

Summary: The synthesis of CpG-reduced plasmid vectors and modifications of CpG amounts deliver a basis for further rational development of DNA vaccine and pDNA vectors for gene therapy approach.

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Impact of CpG-amount of the coding region on the *in vivo* expression of Erythropoietin in a plasmid DNA approach for gene therapy

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Introduction: Plasmid DNA (pDNA) vectors have many advantages regarding safety concerns and the lack of toxicity or infectivity in comparison to viral or viral associated vectors. However, use of pDNA for gene therapy approaches is limited due to low transfection efficiency *in vivo*. Using the murine erythropoietin (mEPO) gene as model transgene, we tested the influence of codon usage optimisation and CpG-dinucleotide amount on the *in vivo* protein expression.

Methods and results: Various optimised mEPO gene regarding CpG-amount and codon usage (wt [14 CpG], opt [20CpG], ΔCpG [0CpG], max [70CpG]) were generated and subcloned into pcDNA5.

All vectors showed substantial transient expression of mEPO in murine (3T3 NIH) and human (293T) cell lines. Various amounts of the different plasmid DNA vectors were injected i.m. into *M. tibialis anterior* of BALB/c mice followed by *in vivo* electroporation. Thereby, high frequency and low voltage electric pulses conditions (50 V/cm amplitude, 50 ms duration, 8 bipolar pulses) were monitored by using a ECM 830 Electro Square Porator from BTX.

In comparison to mEPOwt, single injection of CpG- and codon usage optimised plasmid DNA (mEPOopt and mEPOmax) induced higher levels of therapeutical protein in serum and significantly increased haematocrit levels up to 8 months after application. In contrast, the CpG-depleted EPO-gene (mEPOΔCpG) induced only low EPO levels in serum and didn't increase haematocrit values.

Summary: Optimisation of the coding sequence of therapeutical genes like erythropoietin regarding CpG-modulation and codon usage improvements in combination with optimal application opens new aspects for plasmid DNA approach in gene therapy.

5

Designed New Vaccines Like the Equine Herpesvirus (EHV) Type 1 Based Viral Vectors Effectively Transduce and Activate Monocyte Derived Dendritic Cells (MDDC)

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Objectives: Recently we have proven in a Balb/c mouse model the general capacity of EHV-1 derived vectors to inducing strong cellular, humoral and mucosal responses to HIV immunogens. The primary goal of the current study is to compare a clinical trial lot of a recombinant New-York-Vaccinia Virus based HIV vaccine candidate (NYVAC-C; expressing Gag/Pol/Nef) with a corresponding EHV-based vaccine construct (EHV-C) regarding their capacity to induce maturation and activation of monocyte derived dendritic cells (MDDC).

Methods: A recombinant EHV-1 C-GagPolNef (EHV-C) was generated using BAC-technology and RED-Recombination. Viral mutants were verified by Immunofluorescence, Western- and Southern-Blot analysis. MDDCs were infected with EHV-C and NYVAC-C at different MOIs. Expression of the transgenes was monitored by FACS and Western Blot analysis. Maturation of MDDCs was determined by FACS analysis of differentiation markers (CD80, CD83, CD86, HLA-DR, CD40, CCR7) and in ELISA assay measuring secreted proinflammatory cytokine levels (IL-6, IL-10, IL-12, TNF- α)

Results: Depending on the used MOI a substantial fraction (~40%) of EHV-C infected MDDCs displayed expression of significant amounts of GagPolNef immunogens. MDDCs infected with EHV-1 show various markers for DC maturation and activation as monitored by release of cytokines and surface expression of costimulatory signals. In contrast, MDDCs infected with NYVAC-C only weakly express HIV transgenes and do, upon direct infection, not support DC maturation or activation.

Conclusion: EHV derived vectors support efficient transgene expression and provide signals required for DC maturation and activation.

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Improving DNA based therapeutics by modulating transgenic CpG content

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Introduction: Different plasmid DNA applications like DNA vaccination or gene therapy are hampered by silencing gene expression, often correlating with epigenetic control mechanisms such as methylation of CpG dinucleotides. This study aimed to determine the contribution of intragenic CpG content on the protein and RNA level in transfected and recombinant cells.

Methods: Two humanized GFP gene variants were generated containing 60 and 0 CpGs, respectively. Their reporter activity was quantified by FACS analyses in transiently transfected H1299 and in recombinant CHO and 293T cells. Protein expression was regularly analyzed by fluorescence quantification for more than one year. Additionally protein and RNA levels were analyzed by standard western blot and quantitative RT-PCR (Lightcycler) analyses, respectively. Moreover for both GFP variants the amount of new synthesized mRNA (nuclear run on-assay) and mRNA stability (*ActinomycinD* experiments) were determined.

Results and Discussion: A positive correlation of transgenic CpG content with protein expression and mRNA level could be demonstrated. The individual expression levels were constant for a period of 56 weeks. The mfi (mean fluorescence intensity) was reduced 6-9-fold (CHO cells) and 10-20-fold (293T cells) for the CpG-free GFP gene, respectively. This reduction of reporter function could be correlated to reduced protein levels, RNA copy numbers and decreased amounts of new synthesized mRNA. Regarding mRNA stability or splicing products the CpG modified constructs did not differ significantly. Altogether this observation is contradictory to the general understanding that removal of CpGs is beneficial for long term protein expression. Moreover this phenomenon has been shown for a number of unrelated genes (cytokines, *erythropoietin* and HIV *gag*), supporting the validity of this observation. Therefore we imply a general transcriptionally based mechanism of gene expression regulation via CpGs. Thus optimizing transgenic CpG content serves as appropriate strategy to improve DNA based therapeutic applications by enhancing transgene expression.

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Impact of a synthetic transdominant negative HIV-1 Gag and the Gly-Ala repeat of EBNA-1 on the inhibition of HIV-1 replication and the survival of transduced cells for gene therapy approaches

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Introduction: Transdominant negative HIV-1 Gag mutants (TDgag) have been shown to inhibit HIV-1 replication effectively by interfering with the assembly. The main problem of gene therapy approaches using TDgag derivatives is the host's immune response to the transgenes. The objective of this study was (i) to determine the capacity of a 24 aa Gly-Ala stretch derived from EBNA-1 to overcome proteasomal degradation of GA-TDgag fusion proteins and (ii) to prevent recognition of transduced cells by CD8⁺ T cells.

Methods: PM1 cells were transduced using retroviral vectors and inhibition of HIV replication was determined. Differences in mRNA levels were analysed by Light Cycler and Northern blot analysis. Expression studies were performed to test the influence of proteasome- and translation-inhibitors on protein stability. The immunological impact of the GA stretch was tested by determining the CTL responses after plasmid immunisation and additionally by *in vitro* and *in vivo* cytotoxicity tests.

Results: GA-TDgag was shown to strongly interfere with virus replication. Although N-terminal fusion of the GA-Linker slightly reduced specific mRNA levels, intracellular protein levels were increased, which could be attributed to a diminished rate of proteasomal degradation. The intramuscular immunisation of BALB/c mice with plasmid DNA coding for Gag and TDGag with and w/o GA revealed that recognition of cells expressing GA-TDgag by CD8⁺T cells is dramatically reduced.

Conclusion: GA fusion does not influence the transdominant negative properties of TDgag and counteracts immune recognition by CD8⁺ T cells, a prerequisite for potential *in vivo* gene therapy application of TD-negative polypeptides.

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Regulating cytokine expression via modulation of the coding sequence

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Introduction: In the past, chemokines and cytokines have been proven to play an important role as natural adjuvants regarding the development of vaccines, gene therapy or cancer therapy formulations. Their ability to modulate the immune system in a desired manner opens up new perspectives in the field of a rational vaccine or gene therapy design. Herein we want to evaluate new possibilities to increase the levels of cytokine expression by applying various strategies of RNA- and codon optimization. The investigation of molecular mechanisms leading to an altered cytokine expression pattern after gene optimization also is object of this study.

Methods: The nucleotide sequence of the cytokine genes was modified by applying multi-parameter RNA and codon optimization strategies without changing the encoded amino acid sequences. The impact of the different optimization strategies concerning transcriptional and translational activity of the cytokine genes, as well as RNA-stability and -export were compared in different transient and stable transfected cell lines. EMSAs with an already known, sequence specific DNA binding and transcription activating protein were carried out.

Results and Discussion: The quantitative comparison of chemokine expression after transient transfection clearly demonstrated a correlation of protein expression and the algorithm used for gene optimization. This bias was even more obvious in stable cell lines. Especially the content of CpG dinucleotides within the coding sequence seemed to be strictly interrelated to the levels of chemokine expression. Investigations on the transcriptional level showed that the amount of expressed chemokine is reflected by steady state RNA levels, while none of the gene variants exhibited a changed RNA-export pattern. Competition and methylation experiments indicate that an improved recruitment of cellular factors binding to CpG dinucleotides accounts for enhanced chemokine transcription and subsequent cytokine expression.

Conclusion: The possibility to achieve a more stable and enhanced chemokine/cytokine expression upon delivery of molecular adjuvants and the knowledge of the interrelationship between nucleotide sequence and transcriptional regulation will have a great impact on the development of vaccine or gene therapy combinations by directly influencing magnitude and quality of immune responses *in vivo*.

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Advances in the Application of Combinatorial Technology for the Optimization of Adeno-Associated Virus (AAV) Vectors

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In vitro evolution protocols have been successfully applied to screen combinatorial AAV libraries in order to obtain mutants that escape neutralization by human sera (Perabo et al. JGM 2006; Maheshri et al. Nat Biotechnol 2006).

We have further optimized panning protocols by monitoring selection rounds with Real Time PCR to follow the evolution of the viral pools after each selection round. Application of this technology to new screening experiments allowed in shorter times and at reduced costs the identification of novel capsid variants with higher transduction efficiency and improved ability to circumvent antibody neutralization at the same time. Packaging ability was unaffected by the introduced mutations. Beside providing proof of principle for the advantages of this monitoring procedure, these new variants enrich the pool of alternative vectors that can be employed for the treatment of patients with preexisting immunity to AAV-2 and further extend our knowledge of capsid biology. Our results contribute to the development of combinatorial technology for the engineering of viral particles. This emerging approach is gathering interest as alternative to rational design, overcoming the limit posed by our incomplete understanding of the infectious process, and at the same time offering a powerful tool to dissect viral biology by reverse genetics.

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Correction of β c-deficient Pulmonary Alveolar Proteinosis (β c-PAP) by hematopoietic stem cell gene transfer: Studies in a murine knockout model

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Pulmonary Alveolar Proteinosis is caused by a deficiency of the common signal transduction chain (β c) of the IL3/IL5/GM-CSF receptor superfamily is a rare and rapidly fatal monogenic disease with functional malfunction of pulmonary macrophages. While hematopoietic stem cell gene therapy represents a potentially curative therapy the defect is not functionally manifested in stem cells and stable enrichment of genetically corrected cells *in vivo* will require an additional selection system. Therefore, we have generated a retroviral vector construct (SF91-m β c-IRES-MGMT^{P140K}) expressing the murine β c (m β c) cDNA in combination with the *in vivo* selectable drug resistance gene MGMT^{P140K} coding for an O⁶-benzylguanine (BG) resistant point mutation of the DNA repair protein O⁶-methylguanine-DNA-methyltransferase and utilized this construct to transduce hematopoietic progenitor and stem cells in a murine model of m β c-deficient (m β c^{-/-}) PAP. Our construct functionally restores m β c activity as demonstrated by significantly enhanced colony formation in the presence of GM-CSF upon m β c transduction of m β c^{-/-} bone marrow cells (1 ± 0.5 vs. 19 ± 5 CFU-C/ 1×10^5 , $p = 0.002$; $n = 11$). Control cultures set up with G-CSF, EPO and SCF indicated the GM-CSF specificity of the restored cytokine sensitivity. The GM-CSF sensitivity profile of genetically corrected m β c^{-/-} cells matched that of non-transduced but also of m β c-transduced wildtype bone marrow cells. Functional expression of MGMT^{P140K} was demonstrated by increased resistance of progenitor cell-derived colonies to 10 μ M BG plus 25 to 200 μ g/ml TMZ following SF91-m β c-IRES-MGMT^{P140K} transduction. Significant *in vitro* enrichment of genetically corrected m β c^{-/-} cells was shown when SF91-m β c-IRES-MGMT^{P140K} transduced cells were exposed to 10 μ M BG plus 0, 50 or 100 μ g/ml TMZ before clonogenic culture ($11.4 \pm 4.3\%$ (0 μ g/ml TMZ) versus $36.4 \pm 9.6\%$ (50 μ g/ml TMZ, $p = 0.034$) or $73.7 \pm 19.2\%$ (100 μ g/ml TMZ, $p = 0.015$) ($n = 6$)). To analyse the effect of m β c gene transfer in our *in vivo* PAP model, SF91-m β c-IRES-MGMT^{P140K} transduced β c^{-/-} cells were transplanted into lethally irradiated β c^{-/-} recipient mice which upon hematopoietic recovery were treated with BG/TMZ for 5 weeks. When animals were sacrificed at the end of the experiment, dramatic improvements in lung pathology were observed. Lung sections showed reduction of PAS-positive regions in genetically corrected animals to near normal levels and, furthermore, a radical reduction of lymphoid infiltration. GM-CSF-dependent colony function was restored by bone marrow cells and functional as well as flow cytometric analysis demonstrated substantial enrichment of genetically corrected cells in the bone marrow by BG/TMZ application (from 10 - 20% to 50 - 90%). Our data clearly demonstrate functional correction of the β c-PAP phenotype by m β c gene transfer. Furthermore, these studies suggest MGMT^{P140K} as a candidate selection marker to enrich for therapeutic levels of genetically corrected cells in the context of hematopoietic stem cell gene therapy.

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Physical Incorporation of a Single-stranded Oligodeoxynucleotide During Targeted Repair of a Human Chromosomal Locus

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Introduction The correction of (point-)mutated genes at the level of the cellular DNA is an ambitious goal currently pursued by two basic strategies which are either viral or non-viral. Using a Moloney retrovirus derivative to introduce into the target cell a functional copy of the defective gene, success at the clinical stage has been reported in treating children suffering from SCID-X1 [1]. Unfortunately, however, cases of acute lymphatic leukemia (ALL) developed due to insertional

mutagenesis [2]. This highlights the demand for complementing targeting strategies. An interesting alternative to the 'gene replacement' strategy is the correction of the defective gene at its endogenous chromosomal locus. To this end, single-stranded (ss) oligonucleotides have been designed with various biochemical compositions. These correction molecules have been tested in targeting different types of mutations located in numerous genes and loci. From earlier studies, it had been concluded that some type of DNA repair, e.g., mismatch repair (MMR), might be involved as the step following binding of a correction oligonucleotide to its target site in a D-loop [3,4]. In the work presented, this hypothesis has been challenged by asking whether as a target intermediate an ss oligodeoxynucleotide (ODN) might directly be incorporated into the targeted genomic site. In addition, to aid in the understanding of the mechanism of targeted repair, ODN incorporation is of general interest in the context of ODN-based techniques such as ODN-supported vaccination or genome-modifying technologies.

Methods Single-stranded 21mer oligodeoxynucleotides (ODNs) of sense orientation were directed towards point-mutated enhanced green fluorescence protein transgene loci in HEK-293-derived cell clones. First gene repair assays compared ODNs carrying the canonical termini 5'-phosphate and 3'-OH with their respective variants harbouring non-canonical termini (5'-OH, 3'-H). Second, a protocol was established to allow efficient recovery of integrated short biotin-labelled ODNs from the genomes of gene-corrected cells using streptavidin-coated beads in order to test directly whether transfected ODNs become *bona fide* parts of the target locus DNA.

Results Oligodeoxynucleotides with canonical termini were about 34-fold more efficient than their counterparts carrying non-canonical termini in a phosphorothioate-modified backbone. Furthermore, biotinylated fragments were successfully recovered from genomic DNAs of gene-corrected cells.

Conclusions The experiment showed that ODNs are incorporated into a mammalian genome. This unravels one early repair step and also sets an unexpected example of genome dynamics possibly relevant to other ODN-based cell techniques.

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Use of MGMT^{P140K} as a selection marker in the therapy of monogenic diseases: Efficient selection independent of the position of the MGMT^{P140K} gene within the vector construct

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Hematopoietic stem cell gene transfer of MGMT^{P140K}, an O⁶-benzylguanine (BG) resistant mutant of the DNA repair protein O⁶-methylguanine-DNA methyltransferase (MGMT) protects transduced cells from the hematotoxic effects of alkylator chemotherapy and allows stable selection of these genetically altered cells by combined O⁶-BG/alkylator chemotherapy. Therefore, MGMT^{P140K} represents a promising candidate selection marker in gene therapy of monogenic diseases. This application will, however, most likely require efficient coexpression of MGMT^{P140K} with the respective therapeutic gene from a single bicistronic vector. We have investigated how the position, and accordingly, the expression efficiency of the MGMT gene located either 5' or 3' of an IRES site within an MESV/SFFV-based retroviral construct, affects the protection levels attained and the selectability of the genetically modified cells.

In lin⁻ murine BM cells MGMT expression levels determined by flow cytometry were 2-fold higher for the 5'- versus the 3'-MGMT vector. Subsequently, both vectors were investigated in a murine *in vivo* gene transfer model. While efficient enrichment after BG/TMZ therapy was observed for both vectors, there was a tendency for better selection with the 3'-vector (3'-MGMT: 85±3% of granulocytes and 71±7% of lymphocytes; 5'-MGMT: 67±12% of granulocytes and 66±10% of lymphocytes). Secondary transplants showed stable presence of transduced granulocytes for at least 6 months for both vectors, but again, levels of gene modified granulocytes were higher for the 3'- compared to the 5'-MGMT vector, e.g. 39±7 vs. 26±9% (Expt. 1) and 24±7% vs. 4±1% (Expt. 2). When MGMT activity was determined in BM cells of primary recipients, high levels of functional expression were observed for the 5'- and the 3'-MGMT vector (1,670±125 and 1,360±100 fmol/mg, respectively), as compared to non-transduced cells (35±16 fmol/mg). Likewise, we measured substantially decreased levels of O⁶-methylguanine in the DNA of BM cells expressing the 5'- or 3'-MGMT vector 2 hours after *in vivo* BG/TMZ challenge compared to untransfected controls (2.4 or 3.3 versus 9.2 units). Sister chromatid exchange (SCE) analysis performed at the end of the experiment revealed significantly higher SCE/cell for the 3'- versus 5'-vector in BM (4.6 ± 0.3 vs. 2.7 ± 0.2) as well as spleen cells (6.6 ± 0.3 vs. 4.3 ± 1.1).

In summary, these data demonstrate efficient selection of transduced cells by MGMT^{P140K} expressed from the 5' position of an IRES site in an SFFV/MESV based vector construct. This is of great importance in terms of

developing clinical protocols, since current gene transfer technologies may not allow to deliver high enough transduction rates for therapeutic efficacy *per se* while high initial transduction rates may increase the risk of insertional mutagenesis. The apparently inferior enrichment but higher functional MGMT activity for the 5' versus the 3' IRES-position is subject of further analysis, since a toxic effect of very high MGMT^{P140K} expression levels can currently not be ruled out.

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Selective Targeting of Orthotopically Growing Medullary Thyroid Carcinoma in *RET* transgenic mice by a newly identified peptide: New Implications for Systemic Cancer Gene Therapy

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The development of agents that specifically bind to tumor cells and their metastases is crucial for improved cancer detection and therapy. Peptides possess appropriate properties to serve as tumor targeting agents. Thus, finding new cancer selective peptides is a central goal for molecular therapies. Medullary thyroid carcinoma (MTC) represents an attractive focus for gene therapeutic strategies needed to cope with metastases or recurrent disease. We have previously reported identification of a peptide (HTFEPGV) that selectively binds to human MTC-derived TT cells *in vitro* and transplanted tumor xenografts *in vivo* using phage display (Böckmann et al., 2005; *J Gene Med* 7:179-188). In the present study, we have performed this approach in primary orthotopically growing murine MTCs of RET-C634R transgenic mice as a clinically relevant model for thyroid cancer by intravenous injection of a complex peptide library. Two rounds of screening on primary tumors yielded multiple copies of a phage that displays a cyclic 7-amino-acid peptide, SRESPHP, with a 3000-fold increase in titer between round one and two. The selected phage showed a highly specific binding to the tumor after systemic administration, whereas binding to other organs such as lung, liver, kidney, and heart was reduced up to 90%. After tail vein injection, homing to the tumor was substantially reduced in the presence of synthetic SRESPHP peptide, indicating that tumor phage interaction strictly depends on the displayed peptide. Immunohistochemical analysis of paraffin sections from mouse tissues revealed direct binding of the SRESPHP peptide to MTC tissue. Moreover, this peptide also mediates binding to human MTC cells *in vitro* and *in vivo*, suggesting abundant expression of its cognate receptor in murine and human medullary thyroid carcinoma. Because the SRESPHP peptide is also efficiently internalized into MTC cells, it likely provides the basis for a new selective therapy of medullary thyroid carcinoma.

This work was supported by grant PU188/3-2/3-3 and PU188/5-1/5-2 from the Deutsche Forschungsgemeinschaft.

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TA-p73 β Acts as an Anticancer Agent by Sensitizing Human Malignant Melanomas to Chemotherapy

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Malignant melanoma is the most aggressive form of skin cancer and has proven to be highly resistant to conventional chemotherapy. Intriguingly, the p53 tumor suppressor, a main mediator of chemoresistance in other tumor types, is rarely mutated in melanoma. However, we have previously shown that anti-apoptotic isoforms of p73 (Δ TA-p73), another member of the p53 family, are overexpressed in metastatic melanomas. Δ TA-p73 can oppose the pro-apoptotic functions of p53 and full length p73, and thus it could contribute to melanoma chemoresistance. In this study, we used an efficient adenoviral-based gene transfer approach to introduce a transcriptionally active form of p73 (TA-p73 β) in melanoma cells, with the objective of overcoming drug resistance. Interestingly, TA-p73 β significantly sensitized 5 out of 7 aggressive melanoma cell lines to the standard therapeutic agents adriamycin and cisplatin. More importantly, TA-p73 β displayed a synergistic effect *in vivo* allowing adriamycin or cisplatin to block melanoma cell growth in mouse xenograft models ($p < 0.05$). In summary, our data show that Ad-mediated TA-p73 β gene expression can markedly sensitize a subset of melanoma cell lines to adriamycin and cisplatin *in vitro* and *in vivo*, suggesting a new chemosensitization strategy for malignant melanomas.

Work was supported by grant 10-1934-Pü3 from the Deutsche Krebshilfe, Dr. Mildred Scheel Stiftung and by the FORUN program of the University of Rostock.

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Eradication of Advanced Hepatocellular Carcinoma by Transarterial Infusions of Oncolytic Vesicular Stomatitis Virus in Rats

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Primary hepatocellular carcinoma (HCC) represents one of the most lethal neoplasms worldwide and urgently warrants the development of novel therapeutics. Vesicular stomatitis virus (VSV) is a negative-strand RNA virus with potent oncolytic properties that is exquisitely sensitive to the anti-viral actions of type I interferons (IFN) in normal but not in cancer cells. Here we report that VSV, after a single transarterial infusion at the maximal tolerable dose (MTD), was capable of accessing multifocal lesions of HCC in the livers of rats, which resulted in tumor-selective viral replication, oncolysis, and prolonged survival of the treated animals. To enhance the oncolytic potential of VSV, we constructed a novel recombinant VSV vector capable of inducing syncytia formation between tumor cells through membrane fusion at neutral pH, which led to efficient intratumoral viral spread and improved oncolysis of HCC in the livers of rats. We further demonstrate that repeated administrations of the syncytia-inducing VSV, through a permanent catheter surgically implanted into the hepatic artery, led to sustained tumor-selective virus replication and this treatment regimen also achieved long-term and tumor-free survival in 18% of the treated animals with advanced HCC. When administered at doses above the MTD, VSV caused neurotoxicity as manifested by limb paralysis and/or acute lethal hepatotoxicity in immune-competent rats. We show that exogenous administration of rat IFN- α at a clinically relevant dose allows VSV treatment at doses above the MTD but is still highly oncolytic in multifocal HCC-bearing rats, thereby improving its therapeutic index. Further development of this approach may lead to a safe and effective therapeutic modality for patients with advanced HCC in the future.

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Transposition from a gene-deleted adenoviral vector results in phenotypic correction in a canine model for hemophilia B.

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Many approaches for treating hemophilia via gene transfer have been attempted in large animal models but all have potential drawbacks. Helper-dependent (HD) adenoviral vectors devoid of all viral coding sequences offer high transduction efficiencies but were hampered by transient phenotypic correction at a non-toxic dose. For persistent hemostatic correction in hemophilia B dogs, our current study utilized a novel gene-deleted adenoviral vector system which combines adenoviral vectors for high transduction efficiencies and for the first time in a larger animal the *Sleeping Beauty* (SB) transposase for somatic integration and stable transgene expression. We generated the gene-deleted adenoviral vector FTC/TcFIXattB-(FRT)2 in which a canine factor IX (cFIX) transgene expression cassette with the transposase inverted repeats (IR) is flanked by FRT sites for Flp mediated circle formation. In the presence of Flp recombinase and the very recently developed hyperactive SB transposase HSB5 (Yant et al., unpublished), the transgene expression cassette undergoes Flp mediated excision followed by HSB5 mediated integration into the host genome. To analyze transgene persistence in vivo, C57Bl/6 mice were co-transfused with 2x10⁹ transducing units of the HD vector FTC/TcFIXattB/(FRT)2 and 7x10⁸ transducing units of a second HD vector which encodes the hyperactive transposase HSB5 and Flp recombinase. Control mice received an inactive version of the SB transposase, respectively. For all groups serum levels of up to 5000 ng/ml of cFIX were detected three weeks post-injection. To analyze transgene persistence rapid cell cycling of mouse hepatocytes was induced by performing a two-thirds partial hepatectomy and by injecting CCl₄ i.p. Seventy-five days post-injection we detected serum levels of cFIX of up to 2000 ng/ml in the active SB group and no cFIX in the serum samples of the control mice. This indicated that integration of the transgene expression cassette from the episomal adenoviral vector genome into the host genome occurred.

To test for persistence of hemostatic correction of the bleeding diathesis in hemophilia B dogs we have co-injected 5.4x10¹¹ transducing units of the HD adenoviral vector FTC/TcFIXattB/(FRT)2 and 2.6x10¹¹ transducing units of the second vector which encodes HSB5 and Flp recombinase. This equals a total dose of 1.6x10¹³ viral particles (1x10¹² viral particles per kg body weight). We measured plasma cFIX levels of up to 3900ng/ml (normal level = 5000ng/ml) and observed complete phenotypic correction of the factor IX deficiency. The whole clotting time (WBCT) was reduced from >60min to 18.0 min on day 128, the length of the study to date. This was in sharp contrast to our previous studies in hemophilia B dogs in which we used a non-integrating gene-deleted adenoviral vector. In that study we observed a 3-fold increase of the WBCT already 35 days post-injection which indicates that in our current study transposition stabilized phenotypic correction. In contrast to other studies using first or second generation adenoviral vectors and keeping in mind that for the first time the in

vivo performance of the SB transposase in a larger animal was evaluated, we observed no vector-related elevation of liver enzymes and no fall in platelet counts. Taken together, this study demonstrates that this adeno-transposon hybrid vector system will be important for treating genetic diseases.

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CELL KILLING AND GENE TRANSFER BY PARVOVIRUS H1 AND DERIVED VECTOR IN DIFFERENT HUMAN TUMOR CELLS THAT LACK P53

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Key words: Autonomous parvovirus, apoptosis, human, hepatocellular carcinoma, hepatocytes

Running title: PARVOVIRUS H1 IN HUMAN TUMOR CELLS

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Because of their natural oncotropic and oncolytic properties autonomous parvoviruses deserve to be considered as potential antitumor vectors. Parvoviruses preferentially replicate in and kill *in vitro*-transformed cells and reduce the incidence of spontaneous and implanted tumors in animals. Mutations in the tumor suppressor p53 are a common event in human tumors. Abrogations of p53 function lead to more aggressive cancer phenotypes and a worse clinical outcome. Therefore, a major goal of molecular oncology is to identify means to kill cells lacking p53.

Methods: We explored the role p53 in regulating the pathway of cell killing and the gene transfer of wild-type (wt) and recombinant parvovirus H1 in three pairs of cell lines with different p53 status. Two of them were isogenic human hepatocellular cell lines. In p53-negative Hep3B, an inducible p53 was introduced using a tamoxifen-regulated p53-estrogen receptor chimera (Hep3B BT-4P). The p53^{+/+} HepG2 cells were transfected with a dominant negative p53 (HepG2 dn-p53) to abolish wt p53 function. The third cell line, MCF7 is derived from a human breast adenocarcinoma and p53-positiv. MDD contains a dominant negative variant of the p53 protein. Furthermore we explored the influence of treatment with cytostatic drugs in combination with H1 virus-infection to the p53 different cells HepG2 and MCF7. Apoptosis rate of HepG2 and MCF7 cells after infection with H1 and/or treatment with cytostatic drugs was determined.

Results: In all cell lines, induction of transcriptionally active p53 was confirmed by assessing p53 or its dependent targets. Despite all human HCC cells were susceptible to H1-induced apoptosis, cell toxicity was more pronounced at low virus multiplicities in wt Hep3B and HepG2 dn-p53 than in Hep3B BT-4P and wt HepG2 cells (Viability assay and FACS-scan). Apoptotic cell death correlated with viral non-structural protein expression (Western). The apoptosis rate of the p53 negative cell lines were enhanced after combined treatment with cytostatic drugs and H1 virus-infection.

Conclusions: H1 kills human tumor cells independently of p53. However cells lacking p53 were more susceptible to H1 at low virus concentrations. Thus, H1 virus and its derived vectors may be considered as therapeutic options for HCC and breast cancer, especially for p53 negative tumors. Furthermore, H1 parvovirus infection enhances cytostatic drug therapy in p53 negative tumors.

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Selection for tumour homing of retroviruses displaying a human antibody library

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The specific delivery of anti-cancer drugs to tumour tissue remains a major challenge for the development of more efficient cancer therapies. Tumour growth is linked to angiogenesis and tissue remodelling. Laminin is a key component of the extracellular matrix which is involved in angiogenesis and thought to be exposed in the vasculature of the tumour tissue. Recently, the laminin-specific single chain antibody L36 has been selected from a phage display library that has anti-angiogenic properties and is able to reduce tumour growth (1). We used laminin as target molecule to set up a retrovirus based eukaryotic antibody display library system. Selection of this library (scFv-x-Mo) resulted in viruses L28-Mo and L6-Mo that when bound to laminin remained infectious (2). Here we describe the evaluation of the *in vivo* tumour targeting capacity of these viruses and the set up of an *in vivo* system that selects the scFv-x-Mo library for efficient tumour targeting upon *i.v.* delivery. The wildtype Moloney murine leukaemia virus (MoMLV) as well as viruses L6-x-Mo, L28-x-Mo and L36-x-Mo (10⁶ i.u each) were injected into the tail vein of SCID mice bearing a subcutaneous tumour derived from the human fibrosarcoma cell line HT1080-Rec1, respectively. After two weeks mice were sacrificed, tumour cells were recultivated and genomic DNA of different organs isolated. All virus types became detectable in the tumour tissue with the highest fraction of virus positive cells for L36-X-Mo (47%). PCR analysis of the viral biodistribution confirmed these data showing strongest signals in tumour tissue for L36-X-Mo while MoMLV was mainly detectable in bone marrow and spleen. We then followed basically the same protocol to select the scFv-x-Mo

library in these mice, with the only difference that three injections of the library were performed within six days. Immunostaining of recultivated tumour cells showed an infection rate of below 1% for the first but of about 7% for the second injected mouse. PCR analysis of isolated genomic DNA showed strongest signals in tumour, but lung and spleen also showed PCR fragments of the expected size. Fingerprint analysis of the cloned PCR fragments indicated the selection of three different antibody variants. Further characterisation of the selected variants is underway. The data described demonstrate that retroviruses displaying laminin-specific antibodies show significantly enhanced tumour tropism as compared to the wild type virus. Most likely, these viruses accumulate at vascular sites of tumour growth due to binding to exposed laminin, followed by infection of activated endothelial cells and budding into the tumour tissue. These viruses will be prime candidates for targeted tumour therapy and can serve as a unique platform for the in vivo selection of antibodies.

(1) Sanz et al., 2003; EMBO J. 22, 1508

(2) Urban et al., 2005 ; NAR 33, e35

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Induction of apoptosis by ectopic expression of BH3-only member Noxa in melanoma cell lines

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Background: Although melanoma cells can undergo self-destruction via programmed cell death (apoptosis) the most genotoxic agents act primarily via p53 that is not frequently mutated in melanoma, the resistance of metastatic melanoma to apoptotic stimuli including radiotherapy and chemotherapy still remains an obstacle to treatment. Therefore, we developed a novel therapeutic approach based on the activation of apoptotic effectors downstream of p53 to overcome melanoma resistance to anticancer agents is considered. In this study, we provided evidence for induction of melanoma cell death by the ectopic expression of the BH3-only member Noxa.

Methods: Flow cytometry, Western blot, Immunocytochemistry, Kinase assay, electrophoretic mobility shift assay (EMSA)

Results: A375 and BLM cell lines transfected with Noxa caused cell death in melanoma cells in up to 80% as evidenced by immunocytochemistry for caspases and cytochrome c and annexin V-flow cytometry. Cleavage of PARP in Noxa-transfected melanoma cells indicated that Noxa-induced cell death was mediated through an apoptotic mechanism. In addition, the activation of JNK and p38 pathways and the suppression of ERK as well as the activation of the transcription factors AP-1 and ATF-2 suggest the involvement of MAP kinase signaling pathway in Noxa-mediated apoptosis of melanoma cells.

Conclusion: The ectopic expression of Noxa gene causes apoptosis in melanoma cell lines that are intrinsically resistant to conventional therapies.

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Coapplication of vascular permeability factor significantly enhances long-term transduction of cardiomyocytes by retroinfusion of adeno-associated virus serotype 2 into the coronary vein

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Efficient regional delivery and long-term gene expression may be of crucial importance for successful gene therapy of cardiac diseases. Local gene transfer to cardiomyocytes is hampered by the vascular endothelium, which represents a barrier to distribution of vectors via the vasculature. In this study, regional application of vascular endothelium growth factor/vascular permeability factor (VEGF) to achieve acute permeabilization of the microvasculature was combined with retrograde delivery of adeno-associated virus serotype 2 into the coronary vein.

Methods: In pigs 2.5×10^{12} genomic particles of a modified AAV serotype 2 (AAV2.CMVMLC2v.Luciferase) were applied into the target territory by selective pressure-regulated retroinfusion of the coronary vein with (n=5) and without (n=5) coapplication of 100µg rhVEGF165. 4 weeks later the pigs were sacrificed and myocardial reporter gene expression was determined by luciferase activity.

Results: Selective retroinfusion of AAV 2 into the AIV showed moderate reporter gene expression in the targeted distal LAD territory (3034 ± 573 vs. control territory 110 ± 12 RLU/mg protein). Coapplication of VEGF significantly enhanced transduction of myocytes, and significant reporter gene expression in the target LAD territory was measured (31606 ± 5329 RLU/mg protein) compared to animals without VEGF treatment ($p < 0.05$). Significant transgene expression was not detected in other organs than the heart.

Conclusion: Selective pressure-regulated retroinfusion of a modified adeno-associated virus serotype 2 into the coronary vein is feasible and safe. Coapplication of rhVEGF165 significantly enhances AAV-2-transduction in the targeted LAD area of the porcine heart.

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ENHANCER-DELETED GAMMARETROVIRAL VECTORS FOR THE CORRECTION OF CHRONIC GRANULOMATOUS DISEASE

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Chronic Granulomatous Disease (CGD) is a rare inherited immunodeficiency caused by a functional defect in the microbial killing activity of phagocytes. CGD is caused by mutations in any of four genes encoding for subunits of the phagocyte nicotinamide dinucleotide phosphate (NADPH) oxidase complex (gp91^{phox}, p22^{phox}, p47^{phox} and p67^{phox}). Although curable by HSC transplantation, this strategy is usually limited only to patients with HLA-matched sibling or unrelated donors, as mismatched transplantation is associated with high morbidity and mortality due to graft failure and slow immune reconstitution. A therapeutic alternative for CGD patients is the genetic modification of autologous HSC by retroviral vectors. However, retroviral insertion can lead to activation of neighboring genes eventually resulting in altered cell physiology. To minimize the effects of retroviral insertion on gene expression we decided to construct gammaretroviral vectors lacking the enhancer/promoter elements within the viral long terminal repeats (SIN vectors). To this end we have developed a series of SIN gammaretroviral vectors expressing the gp91phox cDNA either from an internal SFFV promoter or from an internal myeloid specific promoter. Several vector backbones were tested and GALV pseudotyped retroviral particles were produced from an improved 293T based packaging system. Titers up to 3x10⁶ TU/ml were achieved in transduced 293T as well as in PLB985 X-CGD cells. This cell line was also used to compare the functionality of the various constructs in cytochrom C assays after differentiation to neutrophil-like cells and stimulation with PMA. Extensive functional and genotox studies revealed that these vectors are safer than conventional MLV-based retroviral vectors and equal or even superior to our current clinical vector SF71gp91phox in reconstituting NADPH oxidase activity in gp91^{phox} deficient cells lines.

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Recombinant immunoreceptors: dissecting and modulating a redirected anti-tumor T-cell response

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During the last years we have shown that differentiated T cells from the peripheral blood can be redirected towards defined target cells by retroviral expression of a recombinant surface molecule (immunoreceptor) that mediates both binding to the predefined antigen and induction of cellular activation. The design of the immunoreceptor has a number of advantages:

- (i) the receptor molecule is modularly composed allowing de novo composition of signalling and binding properties;
- (ii) receptor binding is independent of MHC presentation of antigen allowing T cell targeting towards unconventional T cell targets, i.e., carbohydrates or lipids;
- (iii) the receptor molecule triggers specifically T cell effector functions.

We used the concept primarily to develop a strategy for the immunotherapy of malignant diseases. The concept, moreover, has the power to answer a number of relevant questions in T cell immunology and adoptive immunity.

Our aims are:

- (i) to elucidate the structure-function relationship of signalling receptor molecules and their interaction with the endogenous TCR in order to develop an optimized molecular design for immunoreceptors as prerequisite for clinical use of redirected T cells;
- (ii) to manipulate certain T cell effector functions in a specific way by manipulating secondary stimuli in order to alter an ongoing immune response;
- (iii) to manipulate the tumor environment loco-regionally in order to break the immunological barrier;
- (iv) to target regulatory T cells in order to repress or increase their effector functions.

The high complexity of the recognition and signalling process makes the specific manipulation by receptor triggered T cell functions even more difficult but opens the possibility to use the highly effective machinery of immunological effector functions for a redirected immunological response against predefined target cells. Knowledge we obtain by dissecting the T cell targeting process allows us to manipulate the deregulated immune surveillance as found in a number of malignant and non-malignant diseases.

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Dominant T cell receptors (TCR) can replace other TCR after TCR gene transfer

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T cell receptor (TCR) gene transfer into peripheral blood lymphocytes (PBL) can be used to produce T cell populations with defined antigen specificity for adoptive therapy. TCR gene transfer has been successfully applied to endow T cells with specificities directed against tumor and viral antigens. On $\alpha\beta^+$ T cells, the TCR is expressed as a heterodimer of α and β chains and stabilized by the invariant molecules of the CD3 complex. Engineering of T cells by TCR gene transfer leads to the expression of two different TCR α and TCR β chains. Theoretically, these cells have the ability to express TCR with four different specificities on their surface, the endogenous and the transgenic ones and two mixed TCR heterodimers consisting of endogenous and transgenic TCR chains. Mixed TCR heterodimers possibly acquire new specificities, which could cause unwanted reactions in patients after adoptive T cell transfer. Moreover, the expression of additional TCR molecules with different specificities could reduce the expression level of the transgenic TCR and affect T cell function. We examined the possibility of mixed TCR heterodimer formation using defined conditions of single and double TCR chain transfer into mouse and human T cells. In a mouse model, we transferred TCR chains of the LCMV-gp₃₃-specific P14 TCR into the T cell line B3Z and into splenocytes of OT-I mice. In a model with human cells, we transferred chains of the gp100 melanoma-reactive TCR into a CMV-specific cytotoxic T lymphocyte (CTL) clone. Using flow cytometry after staining with TCR β -specific antibodies, we showed that mixed TCR heterodimers were expressed on the cell surface. As the specificity of those mixed TCR heterodimers is not known, they have to be considered as a risk factor in gene therapy approaches with TCR gene-modified T cells. Furthermore, we found that some TCR have the potential to replace other TCR. We analyzed this phenomenon on B3Z cells after transduction with the P14 TCR and on the CMV-specific CTL clone after transduction with the gp100 TCR. To exclude the influence of different promoters expressing the different TCR, we used the TCR-deficient cell line Jurkat76 to transfer two TCR in different combinations (e.g. TCR26/TCR53 (both RCC-reactive), TCR53/gp100 TCR, and TCR26/gp100 TCR) into those cells. For some combinations, a TCR replacement could be detected. Such a replacement would reduce the risk of mixed TCR heterodimers and of low expression levels of the desired TCR. Finding out, why some TCR can replace other ones, would have advantages for adoptive T cell therapy as dominant TCR could be selected or TCR could be modified to be more dominant.

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Depletion of gene-modified T cells as a safety mechanism in adoptive T cell therapy

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Cytotoxic T lymphocytes (CTLs) specifically recognize foreign antigens presented on their target cells via MHC class I molecules. Their specificity is determined by the T cell receptor (TCR) expressed on the surface of the lymphocyte. *In vitro* modification of a population of CTLs with TCR genes can provide these cells with a new specificity. This enables them to recognize and eliminate their target cells after adoptive transfer. However, there are possible problems with TCR gene-modified T cells. (i) Since retroviral vectors are exclusively utilized for TCR gene transfer, there is the risk of insertional mutagenesis that might lead to the development of lymphomas as recently reported for genetically engineered hematopoietic stem cells. (ii) It was shown that activation of the transgenic TCR by recognition of target antigens will also allow effector functions via the endogenous TCR. If the endogenous TCR is self-reactive, the therapy might lead to auto-immune side effects which cannot be tolerated. (iii) In the same way, pairing of a transgenic TCR chain with a chain of the endogenous TCR can lead to the generation of mixed TCR heterodimers with unpredictable specificity that may have auto-reactive potential. The aim of the project is to develop a strategy to eliminate adoptively transferred, TCR-engineered T cells *in vivo*. By introducing a tag into the TCR structure, it should be possible to deplete the modified cells by administering the tag-specific antibody to the patient. For this, the murine TCR P14 which recognizes an antigenic peptide on the LCMV gp33 protein has been modified with a myc-tag at nine different reasonable sites. It was then cloned into retroviral vectors, which were used to transduce murine T cells. Transduced cells were analyzed for expression and functionality of the TCR α and β chains via flow cytometry with TCR chain-specific antibodies and multimers. Cells carrying the modified TCR were able to bind to the specific peptide-MHC pentamer and showed activation upon peptide stimulation as shown with the indicator cell line B3Z. Each one construct with a TCR α and TCR β chain modification led to depletion of cells *in vitro* when incubated with a myc-specific antibody and complement. For further studies, it is planned to (i) adoptively transfer myc-tag-TCR-modified T cells into an auto-immune mouse model for *in vivo* proof-of-concept and (ii) reproduce the findings in the context of human TCRs.

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Redirection of T cells using chimeric T cell receptors against HBV surface proteins

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Despite antiviral therapy, hepatitis B Virus (HBV) infected cells often continue to release subviral particles composed of small (S) and to a lesser extent large (L) surface protein. We therefore established chimeric T cell receptors (TCRs), which target HBV infected hepatocytes via single chain antibody fragments (scFv) recognizing S or L protein on their surface. One scFv against S and one against L were selected because they proved specific by ELISA and were functional in the context of chimeric TCRs. Whereby scFv against S bound to native but not to denatured antigen suggesting recognition of a conformational epitope. Chimeric TCRs consisting of an extracellular anti-S or -L scFv and intracellular CD3 ζ and CD28 signalling domains were constructed and retrovirally transduced into primary T cells to redirect them to HBV infected cells. Human and murine TCRs contain species-specific spacer and signalling domains. TCRs directed to carcinogenic embryonal antigen (CEA) and untreated T cells served as control. T cells were cocultured with HBV producing or control cell lines. T cells carrying HBV-specific human TCRs, but not control T cells, released IFN γ and IL-2 upon contact with HBV infected primary human hepatocytes and HBV replicating hepatoma cell lines, and killed these. Respective HBV negative cells were not targeted. Murine TCRs were functional in human T cells and mediated specific cytotoxicity. HBV particles bound to the surface of hepatoma, but not of e.g. Hela cells activated redirected T cells. In comparison, TCRs directed against S were more active than those against L correlating to the abundance of S-protein. *In vivo* studies with intravenous injection of redirected murine T cells into HBV transgenic mice are underway. We are confident that, retargeting of primary T cells by chimeric TCRs is a promising approach to eliminate residual HBV infected cells after antiviral treatment.

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Matrix metalloprotease activatable viruses for the virotherapy of solid liver tumours

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In addition to being one of the most frequent malignancies worldwide, hepatocellular carcinoma patients usually turn up only at advanced tumour stages, which do not allow surgical treatment, anymore. Conventional chemotherapeutic approaches are not able to prolong survival of patients, so far, stressing the fact that new strategies for treatment of liver tumours are urgently needed, at best without any damage to healthy liver tissue. For virotherapy approaches, oncolytic viruses conditionally replicating in cancer cells form an attractive novel class of anti-tumoural agents. We have recently shown that the spreading of retroviruses can be restricted to tumour cells by making them dependent on Matrix metalloproteases (MMP). MMPs are secreted by cancer cells to degrade the surrounding extracellular matrix and are responsible for some hallmarks of cancer. To create this kind of agents, we modified the retroviral envelope protein with blocking domains which prevent cell entry until MMP cleavage of the connecting linker peptide occurs. We also generated libraries by combinatorially diversifying the linker peptide. Selection of such libraries resulted in a series of MMP activatable retroviruses and MMP substrate peptides. The *in vivo* spreading behavior of MMP-activatable retroviruses was monitored in SCID mice transplanted with HT1080 tumour cells. The library selected virus clones showed a strongly enhanced dissemination efficiency spreading through the complete tumour tissue upon intratumoural injection. PCR analysis of tumour cell DNA confirmed their genetic integrity. Interestingly, spread from the infected tumour to a distantly transplanted uninfected tumour was also observed. When systemically administered, the unmodified wildtype virus infected spleen, liver and bone marrow tissue, while the MMP-activatable viruses were not detectable. To transfer the MMP activation concept to oncolytic viruses we have engineered two of the library selected cleavage sites (AKGLYK and PLGLHV) into the measles virus (MV) fusion protein F, which requires activation by the ubiquitous intracellular protease furin. Expression of the F proteins having the two linker peptides inserted at the furin cleavage site showed a selective fusogenic activity in MMP-expressing tumour cells for F proteins having the PLGLHV linker peptide incorporated. Upon reinsertion of these F-variants into the MV genome, the replicative and oncolytic properties of the corresponding MMP activatable viruses will be characterized *in vitro* and *in vivo*, as well as on patient samples. Cell lines and tissue slices from patients with liver tumours can be used to collect preclinical data. So far, zymography analysis revealed moderate activity of proMMP2 in HepG2, Huh7, and Hep3B hepatoma cells. However, strong activity of tissue plasmin activator (tPA) as well as of an unidentified high molecular weight protease was detected in a plasminogen-gelatine zymography in Hep3B cells. In patient samples, high levels of MMP2 and urokinase PA activity were selectively detected in liver tumour sections while surrounding tissue samples were negative. The data demonstrate that MMP activation can efficiently restrict virus spreading to MMP-positive tumour tissue. The protease activity profile of patient samples strongly suggests the application of MMP activatable oncolytic viruses for liver cancer therapy.

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Gene Therapy of CAMT: A Difficult Balance to Cure MPL Deficiency

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CAMT (Congenital Amegakaryocytic Thrombocytopenia) is a severe inherited disorder with an expected life span of one to ten years. The phenotype of the disease is thrombocytopenia, absence of megakaryocytes and impaired stem self renewal

which later leads to aplastic anemia. The only known curative therapy is allogenic bone marrow transplantation. The disease is caused by inactivating mutations in the c-Mpl gene, the receptor for TPO (thrombopoietin). Conversely, activating mutations of c-Mpl may lead to leukemia. To address the consequences of a worst case scenario in which retroviral vectors introduce activating mutations into c-Mpl, we transduced murine bone marrow cells with a constitutively active form of Mpl (caMpl). Following transplantation into irradiated mice, these cells rapidly induced an aggressive leukemia with massive hepatosplenomegaly and leukocytosis (latency of three weeks). The latency of the disease depended on the expression level of caMpl. In line with these observations, caMpl expression led to growth factor independence of murine 32D cells, which normally require interleukin-3 for self-renewal. To address potential side effects caused by ectopic over-expression of wild-type c-Mpl, we constructed retroviral vectors that express high levels of c-Mpl under control of the retroviral long terminal repeats. 32D cells transduced with c-Mpl proliferated in the presence of TPO, but were not growth-factor independent. Interestingly, ectopic expression of c-Mpl in repopulating hematopoietic cells of C57Bl6 mice was found to cause severe anemia in all hematopoietic lineages. Control mice receiving cells transduced with a dominant negative form of c-Mpl that lacked the intracellular signal transduction domain developed a similar phenotype. Some of the animals transplanted with c-Mpl transduced cells became leukemic with a phenotype reminiscent of caMpl. It remains to be tested whether these leukemias contained a mutated form of c-Mpl or were caused by insertional mutagenesis. Interestingly, we also observed mice that showed no severe alterations of hematopoiesis, suggesting the existence of a therapeutic window for ectopic c-Mpl expression. These data reveal that successful gene therapy of CAMT needs to achieve physiological expression levels and avoid activating mutations. This model illustrates the importance of defining tightly regulated expression vectors to correct diseases associated with mutations in signalling molecules.

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Upstream polyadenylation enhancers in the U3 region of self-inactivating retroviral vectors improve titer and gene expression, and prevent transcriptional read-through

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Severe adverse events related to insertional mutagenesis have reinforced the interest in self-inactivating (SIN) retroviral vectors lacking enhancer-promoter sequences in the U3 region of the long terminal repeats (LTRs), thereby decreasing the likelihood of transactivation of neighboring alleles. However, deletion of the 3' U3 region may impede transcriptional termination, increasing the probability of read-through into cellular genes (Zaiss et al., *J. Virol.* 76, 2001). To improve 3' end processing, introducing strong heterologous polyadenylation signals within the R region or downstream of the 3' LTR has been proposed. However, these modifications only improve polyadenylation in transfected packaging cells and are lost in transduced target cells. In contrast, including such sequences into U3 would act both during vector production and in transduced cells. Interestingly, a number of viruses and cellular genes have evolved upstream polyadenylation enhancer elements (USE) to strengthen polyadenylation efficiency. We therefore incorporated 7 different USE elements derived from HIV, SV40, WHV, adenovirus, prothrombin and C2 complement factor, and cloned these into the 3' U3 deletion of gammaretroviral SIN vectors. This resulted in an enhancement of both titer and gene expression in several cell lines and hematopoietic primary cells. Similar effects were observed in the context of 3 internal promoters (CMV, PGK, SFFV). A recombinant 100 bp sequence designed on the basis of the SV40 USE element gave the best results. Compared with the WHV posttranscriptional regulatory element (PRE), this element mediates approx. 50% of the enhancing activity on gene expression and titer. Importantly, the recombinant SV40 USE also prevents transcriptional read-through, in contrast to the PRE. This was confirmed by FACS and Northern blot assays, using a sensitive reporter assay in which a fluorescent protein is expressed from an internal ribosomal entry site inserted downstream of the 3' LTR. Finally, the recombinant SV40 USE also enhanced titers and gene expression and decreased read-through in 3rd generation lentiviral vectors lacking the PRE. Of note, improvement of polyadenylation efficiency was also shown in the context of the clinically relevant IL2 receptor gamma chain (X-SCID). Taken together, the presence of small USE elements in retroviral or lentiviral vectors can substitute for the presence of a PRE element, leading to a gain in titer and gene expression levels. The decreased likelihood of read-through over the termination signal potentially improves biosafety.

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Reverse transcriptase deficient retroviral vectors for transient cell modification

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Short-term, reversible expression of foreign proteins could be useful to modify cell fate. We have previously shown that retroviral vector mutants that are unable to initiate reverse transcription of their plus-stranded mRNA genomes mediate transient and highly efficient delivery of the site specific recombinase Cre into human and mouse fibroblasts (Galla et al., *Mol Cell* 2004). Cre protein activity was detected in >95% of target cells with a single treatment. Cre activity was particle-mediated and required the retroviral mRNA packaging signal as well as the expression of both Gag and Env in the packaging cell. Furthermore, this mode of Cre delivery is restricted by the tripartite motif protein TRIM5 α (collaboration with G. Towers, London), a restriction factor mediating an early

postentry block in human cells. Since TRIM5 α interacts with the incoming retroviral core and acts usually before viral DNA synthesis, this supports the working hypothesis of particle mediated mRNA transfer and excludes plasmid contamination and Cre protein transduction as the underlying mechanism. Our data thus suggest that retrovirally delivered mRNA serves as an immediate translation template if not being reverse transcribed. When delivering the Sleeping Beauty (SB) transposase (cooperation with Z. Ivics, Berlin) by this approach, RT-deficient retroviral vector mutants were sufficient to mediate transposition of transfected plasmids containing transposon DNA. However, the efficiency of stable integration was only 3-fold above background levels, suggesting that the timing and/or expression levels have to be improved. Side-by-side comparison with the delivery of episomal DNA by lentiviral vectors revealed that RT-deficient retroviral mutants express foreign proteins for a much shorter duration (max. 4-5 days in replicating cells as opposed to >7 days for episomal lentiviral vectors) and lower levels (one to two orders of magnitude below the activity provided by episomal lentiviral vectors). As many receptor molecules require relatively low levels of expression to confer biological effects, we tested RT-deficient retroviral vectors to deliver the sialomucin CD34 or the murine cationic acid transporter mCAT-1, which serves as the receptor for the ecotropic murine leukemia virus. ~90% of the exposed cells could be transduced following a single exposure to RT-deficient vectors encoding these receptors, and mCAT-1 expression conferred susceptibility of Jurkat cells to transduction with retroviral or lentiviral vectors pseudotyped with the murine ecotropic envelope protein. We conclude that RT-deficient retroviral vectors hold great promise for applications in which low and transient expression of proteins achieves striking biological effects.

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The Insertional Dominance Database (IDDb): Retroviral vector insertion sites associated with malignant and benign clonal dominance in murine models of bone marrow transplantation

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Insertional side effects of replication-defective retroviral vectors may leave cells unperturbed, confer a selective disadvantage, or induce clonal dominance. To select retroviral vector insertion sites (RVIS) present in prominent clones and neglect clones of minor size, we modified and validated ligation-mediated PCR (LM PCR) originally introduced by Schmidt *et al.* To date we have accumulated >300 RVIS from several murine long-term studies resulting in benign or leukemic clonal dominance. Interestingly, serially transplanted benign clones and leukemias showed some similarities in distribution of RVIS for signalling genes but differences for protooncogenes. About 5% of RVIS affect the Evi1 locus which encodes a Zinc finger transcription factor expressed in primitive hematopoietic cells; 15% are other common integration sites (58% of which are known proto-oncogenes; 32% are signalling genes and 10% are unknown/metabolic genes). The resulting insertional dominance database (IDDb) shows substantial overlaps with the transcriptome of primitive hematopoietic cells and the retrovirus-tagged cancer gene database (RTCGD). In contrast to the RTCGD which collects data obtained with replication-competent retroviruses, the IDDb contains a unique set of genes involved in benign dominance and also shows some proto-oncogenes to be associated with different leukemia phenotypes. The IDDb thus forms an increasingly powerful resource for the identification of genes that stimulate or transform hematopoietic stem cells.

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Gene Therapy of Cystinuria

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Cystinuria is one of the most common genetic disorders with a prevalence of 1 in 7000. It is due to the defective transport of cystine and dibasic amino acids through the kidney epithelium, caused by mutations of an amino acid transporter encoded by the genes SLC3A1 and SLC7A9. This results in the formation of calculi in the urinary tract leading to obstruction, infections and finally renal insufficiency. To restore the transport capacity of deficient epithelial cells in renal tubules, we constructed MLV and FIV vectors carrying an SLC3A1 expression cassette. Using these vectors we were able to transfer the SLC3A1 gene to cells of the proximal tubular kidney cell line HK-2. SLC3A1 gene expression was blocked in HK-2 cells by transfer of an antisense expression construct. Furthermore, we started *in vivo* studies in a mouse model of cystinuria. In a first approach, routes and techniques for delivery of vectors to the kidney were explored in SLC3A1 gene knockout mice. Currently, mice are treated with SLC3A1 expressing vectors and the results are monitored by analysis of metabolic markers, control of the gene transfer efficiency and phenotype analysis.

IMPROVEMENT OF DLI SAFETY IN PEDIATRIC LEUKEMIA PATIENTS BY COMBINING TWO INDEPENDENT SUICIDE STRATEGIES

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Adoptive immunotherapy employing donor lymphocyte infusions (DLI) represents an attractive treatment option for pediatric leukemia patients after allogeneic stem cell transplantation. In case of an increasing mixed chimerism due to either a relapse or a graft rejection, DLI can induce a Graft-versus-Leukemia-Effect (GvL) and is particularly effective to revert a graft rejection. However, application of DLI is hampered by a high risk of life-threatening Graft-versus-Host Disease (GvHD) representing the dose-limiting factor for this treatment. The risk for GvHD can be reduced by genetically modifying alloreactive T cells with suicide genes that allow for their eradication in case of a GvHD. Since a fast and almost complete elimination of alloreactive T cells has to be achieved for pediatric patients, we have combined two suicide approaches, which should allow for both an increased purification efficiency due to two selective markers and an enhanced elimination by means of two independent killing mechanisms. The model T cell line HuT78 was transduced with a retroviral vector encoding the B lymphocyte antigen CD20. CD20-positive cells can effectively be killed by Rituximab, a monoclonal antibody against CD20, by means of complement-dependent cell death. Rabbit serum was used as a source of complement and showed a stronger induction of cell death in comparison to human serum. After positive selection using CD20 microbeads, HuT78 CD20⁺ and wild type cells were transduced with a ganciclovir-hypersensitive mutant of herpes simplex virus thymidine kinase (tk39) fused in frame to a truncated version of CD34 (tCD34) functioning as a second marker gene for purification. Double- and single-transduced HuT78 cells were exposed to 10 µg/ml Rituximab (RTX) and 15% rabbit serum complement in combination with 10 µM Ganciclovir (GCV) for up to 96 hr. Samples were taken at various time points and analyzed by flow cytometry for CD20 and tCD34 expression. In order to determine the absolute number of surviving cells at each time point analyzed, propidium iodide (PI) and thiazole orange (TO) stainings allowing for the discrimination between dead and living cells were performed in combination with liquid counting beads. After 5 hr of treatment, Rituximab-induced cell death was observed in 92% of double-transduced Hut78 cells in comparison to non-transduced cells which showed only little or no cell death. An enhanced elimination of double-transduced cells due to the combined treatment with RTX and GCV was observed after 96 hr (98.3%) in comparison to cells expressing only one suicide gene (92.6% tCD34tk39⁺ and 76.2% CD20⁺). First results suggest an initially fast and subsequent enhanced elimination for double-transduced HuT78 cells after treatment with Rituximab and Ganciclovir. Further studies employing a bicistronic vector encoding both suicide genes are required in primary human and murine T cells to show the effectiveness of the combined suicide system for safety enhancement of DLI.

Supported by GK 1172

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Effects of non-oncogenic deletion mutants of adenoviral E1A on ovarian cancer cells

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Distinct molecular characteristics and the local spread of ovarian cancer, the most lethal gynaecologic malignancy, suggests intraperitoneal gene therapy. Phase I trials have been conducted to investigate the safety and clinical effects of adenoviral E1A in gene therapeutic approaches. However, besides tumor-suppressive effects, E1A is known to transform rodent cells in conjunction with other factors, e.g. an activated ras oncogene. In an effort to eliminate elements favouring malignant conversion, the potential therapeutic effect of E1A deletion mutants on ovarian cancer cells was studied. To avoid any selection bias, a doxycyclin-regulated system was used to express E1A mutants and study their effects on the proliferation of ovarian carcinoma cell lines. As confirmed by Western blot analyses, the expression of the mutant proteins was almost completely suppressed by addition of doxycyclin to the culture medium. A substantial reduction in proliferation was achieved by expression of the wildtype protein and mutants lacking the p105^{RB}-binding motif. Therefore, deletion of the CR2 sequence should increase the safety of therapeutic application of E1A without affecting tumor suppression. The doxycyclin-regulated expression system was established to enable the elucidation of the mechanisms underlying the therapeutic effects of E1A in ovarian cancer cells and to analyze apoptosis of ovarian cancer cells induced by chemotherapeutic agents mediated by E1A.

Insertional mutagenesis by replication-deficient retroviral vectors encoding the Large T oncogene

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Insertion sites of replication-deficient retroviral vectors may trigger clonal dominance of hematopoietic cells in vivo. Here, we tested whether this would also be the case when using vectors that express powerful oncogenes such as the large tumor antigen (TAg) of simian virus 40. TAg inactivates the tumor suppressor proteins p53 and Rb by virtue of a chaperone-like activity. Primary hematopoietic stem/progenitor cells transduced with retroviral vectors encoding TAg induced histiocytic sarcoma or myeloid leukemia in transplanted mice (average survival of 21 weeks). Retrovirally introducing TAg into pretransformed 32D cells generated a monocytic leukemia, with faster kinetics (~8 weeks). Leukemic clones showed retroviral insertions in genes contributing to all known TAg cooperation pathways, acting mitogenic and/or modulating apoptosis (such as *BclX*, *Crk*, *Eras/Pim2*, *Csfr1/Pdgfrb*, *Osm/Lif*, *Axl*, *Fli*, *Sema4b*, *Src-like activity*, *Sox4*). 32D-derived monocytic leukemias showed hits in *Eras/Pim2* and *Max* proto-oncogenes, or the chaperone *Hspa4*, plus additional signaling genes. Vector-mediated insertional mutagenesis thus revealed a broad spectrum of potential TAg complementation genes, in a comparatively small sequence sample (n=31). These findings have important implications for the use of retroviral transgenesis in cancer research, and the expression of signaling genes in somatic gene therapy.

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Stable Gene Transfer into Normal and Leukemic Hematopoietic Stem Cells by using different retroviral Vector Systems

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Physiological hematopoiesis originates from a limited number of self-renewing stem cells which ultimately give rise to all mature blood cells. It has been postulated, that also leukemic malignancies, such as acute myeloid leukemia derive from a minor "leukemic" stem cell population and are organized in a similar hierarchical structure as lifelong normal hematopoiesis. In this context, retroviral marking and xenotransplantation of retrovirally marked cells into immunodeficient mice are powerful technics which can be utilized to investigate the biology of acute leukemias. In this study we have utilised three retroviral gene transfer systems, i.e. foamy-, lenti- and gammaretroviral vectors, and compared their potential for stable gene transfer into leukemic cell lines as well as normal and leukemic primary hematopoietic cells. Foamy- and lentiviral vector preparations were produced using a transient transfection system, while gammaretroviral preparations were generated from a stable packaging line. While high titer lentiviral ($0.5-4 \times 10^6$ particles/ml) and gammaretroviral ($1-3 \times 10^5$ particles/ml) preparations were successfully generated, foamyviral preparations in our hands, in contrast to other investigators, only achieved low titers ($3-5 \times 10^4$ particles/ml) when tested on HT1080 cells. When lenti- and gammaretroviral preparations were used to transduce a T cell lymphoma cell (Jurkat) and an ALL-derived cell line (RS), analysis of eGFP transgene-expression by flow cytometry analysis revealed high transduction efficiencies ranging from 50% to 90% for both vector systems. Subsequently lenti- and gammaretroviral constructs were used for gene transfer into primary normal or malignant hematopoietic cells. Non-prestimulated cells (16h protocol) or cells prestimulated with cytokines for 24h (40h protocol) were transduced overnight in the presence of fibronectin and growth factors. For transduction of normal hematopoietic cells also an 72h protocol employing 48h of prestimulation was used. For the lentiviral vector system transduction efficiencies of $6\% \pm 1\%$ (mean \pm SEM, n=5/5) using the 40h protocol on peripheral blood stem cells were achieved, and results could be improved to 26% with the 72h protocol. This compared favorably with gammaretroviral transduction which yielded $7\% \pm 3\%$ (n=4/4) transgene expression using the 72h protocol. In primary acute myeloid leukemic (AML) cells lentiviral transduction with the 40h protocol resulted in reproducible gene transfer with an efficiency of $14\% \pm 8\%$ (n=9/9). In non-prestimulated cells the gene transfer efficiency was $6\% \pm 5\%$ (n=4/5). Surprisingly, even without prestimulation gammaretroviral vectors showed reproducible transduction of AML cells ($11\% \pm 9\%$, n = 3/3) which was increased by 24h of prestimulation to $14\% \pm 11\%$ (n=8/8). With the foamy viral low titer preparations only low efficient transduction of Jurkat ($7\% \pm 3\%$, n=4) and RS ($6\% \pm 2\%$, n=4) cells, and even inferior results in primary human hematopoietic cells ($1\% \pm 1\%$, n=2/5 samples transduced) were achieved. In summary, here we show that with lentiviral and gammaretroviral preparations can be employed for efficient gene transfer into leukemic cell lines as well as primary normal or leukemic hematopoietic cells. A next step will be the transplantation of transduced leukemic cells into NOD / SCID mice in order to perform retroviral marking studies.

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Generation and application of an efficient Chronic Myelogenous Leukemia (CML)-targeted recombinant adeno-associated virus (rAAV) vector: New therapeutic options?

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Introduction: Despite great advances in vector development, most standard rAAV-2 vectors still lack the target cell specificity required for an efficient in vivo application. In addition, in many promising target cells (e.g.: peripheral blood progenitor cells (PBPC)), the gene transfer efficiency is generally low. Thus, designing gene therapy protocols for diseases like chronic myelogenous leukemia (CML) remain difficult, as standard rAAV-2-based vectors lack both the required gene transfer efficiency and selectivity. A recent advancement in vector development (Muller et al., 2003) now allows the generation of rAAV capsid mutants that offer higher target cell specificity and transduction efficiency. This method was applied to obtain a highly efficient and selective CML-targeted rAAV vector and tested on a panel of leukemic cell lines, as well on primary human CML and PBPC cells.

Material and Methods: Performing latter method on the CML cell line K562 (4 selection rounds), several mutant CML-targeted rAAV2 capsid clones were obtained, cloned into an rAAV2 helper plasmid and for each of the mutants rAAV2-eGFP vector stocks were produced. Titers were verified using our real time PCR-based titration assay. To determine efficiency and specificity, a panel of leukemic (CML and AML) and non-leukemic cell lines, as well primary CML and PBPC cells were transduced with these vectors. Mock, normal rAAV2 and random mutant clone-transduced cells served as controls. Transduction of primary human CML and PBPC cells was confirmed using FACS-sorted GFP⁺ cells with subsequent bcr-abl-FISH or FACS analysis for GFP/CD34⁺ co-expression, respectively.

Results: Transduction of a panel of six leukemic cell lines with the CML-targeted rAAV2 vectors (moi 100 iu) resulted in an over 10-fold increase of gene transfer efficiency compared to random mutant and standard rAAV2 vectors; gene transfer rates of >60% were obtained. In primary human cells, for both CML (n=4) and PBPC (n=2), an increase in gene transfer was observed (**CML:** AAV2: 2.4 ± 0.2 vs AAV2-cml: 5.1 ± 1.6 (p=0.04). **CD34⁺:** AAV2: 3.2 ± 0.9 vs AAV2-cml: 8.5 ± 4.3 (p>0.05)). All transduced cell were positive for either the BCR-ABL fusion gene (CML) or the PBPC marker CD34. In contrast, gene transfer of the mutant CML-targeted rAAV2 vectors into non-leukemic cell lines resulted in a reduction in gene transfer efficiency compared to standard rAAV2 vectors of over 50 percent.

Conclusion: In this study, we were able to generate a CML-targeted rAAV2 vector, which was able to transduce leukemic cell lines and primary human CML cells with significant higher efficiency and specificity than standard rAAV2 vectors. At this moment the AAV capsid mutant library method is applied on primary human CML cells in order to generate a high-efficiency CML-targeted AAV vector. The development of these more efficient and specific mutant rAAV2 vectors holds promise for future clinical applications.

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Restriction of Adenoviral Replication to the Transcriptional Intersection of two Different Promoters for Colorectal and Pancreatic Cancer Treatment

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In our current study, we developed oncolytic adenoviruses which preferentially lyse pancreatic and colon cancer cells by replacing viral E1 and/or E4 promoter with the tumor/tissue-specific promoters COX-2, MK, or the cell cycle-dependent promoter E2F1. We generated three sets of recombinant adenoviral vectors: In the first set, only the native E1A promoter was replaced by the COX-2, MK, or E2F1 promoter, respectively. In the second set, the viral E4 promoter was substituted by these heterologous promoters and the viral E1A promoter by the ubiquitously active CMV-IE promoter. In the third set, we substituted the viral E1A and E4 promoter with the COX-2, MK, or E2F1 promoter, respectively. In our system, transcriptional targeting of solitary viral E1A resulted in 50% enhanced restricted vector replication, when compared to an unrestricted replication-competent adenovirus. Furthermore, a targeted expression of the viral E1A gene products had a greater impact on restricted adenoviral replication than that of the E4 region. With our vectors Ad.COX•MK and Ad.MK•COX, using two different heterologous promoters to control E1A and E4 expression, we demonstrated enhanced viral replication specificity when

compared to Ad.COX•COX or Ad.MK•MK, respectively. In a subcutaneous xenograft tumor model, there was no significant difference in the anti-neoplastic efficacy of the double heterologous promoter controlled vectors when compared to our unrestricted replication-competent control adenovirus or vectors with only E1A transcriptionally driven by a heterologous promoter.

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Inhibition of malignant glioma cell growth by retroviral shRNA-targeting of chromosomal passenger proteins Survivin and INCENP

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Glioblastoma multiforme (GBM) is a highly malignant brain tumour that is resistant to conventional radiotherapy and chemotherapy. This resistance as well as dysregulated apoptosis, and enhanced cellular growth of glioblastoma cells originate from genetic alterations, which often include a loss of function of tumor suppressor p53 or other proteins involved in cell cycle control. So far, concerted efforts to improve survival of patients suffering GBM has remained unsuccessful, with median survival times below two years. In order to develop a new approach for the treatment of GBM, we sought for a new gene therapy strategy using reagents destroying the cell cycle and causing cell death independently of p53 function. Therefore, we constructed retroviral shRNA-targeting vectors for the specific silencing of the chromosomal passenger proteins Survivin and Inner Centromeric Protein (INCENP). We demonstrate here, that transduction of cells with either shSurvivin and shINCENP resulted in polyploidy and growth inhibition of glioblastoma cells. The high degree of polyploidy seen early after transduction was followed by apoptosis as detected by internucleosomal DNA fragmentation analysis. In addition, clonogenic assays verified the strong cytopathic effects mediated by the transduced shSurvivin and shINCENP. This suggests a p53-independent inhibitory effect of shSurvivin and shINCENP on these cells, which might be exploited for the treatment of glioblastoma.

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Targeting PSCA-positive tumor cells: Construction and evaluation of chimeric T cell receptors

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Prostate carcinoma (PCa) is the second leading cause of death among men in the industrialized western countries. The therapy of advanced PCa is limited. Primary tumors can be resected but therapy of the advanced disease is limited to radiotherapy or hormone-ablative therapy. After a short period of approximately two years, androgen-independent tumors arise in most patients, which often metastasised the bones and in later stages the brain. Specific engagement of immune effector functions is a promising approach to target and destroy minimal residual disease (metastasis). Today the strategy to recruit the adaptive immune system for an "immunotherapy" is based on specifically activated cytotoxic T-cells (CTLs), which are reactive to HLA-bound tumor derived peptides, and monoclonal antibodies directed against tumor associated antigens (i.e. Her2/neu, CD30) on the surface of tumor cells. The success of these selective therapies is hindered by the heterogeneity of the tumor cells as well as "Escape mechanisms" (i.e. downregulation of HLA, inefficient processing of antigens) and the often-weak antibody-dependent effector mechanisms (i.e. ADCC, complement). Although the adoptive transfer of tumor-specific T-cells in animal tumor models has been proven to be efficient, similar approaches failed in tumor patients due to limited numbers of reactive CTLs. To overcome these problems and in order to develop an efficient MHC-independent CTL-based immunotherapy against PCa we chose the "Prostate Stem Cell Antigen" (PSCA), a GPI-anchored surface protein, which is specifically expressed in normal prostate and PCa, as a target structure. We successfully developed a single chain antibody fragment (scFv) against PSCA and constructed retroviral vectors encoding chimeric T-cell receptors consisting of the betaC2-CD3zeta-chains of the TCR-complex fused to this anti-PSCA-scFv. To provide costimulatory signals to the T-cells we also constructed a retroviral vector encoding a chimeric anti-PSCA-scFv-CD28 molecule. In transduced T-cells these chimeric receptors were activated after cross-linking with recombinant PSCA and mediated a specific killing of PSCA-positive target cells. This suggests that primary human T-cells, armed with anti-PSCA chimeric T-cell receptors, might be exploited for the treatment of advanced PCa.

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HCMV glycoprotein-specific chimeric TCR for antiviral immunotherapy

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Herpesviruses infection or reactivation in transplant or otherwise immunosuppressed patients is still a major clinical problem. Adoptive transfer of virus specific T-cells has been successfully used to overcome EBV and CMV reactivation. However, it is nearly impossible to expand virus-specific cytotoxic T-lymphocytes (CTL) from Human Cytomegalovirus (HCMV) seronegative donors in vitro. For that reason, we aim at generating CTLs that express chimeric receptors directed against HCMV glycoproteins, which can serve as antiviral HCMV-specific MHC-unrestricted effector T-cells. Two different scFvs were derived from HCMV glycoprotein B specific antibodies, and we can demonstrate preserved antigen recognition of one scFv. The scFVs were then inserted into chimeric TCR constructs that include the CD3zeta cytoplasmatic signaling domain; a further construct also encompasses a CD28 signaling domain to provide simultaneous costimulatory signals. Two different viral vector systems are used for the transduction of human T-cells. The *Herpesvirus saimiri* (HVS) human T-cell transformation system is employed for the generation of T-cell lines that simultaneously express the chimeric TCRs. VSV-G pseudotyped HIV-derived lentiviral vectors are used to transduce T-cell lines and primary T-cells. We have successfully demonstrated transformation of human T-cells using the HVS vectors, and expression of the receptors in human T-cells after transduction with different lentiviral vectors. This will allow to study the targeted lysis of HCMV infected cells by effector T-cells expressing chimeric T-cell receptors.

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Gene transfer by retroviral self-inactivating (SIN) vectors into murine hematopoietic stem cells in vivo: Analysis of clonality and genotoxicity

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Retroviral vectors with long terminal repeats (LTRs), which contain strong enhancer-promoter sequences at both ends of their genome, are widely used for stable gene transfer into hematopoietic cells. However, recent clinical data and mouse models are pointing to insertional activation of cellular proto-oncogenes as a dose-limiting side effect of retroviral gene delivery, potentially inducing leukemia. Self-inactivating (SIN) retroviral vectors do not contain the terminal repetition of the enhancer-promoter, theoretically attenuating the interaction with neighboring cellular genes. In this study we compared different groups of mice transplanted with SIN retroviral vectors expressing IL2RG (n=12) or dsRED (n=6) by the internal SFFV promoter. The cells were transduced with different MOIs and mice observed for up to 11 month. So far there was no evidence of any severe adverse effect due to the gene transfer by gamma retroviral SIN vectors. In a control group of mice transplanted with bone marrow transduced with the LTR vector SF91P-dsRED (n=5) one leukemia developed after secondary transplantation. 2 control groups of mice transplanted with MFG- γ C (n=9) transduced BM or by a lentivirus encoding IL2RG driven by an internal SFFV promoter (n=12) did not develop any hematopoietic abnormalities. Analysis of hematopoietic tissues of SIN vector transplanted mice by Southern blot and LM PCR showed polyclonal hematopoiesis and weaker selection of dominant clones compared to mice transplanted with SF91 transduced bone marrow cells. In summary, so far we could not observe any development of leukemias after gene transfer of IL2RG in a mouse transplantation model using different retroviral vectors whereas it was possible to induce leukemia after high dose gene transfer of dsRED in the context of the LTR driven (SF91) retroviral vector. Furthermore, initial results in mice transplanted with SIN vector transduced BM point to a weaker selection for dominant clones by insertional mutagenesis.

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Hepatitis B Virus Vectors: Variation of Viral Envelope Proteins

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Hepatitis B virus (HBV) targets the liver and infects quiescent hepatocytes. Vaccine escape mutants of HBV have been described which are hardly recognized or neutralized by anti-HBs antibodies due to mutations in the small envelope (S) gene. We have recently shown that the human hepatitis B virus (HBV) can be converted into a vector that allows hepatocyte-specific gene transfer. HBV based vectors containing naturally occurring escape mutations in main immunogenic "a" determinant of the S protein. These vectors might on the one hand be useful as a gene shuttle avoiding a possible neutralization by vaccine induced antibodies. On the other hand, they may serve as a molecular tool to investigate the properties of escape variants of HBV. We produced HBV based

vectors by transfection of a helper-plasmid providing all viral proteins and a transfer-plasmid, which produces a pregenomic RNA with the S-gene replaced by *Renilla* luciferase reporter gene. To prevent recombination possibly resulting in the generation of replication competent HBV, HepG2 producer cell lines were generated by stable transfection of the transfer-plasmid. In order to create „escape“ vectors, we introduced a conformational alteration of the S-protein by exchanging codon 144-Asp to 144-Ala or codon 145-Gly to 145-Arg in the helper plasmids. Although sufficient viral proteins and recombinant vector genome were produced, the amount of vector particles released was very low when single „escape“ helper plasmids were used. Combining both „escape“ helpers lead to secretion of HBV vectors at titers comparable to wild type vectors. In the presence of neutralizing antibodies, „escape“ vectors were not blocked as observed for unmodified vectors. Molecular analysis of the secretion defect is under way. Taken together, HBV derived vectors containing a mutation in the envelope S protein seem to escape their neutralization by antibodies, although their production proved to be complex. The observed secretion defect of the escape variants could be one reason for the limited spread of escape variant HBV *in vivo*.

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Targeting viral entry in gene therapy of HIV-infection

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Despite significant improvements of the survival of HIV-infected patients during HAART, treatment limitations such as the development of drug-resistant HIV strains and long-term toxicities call for innovative treatment strategies. In this study, a novel antiviral gene that effectively inhibits virus entry was tested in a phase I/II clinical trial. The antiviral gene is expressed from a retroviral vector (M87o) and encodes for the membrane-anchored antiviral peptide C46. C46 comprises 46 amino acids, is derived from the second heptad repeat of the HIV-1 envelope glycoprotein gp41 and effectively inhibits fusion of the viral and cellular membranes during virus entry. The membrane-anchored form (maC46) was shown to effectively block entry of a broad range of HIV isolates, including viruses resistant to the fusion inhibitory peptide C36 (T20). C36 (T20) corresponds to the 36 C-terminal amino acids of C46. The gene was introduced into autologous CD4 T lymphocytes from 10 patients with severe immunodeficiency and HAART failure. Cells were expanded by co-stimulation with anti-CD3 and anti-CD28 immobilized on beads. Between 2 and 15 x 10E9 cells were infused. No major toxicities were observed and neither cellular nor humoral immune responses to the transgene product were detected. While a significant rise in T helper cell counts was seen, viral load was not affected. Gene marking could be detected throughout the one-year follow-up, but the levels were too low to account for the marked rise in CD4 counts.

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Gene Therapy of Glioblastoma with Adult Bone Marrow Derived Tumor Infiltrating Cells (BM-TIC)

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Bone marrow derived multipotent adult progenitor cells – termed here bone marrow derived tumor infiltrating cells (BM-TIC)- are an attractive alternative to neural or mesenchymal stem cells for the gene therapy of gliomas, as they can be passaged for an extended time without requiring exogenous immortalization. This high passaging capacity is a prerequisite for their clinical use as it allows the generation of viral packaging cell lines and large-scale production of genetically modified cells. In this study, we evaluated the migratory behaviour and the therapeutic efficacy of gene-modified BM-TIC in a 9L rat glioma model. Upon injection into the tumor/ vicinity of the tumor, BM-TIC infiltrated 9L tumors similar to the neural stem cell line C17.2. BM-TIC, genetically modified to express the thymidine kinase of the Herpes simplex virus (BM-TIC-tk-GFP), mediated a significant therapeutic effect with 67% of long-term survivors. Histology of brain sections revealed even distribution of BM-TIC within tumors, indicating that genetic modification did not impede their capability to migrate. Furthermore, we were able to locate the cells within the tumor by non-invasive positron emission tomography (PET). Based on BM-TIC we created a stable packaging cell line (BM-TIPC) for retroviral vectors pseudotyped with glycoproteins of the lymphocytic choriomeningitis virus (LCMV). This pseudotype was shown to efficiently and specifically transduce glioma cells *in vivo*. Upon injection into 9L glioma, BM-TIPC disseminated intratumorally and mediated transduction of glioma cells in different tumor areas. In conclusion, gene-modified BM-TIC may be a useful tool to

enhance the distribution of therapeutic drugs and vector particles as well as the specificity and efficacy of gene transfer to gliomas in patients.

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Deletion of Y2-Receptors in the CNS of Y2lox/lox Mice Induced by Stereotactic Application of AAV Vectors Expressing Cre-Recombinase.

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AAV vector mediated gene expression has been established as a powerful tool for the *in vivo* analysis of gene function. Due to the absence of AAV-specific, viral gene expression, the transgene effects on the cellular gene of interest can be studied in the absence of interfering vector-mediated gene expression. Furthermore, stereotactic and site-specific vector application opens the possibility to differentiate gene function in very circumscribed and neighbouring areas. In the brain direct comparison of treated and untreated side further helps to interpret the experimental phenotype. Neuropeptide Y (NPY) is abundant in the central and peripheral nervous systems. It acts through Y1, Y2, Y4 and Y5 receptors and is involved in a variety of brain functions, including regulation of appetite and anxiety. When applied locally into the amygdala, NPY exerts an anxiolytic action, presumably mediated by Y1 receptors. In contrast, stimulation of Y2 receptors causes anxiety. Depletion of Y2 receptors induces an anxiolytic phenotype, possibly by abolishing the release-inhibiting action of presynaptic Y2 receptors. We now established site-specific deletions of Y2 receptors in conditional Y2lox/lox mice by local injection of an AAV2/2-Cre-recombinase vector into the hippocampus, septum or amygdala. As a control, an AAV2/2-GFP vector was injected in Y2lox/lox mice at the same sites. Expression of Cre and GFP was demonstrated by *in situ* hybridization and immunohistochemistry. Deletion of Y2 receptors and Y2 mRNA was visualized by receptor autoradiography and *in situ* hybridization, respectively: It was neuron-specific and restricted to the injection sites. After bilateral injection of AAV2/2-Cre or AAV2/1-Cre vector into the amygdala, mice showed a tendency towards an anxiolytic phenotype in the light-dark test for anxiety. No anxiolytic effect was detected in mice after intra-hippocampal or intra-septal injections of AAV vectors. The experiments indicate that the anxiolytic effect if Y2 receptor deletion may be generated in the amygdala. The analysis of brain function by stereotactic AAV vector-mediated transgene application helps to divide concepts for both, tailored gene therapy and intervention by pharmacology.

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Lentiviral SIN- vectors affect target site selection and clone survival

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The occurrence of three cases of leukemia in patients in a clinical SCID-X1 gene therapy trial, induced by insertional mutagenesis, uncovered the necessity to develop safer vectors for further clinical trials. Lentiviral SIN LTR HIV-based vectors present one group of these new and promising vectors. In earlier studies it was observed that different type of vectors and LTRs show various integration site patterns. In our study we investigated the influence of various internal vector elements on integration target site selection and clone survival. Therefore we transduced HeLa cells with three distinct SIN-HIV-based vectors differing in their promoters (CMV, SFFV) and transgenes (eGFP, MERTK). In our analysis we compared 1439 integration sites at three different time points (1d, 30d, 60d). We confirmed the observation that lentiviral vectors favor integration in genes. 63% of the integrations were detected in RefSeq genes without any significant differences among the distinct vectors. We further observed an equal chromosomal distribution of these vectors. Interestingly we could detect differences in gene ontology between the first time point (1d) and the later time points. In freshly transduced cells, we found a significant overrepresentation of genes involved in cell cycle, whereas at the later timepoints there was an overrepresentation of genes involved in ATP binding, kinase and transferase activity. Moreover, we observed an increased significance over time (30d to 60d) in gene ontology of these special molecular functions. This points to a clonal selection induced by lentiviral SIN vectors *in vitro*. The occurrence of vector-induced target site selection and clone survival was further highlighted by the fact that up to 20% of the identified integrations were clustered as common integration sites (CIS). Our findings indicate that lentiviral SIN-HIV-based vectors may influence the clonal growth of modified cells independently

of internal vector elements. The character of such effects as well as any putative relevance of such genotoxicity for the in vivo situation will have to be investigated in detail for the role of individual vector / cell type configurations.

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RRE-deficient lentiviral vectors reduce the risk of mobilization by decreasing vector packaging

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Mobilization of HIV-1 vectors is reduced by at least a factor of 10,000 after mutation of the Rev responsive element (RRE). Although the Rev/RRE system is known to be responsible for transfer of genomic RNA from the nucleus to the cytoplasm, our previous study showed only small effects of Rev on cytoplasmic vector RNA levels¹. These results indicate further functions of Rev beyond nuclear export of viral RNA. Therefore we analyzed the influence of Rev on vector packaging. HIV-1 and SIV vectors were constructed containing wild type or mutated RRE. These vectors were cotransfected with Rev-independent packaging plasmids in the presence or absence of a *rev*-expression plasmid. Vector titers were determined and packaging of the vector constructs was analysed by determining genomic vector RNA levels in the cytoplasm and the vector particles using a quantitative RT-PCR. Omitting Rev reduced HIV-1 and SIV vector titers around 50-fold, while cytoplasmic vector RNA-level decreased approximately 2-fold. Despite substantial cytoplasmic vector RNA levels, its packaging was severely reduced and comparable to the decrease in vector titers. RRE-mutated vectors showed reduced packaging independent of Rev. In addition to nuclear export, the Rev/RRE system plays an important role in the packaging of genomic lentiviral vector RNA. Dependence on particular nuclear export pathways or compartmentalization of cytoplasmic RNA might explain the effects of the Rev/RRE system on packaging.

¹ Susann Lucke, Thomas Grunwald and Klaus Überla (2005). Reduced mobilization of Rev-Responsive Element-deficient lentiviral vectors. *J. Virol.* 79: 9359-9362.

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Efficient knock-down of prion protein by lentivectors expressing short hairpin RNAs

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Transmissible spongiforme encephalopathies (TSEs, or Prion diseases) are fatal neurodegenerative diseases that are characterized by the accumulation of PrP^{Sc}. The infectious agent, PrP^{Sc}, is the protease-resistant form of the normal cellular prion protein (PrP^C). Since PrP^C-deficient (Prnp0/0) mice are resistant to prion disease and do not propagate infectious PrP^{Sc}, targeting of PrP^C by RNA interference (RNAi) is a promising approach for the treatment of TSEs. RNAi is a conserved mechanism by which small interfering RNAs (siRNAs) specifically silence target genes. Although RNAi can be induced in mammalian cells by transfection of siRNA molecules, this approach allows only for transient silencing of gene expression and requires chemical or enzymatic synthesis of siRNAs. Intracellular transcription of siRNAs can be achieved by incorporation of H1 or the U6 RNA polymerase III promoters in lentiviral vectors. To induce RNAi in vitro and in vivo, siRNA design: lentiviral vectors (LVshPrP) were designed to express short hairpin RNAs (shRNAs) directed against murine PrP^C under the control of the H1 promoter. LVshPrP also carries EGFP as a reporter gene to identify infected cells and tissues. Transduction of murine N2a neuroblastoma cells with LVshPrP (MOI=10) resulted in a 97% knock-down of PrP^C. In contrast, lentivectors carrying scrambled shRNA had no significant effect on PrP^C expression. Next, we analysed the effect of LVshPrP in primary neuronal cells. LVshPrP transduced granule cells demonstrated a 85% reduction of PrP^C expression within 3 days after infection. To analyse the efficacy of LVshPrP in vivo, we generated lentiviral transgenic mouse lines by infection of embryonic stem (ES) cells. In the brain of ES-cell derived chimeric mice (80-90% coat color chimerism), a reduction of PrP^C levels of up to 71% was observed. Taken together, lentiviral shRNA vectors are versatile tools to efficiently silence PrP^C expression in vitro and in vivo.

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Selective expansion of MDS1/EVI1, PRDM16 and SETBP1 integration clones in successful chronic granulomatous disease (CGD) gene therapy trial

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The potential of gene therapy to cure genetic diseases of the lymphoid compartment has been shown in patients suffering from ADA-SCID as well as X-linked SCID. The first successful correction of a genetic disease concerning the myeloid compartment could be achieved in the German-Swiss chronic granulomatous disease (CGD) gene therapy trial. In this trial, two patients received CD34+ autologous bone marrow cells, transduced with a SFFV-based retroviral vector encoding the therapeutic transgene gp91phox. Before transplantation, both patients received nonmyeloablative conditioning. 3 months after reinfusion, the proportion of marked granulocytes was 20% and 10% in patient 1 and patient 2, respectively. The proportion of gene-corrected granulocytes expanded 4 fold 5 to 9 months after therapy in both patients and was then stable until 542 days post transplantation in patient 1 and 343 days post transplantation in patient 2. We accompanied this trial with a prospective monitoring by LAM-PCR analysis to define the clonality of the hematopoietic repopulation and accomplished large scale sequencing and mapping of the insertion sites. We analysed peripheral blood leukocytes and sorted cells from different time points post transplantation. Mapping of 435 unique integration sites derived from patient 1 and 330 from patient 2 identified the zinc finger transcription factor homologues MDS1/EVI1 and PRDM16 as common integration sites (CIS) in both patients and SETBP1 as third CIS in patient 1. RNA analysis of both patients showed an activating influence of the retroviral vector on these individual CIS genes. Insertion site analysis performed on CFU-GM and BFU-E derived colonies demonstrated that 1 to 4 vector insertions are present per cell and confirmed a dominance of up to over 80% of one MDS1/EVI1 clone in patient 1 originally discovered in the peripheral blood LAM-PCR pattern. Our data show that upregulation of endogenous genes by integrated retroviral vectors may lead to an *in vivo* expansion of the affected cell clones at this time considered beneficial for the clinical outcome of affected patients requiring to be closely monitored or altogether avoided to prevent untoward side effects in the future.

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The inner tegument promotes herpes simplex virus capsid motility along microtubules *in vitro*

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Neurotropic viruses such as herpes simplex virus type 1 (HSV1) require efficient, active axonal transport during pathogenesis. HSV1 is considered a promising vector for the treatment of neurodegenerative diseases, pain and skin and brain tumors. HSV1 enters cells by fusion at the plasma membrane, and incoming cytosolic capsids use the minus-end directed microtubule motor dynein for transport to the nucleus. The capsids dock at the nuclear pore and release the viral genome for transcription and replication into the nucleoplasm. During virus assembly, outgoing cytosolic capsids are transported, presumably again on microtubules, to the organelle of secondary budding, and axonal capsids colocalize with the plus-end directed microtubule motor kinesin-1. Using (1) capsids tagged on the small capsid protein VP26 with GFP, (2) Cy3-labelled microtubules and (3) membrane-free cytosol, we have reconstituted viral capsid transport *in vitro*. In the presence of ATP, capsids moved along microtubules up to 30 μ m. Blocking the function of dynactin, a cofactor of dynein and kinesin-2, inhibited the transport. Removing outer tegument proteins from the capsids increased *in vitro* motility. In contrast, capsids devoid of inner and outer tegument did not interact with microtubules. VP26 can interact with dynein light chains (Douglas et al. 2004, JBC 279:28522). To compare the cell entry of different HSV1 strains, we characterized the inocula regarding their infectivity, viral genome content, protein and particle composition. Only preparations with a low particle to PFU ratio showed efficient nuclear targeting. When cells were infected with such preparations of HSV1- \square VP26 or HSV1-GFPVP26, capsids were transported along MT as reported for HSV1 wild type. When dynein function was inhibited, fewer capsids of HSV1 as well as HSV1- \square VP26 and HSV1-GFPVP26 arrived at the nucleus. Moreover, naked capsids exposing VP26 on their surface were unable to recruit dynein or dynactin in biochemical binding assays, whereas capsids of HSV1, HSV1- \square VP26 or HSV1-GFPVP26 and covered with inner tegument proteins bound dynein and dynactin. Thus, even in the absence of the potential dynein receptor VP26, HSV1 used MT and dynein for efficient nuclear targeting, and *in vitro* dynein binding and microtubule motility required inner tegument proteins.

Döhner et al. (2002), Mol Biol Cell 13:2795-2809; Wolfstein et al. (2006), TRAFFIC 7:227-237; Döhner, Radtke, Schmidt & Sodeik (2006), JVI, resubmitted.

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mRNA-Stabilisation as a tool to improve recombinant FVIII expression

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Aim of the study: Considering the immediate access to the bloodstream and the involvement in coagulation, megakaryocytes and monocytes would be ideal targets for gene therapy of hemophilia A. However, several attempts to show recombinant expression of factor VIII (rFVIII) by primary hematopoietic cells using an ubiquitously expressed CMV-promoter have shown to be ineffective. In part, this failure was attributed to the inefficient transcription from the CMV promoter in hematopoietic cells. This study explores the potential of

megakaryocyte and monocyte specific FXIIIa transcriptional elements to enhance the expression of FVIII in hematopoietic cells.

Material and Methods: The enhancer region (enh), the 5' untranslated region (5'UTR) both of the FXIIIa were cloned in combination or as a single element in 5' to 3' direction of the CMV-promoter into the mammalian expression plasmid pcDNA3.1 upstream of either FVIII B-domain deleted (FVIII-DB) or the full length FVIII (FVIII-FL) cDNA. These vectors were transfected into CD14+monocytes, the megakaryocytic cell line K562 and human embryonic kidney cell line 293T using nucleofection (Amaxa). FVIII expression was analysed by a chromogenic assay and RT-PCR. Transfection efficiency was compared by measuring green fluorescent protein (GFP) expression from a GFP encoding pcDNA3.1 Vector.

Results: FVIII-DB expression was most effective in K562 cells and primary monocytes when transfected with the 5'UTR containing vector (in mIU/ml: 20 for Monocytes and 150 for K562 compared to 7 and 100 in these cells using the vector without the 5'UTR); the FXIIIa-enhancer element containing plasmid proved to be equally effective in K562 cells whereas the combination of both, enhancer and 5'UTR, resulted in reduced FVIII levels compared to the vector with the basal CMV promoter alone.

Conclusion: The inclusion of the 5'UTR of the FXIIIa gene upstream of the FVIII coding sequence results in a significantly improvement of FVIII expression levels from a CMV driven expression vector in primary monocytes and K562 cells. Further experiments have to rule out if the FXIII 5' UTR element may be beneficial for hematopoietic lineage restricted expression of in animal models that could ultimately translate into safe gene therapy approaches for hemophilia A.

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Coagulation and Viral Transduction: Protease-Activated Receptors modulate AAV and Adenoviral Gene Transfer Efficacy

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Adeno-associated viral (AAV) vectors hold promise for the treatment of several diseases and were already tested in clinical Phase I/II studies. Previously, we determined an unexpected inhibitory effect of anticoagulative drugs, direct thrombin- and factor Xa inhibitors, on AAV-2 and adenovirus mediated gene transfer. Thus we hypothesized that coagulation proteases may influence AAV-2 gene transfer. Cellular responses triggered by proteases are mediated by protease-activated receptors (PAR) and PAR-1 activation by thrombin enhances alphaVbeta5 integrin (co-receptor for AAV-2 and adenovirus)-dependent cellular function. Therefore we sought to test whether PAR activation could modulate AAV-2 and adenoviral gene transfer. AAV-2 vectors were delivered to PAR-1, PAR-2, PAR-4, and beta5 deficient mice. Mice homozygous (-/-) or heterozygous (+/-) for PAR-1 (n=11 or 18), PAR-2 (n=13/13), or PAR-4 (n=8/10) received tail vein injections of AAV-2 vectors expressing coagulation factor IX (FIX) by a liver specific expression cassette and were compared to littermate controls (+/+). FIX levels among PAR-1 controls were 2-fold higher than in PAR-1 (-/-); p<0.02. Similarly, PAR-4 deficient mice presented 3-fold lower FIX levels than controls (0.4 vs 1.2 ug/ml, p<0.05), while PAR-2 deficiency did not influence gene transfer. Moreover, upon activation of receptors by PAR-1, -2, or -4 -specific peptide agonists in normal C57Bl6 mice, FIX levels increased 1.5 to 5-fold in a dose-dependent manner for PAR-1 and PAR-4. Gene copy measurement and immunofluorescence staining revealed low vector uptake by livers of PAR knockout mice while PAR activation increased uptake and lead to widespread expression. PAR activation or a PAR null allele had a similar effect on adenoviral vector transduction, but no effect on AAV-5 or AAV-8-mediated gene transfer, which use other cellular receptors. Interestingly, beta5(-/-) mice showed lower FIX expression than controls (0.5 vs 2.0 ug/ml, p<0.0005; total n=26) for AAV-2 but not following AAV-5 gene transfer. Further, the influence of PARs on AAV-2 transduction was not present in beta5 deficient cells or mice, which all suggest a receptor dependent mechanism by PARs. In conclusion, these data demonstrate a novel role of coagulation proteases and PARs on viral vector (AAV-2 and adenovirus)-mediated gene transfer and provide an alternative target to modulate gene therapy strategies.

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Evaluation of the therapeutic effect of oncolytic Herpes simplex viruses by bioluminescence imaging

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Glioblastomas (GBM) are aggressive brain tumors with a poor prognosis. The average life span of patients with GBM is approximately one year and so far conventional anticancer therapies have failed. Thus, alternative treatment options such as virotherapy should be followed. Herpes simplex virus type 1 (HSV-1) is a neurotropic DNA virus, which efficiently infects tumor cells derived from the nervous system. By specific modification of the HSV-1 genome oncolytic viruses can be generated, which selectively replicate in and lyse tumor cells, but spare normal cells. Bioluminescence imaging represents a non-invasive method for monitoring tumor growth and

therapeutic efficacy of oncolytic viruses in mice. To this end, tumor cells and the oncolytic HSV-1 were tagged with different luciferase reporter genes. Proliferative capacity and sensitivity to infection with oncolytic HSV-1 was determined in human U87MG glioma cells stably expressing luciferase reporter genes by the MTT assay. The HSV-1 strain 17 genome cloned as a bacterial artificial chromosome (BAC) in *E.coli* was manipulated by the powerful methods of bacterial genetics. Several viral genes, which target cellular pathways activated in tumor cells were deleted from the HSV-1 genome. HSV-1 most likely is not dependent on these genes for replication in tumor cells, because the activated cellular pathways will substitute for viral functions. Accordingly, the HSV-1 mutants should replicate and destroy the tumor cells, but will be unable to grow in normal cells. This hypothesis will be tested in various glioma cell lines in cell culture, followed by the evaluation of oncolytic HSV-1 therapy in subcutaneous and orthotopic mouse models of GBM.

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Expression of a neutralizing antibody against HIV in Lymphocytes using a retroviral vector

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The HIV entry mechanism is an attractive target for gene therapy approach. HIV-infection of CD4⁺ lymphocytes proceeds via binding of the viral surface receptor gp120 to the CD4 receptor and subsequently to a co-receptor, mostly CCR5 or CXCR4. Fusion is brought about by conformational changes with gp41. The N- and C-heptad repeats of the trimeric gp41 form a coiled-coil structure prior to fusion. Binding of gp120 to receptor and co-receptor lead to the rearrangement of the gp41 coiled coil into a six-helix hairpin bundle. Peptides derived from the C-heptad repeat (eg T-20, C36, C46) prevent six-helix bundle formation and inhibit HIV entry. Additionally, antibodies directed against epitopes of gp41 (eg C2F5, 4E10) act in a similar way. Two major aims should be achieved with an effective HIV entry inhibitor: Protection of the transduced (selective advantage) and the untransduced cells (bystander-effect). In an earlier study we have demonstrated that membrane-anchored fusion inhibitor C46 leads to protection from HIV infection of the transduced T cell. The disadvantage of this approach is that only a fraction of T cells can be transduced and no bystander-effect is achieved. A secreted principle for HIV entry inhibition could enable effective protection of the transduced cells and also give a bystander effect. In the present study, we have pursued the aim to generate a secreted entry inhibitor by cloning a retroviral vector that expresses the HIV neutralizing antibody C2F5. Here we show that both T and B cells can express functionally active C2F5 antibodies. This approach is especially interesting as transduced T cells can migrate into tissues like the lymph nodes in which HIV infection is elevated. Our results also indicate a high expression of C2F5 after transduction of murine stem cells. After transplantation of irradiated Rag-1 mice, significant titers of C2F5 in mice sera were detected. We propose that a bystander-effect renders high transduction efficacy unnecessary, thus lowering the risk of insertional mutagenesis.

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Recombinant adenovirus transfects and activates plasmacytoid dendritic cells in vitro and in vivo

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Recombinant replication-deficient adenovirus (Ad) induces short term expression of transgenes in an immunogenic form. While long term gene expression is desired for the use in gene therapy, immunogenic short term expression is sufficient for DNA vaccination approaches against infection and cancer. Here we further explore the interaction of Ad with the network of dendritic cells (DC) which control adaptive immunity. We show antigen expression not only in conventional dendritic cells (cDC) but also in pDC with subsequent phenotypic maturation and secretion of IFN- α and IL-6 in both DC populations thus bridging the innate and the adaptive immune system. In vitro recombinant Ad activates DC and induces production of cytokines largely independent of toll-like receptor 9 (TLR9) which is capable of recognizing double-stranded DNA viruses. However, a detailed comparative analysis shows that recognition of recombinant adenovirus by TLR9 does occur. Most important in vivo the induction of CD8⁺ cytotoxic T lymphocytes (CTL) is largely TLR9-independent. In conclusion, our results provide important mechanistic insight how recombinant Ad stimulate cytotoxic cellular immune responses and highlights the advantages of further developing recombinant Ad as a vector for DNA vaccination approaches.

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Cell type specific activity and promoter regulation of HERV-Elements

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The human genome contains more than 450,000 endogenous retroviral LTR sequences that can be regarded as mobile regulatory modules for gene expression. We have analyzed over 100 randomly selected human endogenous retroviral (HERV) LTRs in a transient transfection assay using luciferase as a reporter gene. About two thirds of these sequences were still active and able to promote the expression of any gene. Most of these LTRs showed differential activities depending on the cell type. For example, proviral and solitary LTRs of class III elements (HERV-L) proved to be specifically active in human skin keratinocytes (HaCaT, NHEK), HeLa cells, and/or uterus cells (KLE). To define the regulatory sequences involved in tissue specificity, a number of HERV-L LTR sequences with different specificities were compared and potential transcription factor binding sites identified. Computer analysis using the program GEMS launcher (Genomatix) suggested the presence of partially overlapping binding sites for the AP1, SP1F, EGRF, AP4R/NEUR, SMAD, PAX8 and HOXF families of transcription factors. Site-directed mutagenesis revealed the importance of PAX8, Sp1, AP1, Egr-1, HOXF, AP4R binding sites in KLE and HaCaT cells, whereas an additional SMAD binding site appears to be essential for the transcriptional activity of HERV-L LTRs in keratinocytes. Furthermore the binding of the transcription factors Sp1, AP1, Egr-1 and HOXF could be confirmed by electrophoretic mobility shift assays. The cell type specific promoter activities of many HERV-LTRs could make them suitable for the construction of retroviral vectors with specified expression patterns. Furthermore, using HERV elements in retroviral vectors might alter preferred integration sites.

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IntegrationSeq and IntegrationMap : Tools to Rapidly Characterize Integration Sites of Gene Therapy Vectors

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Increasing use of retroviral vector-mediated gene transfer created intense interest to characterize vector integrations on the genomic level. Techniques to determine insertion sites, mainly based on time consuming manual data processing, are commonly applied. Since a high variability in processing methods hampers further data comparison, there is an urgent need to systematically process the data arising from such analysis. To allow large-scale and standardized comparison of insertion sites of viral vectors we developed two programs, IntegrationSeq and IntegrationMap. IntegrationSeq can trim sequences, and valid integration sequences get further processed with IntegrationMap for automatic genomic mapping. IntegrationMap retrieves detailed information about whether integrations are located in or close to genes, the name of the gene, the exact localization in the transcriptional units and further parameters like the distance from the transcription start site to the integration. We validated the method using 259 files originating from integration site analysis (LM-PCR). Sequences processed by IntegrationSeq led to an increased yield of valid integration sequence detection, which has shown to be more sensitive than conventional analysis and 15 times faster, while the specificities are equal. Output files generated by IntegrationMap were found to be 99.8% identical with results retrieved by much slower conventional mapping with the ENSEMBL alignment tool.

Using IntegrationSeq and IntegrationMap, a validated, fast and standardized high-throughput analysis of insertion sites can be achieved for the first time.

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Replication properties of human adenovirus in vivo and in primary cell cultures from different animal species

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Oncolytic adenoviruses have emerged as a promising approach for the treatment of tumors resistant to other treatment modalities. However, preclinical safety studies are hampered by the lack of a permissive non-human host. Screening of a panel of primary cell cultures from different laboratory animals revealed that porcine cells support the replication of human adenovirus 5 (Ad5) as efficiently as human cells, while release of infectious virus

by murine and other rodent cells is diminished by several orders of magnitude. Restriction of Ad5 replication in rodent cells seems to act primarily at a post-entry step. Replication efficiency of adenoviral vectors harboring different E1 deletions in porcine cells was similar to that in human A459 cells. Side by side comparison of viral load kinetics in blood of mice and pigs injected with Ad5 or a replication-deficient adenoviral vector failed to provide clear evidence for virus replication in mice. In contrast, adenoviral late gene expression, a 13.5-fold increase in viral load in an individual pig from day 3 to day seven, and 100-fold higher viral DNA levels in the Ad5 infected swine than in the animal receiving Ad.TK suggests adenovirus replication in swine. Lung histology of Ad5 infected pigs revealed a severe interstitial pneumonia. Although the results in swine are based on a small number of animals and need to be confirmed, our data strongly suggest that infection of swine with human adenovirus or oncolytic adenoviral vectors is a more appropriate animal model to study adenoviral pathogenicity or pharmacodynamic and toxicity profiles of adenoviral vectors than infection of mice.

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Establishment of multilineage hematopoietic readout systems in a nonhuman primate model (*Callithrix jacchus*) for comparative in vivo/in vitro analysis.

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To investigate the properties of human hematopoietic stem and progenitor cells for therapeutic safety and efficacy after in vitro manipulation, reliable readout systems are necessary for preclinical evaluation. Although stroma dependent long-term in vitro systems are capable of measuring human progenitor properties up to a single cell level, they fail to predict stem cell engraftment. Similarly, the low engraftment frequency as well as the non-species specific microenvironment limits the use of xenogeneic transplantation models. Since the commonly used murine models differ greatly in pathophysiology and pharmacology to the human system, nonhuman primate models have emerged as highly desirable experimental systems to assess the properties of modified hematopoietic stem cells closely related to the clinical setting in humans. However, these animals are very expensive and in addition require expensive labor efforts as well as large facilities. A small new world monkey, the common marmoset (*Callithrix jacchus*) could overcome these disadvantages. It is small, easy to breed, inexpensive and does not require special facilities. Here we report the establishment of long-term hematopoietic in vitro readout systems to functionally characterize and enumerate primitive and stem cell equivalent progenitors of numerous hematopoietic lineages from the common marmoset. The animals (n=3) have been put into short term general anaesthesia and approximately 1 ml of bone marrow was aspirated from the femur. After density centrifugation CD34+ cells were enriched by magnetic separation using a recently described monoclonal mouse anti-marmoset CD34-antibody (MA24). To further characterize the CD34 marmoset-population several human-specific antibodies, such as AC133 have been evaluated. After an initial short-term expansion culture (using flt-3L, SCF and IL-7) frequency assessment and functional characterization of CD34+ progenitors were performed by transferring equal aliquots of cells from the primary cultures into human specific myeloid as well as lymphoid readout systems. Myelo-erythroid progenitors were assessed as Long-Term Culture Initiating Cells (LTC-IC), more committed cells were enumerated as Colony Forming Units (CFU) that can be generated within 2 weeks in clonogenic methylcellulose cultures. Alternatively, the lymphoid differentiation potential was assessed by measuring Natural-Killer-Cell Initiating Cells (NK-IC) under human specific in vitro conditions. Mature NK-cells were further characterized by flow cytometry to assess the reactivity of NK-cell specific anti-human antibodies in the marmoset system. We demonstrate that similarly to the human system LTC-IC and NK-IC can be enumerated after 8 weeks of in vitro culture with an initial inoculation of only 500-1000 total CD34+ cells. After approximately 4fold initial expansion the LTC-IC frequency in marmoset CD34+ cells was assessed between 10 to 59% (35±14%), the NK-IC-frequency was enumerated between 10 and 43% (25±9%). Further characterization of NK-cell maturation revealed that marmoset NK-cells can be evaluated using specific clones of anti-human CD56, NKp30 and NKp46. The frequency of colony forming cells (CFC) was measured between 23 and 61% (42±11%) after primary expansion. Thus, this marmoset readout system can easily be adapted to our previously described human single cell setup. This will allow comparative in vivo/in vitro analysis as well as clonal studies i.e. for the preclinical evaluation of novel stem cell-directed gene therapy approaches.

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Regulating HLA Expression to Decrease Immunogenicity of Cellular Therapeutics

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HLA polymorphism is the most relevant barrier to the development of cell-based therapies for regenerative purposes. To overcome this limitation, we used RNA interference (RNAi) to specifically knock down HLA class I transcripts. Regions susceptible to the action of small interfering RNAs (siRNAs) were identified in HLA-A heavy chain and β 2-microglobulin (β 2m) transcripts to achieve a gene or class I specific HLA silencing, respectively.

Lentiviral vectors were designed to express short hairpin RNA sequences (shRNA) targeting HLA-A heavy chain or $\beta 2m$ constitutively or controlled by a doxycycline-inducible promoter system. The level of HLA suppression in HeLa cells, B-LCLs and peripheral blood monocytes was detected by flow cytometry, real-time RT-PCR and Western Blot. The capacity of this silencing approach to resist IFN- γ -mediated HLA class I up regulation was tested in stimulation assays. Complement-dependent cytotoxicity assays were performed to evaluate the protective effect of HLA suppression against immune response. CD8+ T cell response was tested in proliferation and IFN- γ secretion assays. The transduction of inducible RNAi cassettes containing the sequences for shRNAs targeting $\beta 2m$ suppressed HLA class I expression by up to 90% in HeLa, B-LCLs and primary monocytes in a fully reversible manner. Similarly, conditional delivery of short hairpin RNA targeting HLA-A heavy chain transcripts silenced HLA-A expression by up to 90%. It was shown that HLA silencing was maintained even under inflammatory conditions. In cytotoxicity and proliferation assays, it was demonstrated that HLA class I knockdown was effective in preventing antibody-mediated cell lysis, CD8+ T cell response and interferon-gamma secretion, while the residual HLA expression in HLA-silenced cells was protective against NK cell-mediated lysis. In conclusion, we demonstrate the feasibility of controlling HLA expression by genetically modifying cell-based therapeutics to overcome the limitations of immunological rejection, bringing cellular therapies closer to reality.

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Foamyviral Vectors Efficiently Transduce Nonhuman Primate Hematopoietic and Embryonic Stem Cells

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Preclinical animal models are important for evaluating the safety and therapeutic efficacy of new therapeutic modalities such as gene therapy. From the different large animal models, nonhuman primate models have emerged over the last decades as highly desirable experimental systems from both a pathophysiologic and pharmacokinetic viewpoint and the study of nonhuman primates has provided important information on the efficacy and safety of gene therapy systems *in vivo* prior to human trials. The common marmoset (*Callithrix jacchus*) has the advantage that it is a small, and thus relatively inexpensive nonhuman primate model. Currently, very little data on the transduction efficiency of foamyviral vectors for the transduction of marmoset stem cells exists. We therefore performed a direct comparison using identically designed gammaretroviral, lentiviral and foamyviral vector constructs expressing the enhanced green fluorescent protein (EGFP) from the spleen focus forming virus (SFFV) promoter pseudotyped with either a modified prototype foamy virus (PFV) envelope or the G-protein of vesicular stomatitis virus (VSV-G) for the transduction of marmoset CD34+ hematopoietic progenitor cells as well as common marmoset embryonic stem cells (CMES). Three different target cell populations were transduced: Previously cryopreserved CD34-enriched cells from bone marrow of a common marmoset either (1) after a two-day prestimulation in the presence of IL-6, FLT3L, cSCF and TPO at a concentration of 100 ng/mL each, (2) after overnight incubation with 100 ng/mL SCF only and (3) common marmoset embryonic stem cells (ESC). Equal numbers of cells were exposed to the four different vector preparations for 16 hours in CH-296-coated 12-well dishes. The read-out was based on fluorescence microscopy of colonies plated in methyl cellulose as well as flow cytometry (FACS). Foamyviral vectors with the foamyviral envelope were the most efficient gene transfer tool for marmoset hematopoietic CD34-positive cells with stable transduction rates of over 80% as assessed by flow cytometry at both 2 or 7 days after the end of transduction and on average 88% transduction efficiency into colony forming cells (CFU-C). Transduction of CFU-C with the other vectors was always below 60%. In CMES, initial gene transfer rates of over 80% were achieved with the VSV-G pseudotype lentiviral vector, however, expression decreased to 13% after 7 days. In contrast, the foamyviral vector pseudotyped with the foamyviral envelope decreased only from 49% to 24% after 7 days. In conclusion, we achieved stable viral gene transfer and expression in CMES cells as well as highly efficient gene transfer into common marmoset hematopoietic CD34 positive cells using foamyviral vectors. In conclusion, these results suggest that foamyviral vectors may be highly feasible vectors for stem cell gene transfer and thus set the stage for a more detailed analysis of this vector system in transplantation studies in this nonhuman primate model.

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Risk assessment of AAV-mediated gene repair

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Vectors based on adeno-associated viruses (AAV) have attracted much attention as gene transfer vehicles and, more recently, as a tool to specifically alter complex genomes through homologous recombination (HR). In contrast to wild-type AAV, AAV vectors predominantly persist in an episomal form. However, the few integration events that occur randomly may play a role in insertional mutagenesis. For gene repair by HR, an AAV repair vector is specifically designed to recombine with the mutant target locus. HR is a rare event in mammalian cells

but the process can be stimulated some 1000-fold by creating a DNA double-strand break (DSB) within the target locus. Local stimulation of HR through creation of a DSB can be realized by expressing a site-specific endonuclease. Here, we assessed the risk of AAV-mediated gene repair with regard to insertional mutagenesis by determining simultaneously the number of gene repair (GR) and random integration (RI) events. The experiments were performed in p53+ and p53- human cell lines, in the presence or absence of a DSB. Titration experiments with an AAV repair vector and an endonuclease expression vector showed that both GR and RI increased with vector dose. At the highest amount, up to 10% of p53+ cells underwent HR at the target locus. Analysis of the kinetics of recombination events showed that GR events remained constant over a period of 36 days, while the number of cells carrying AAV episomes decreased from day 7 to 36. The targeting ratio (corrected cells per RI event) is a risk/benefit indicator and ranged from 1:10 to 20:1, depending on vector dose, the presence of a DSB and cell type. To verify our functional analyses, we are currently establishing a real-time PCR assay to quantitate the targeting ratio on the genome level. In summary, our results show the potential of AAV-mediated gene repair as a therapy for inherited disorders, but also reveal the risk associated with this approach, asking for a careful risks/benefit analysis for every single target tissue.

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HSPG binding properties of Adeno-Associated Virus (AAV) retargeting mutants and consequences for their in vivo tropism

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Different technologies have been developed to control or redirect tropism of adeno-associated virus of type 2 (AAV-2) by genetic capsid modification. Many of these approaches are based on the introduction of peptide sequences on the viral capsid in order to provide the vector with the ability to bind particular receptors. To obtain selective vectors however, simultaneous abrogation of capsid usage of natural receptor is essential. Insertions at the amino acid position 587 of the major capsid protein VP1 ablated the heparan sulphate proteoglycan binding of wild-type AAV in some, but not all previously described AAV targeting vectors. By panning an AAV Display library (Perabo et al., 2003) on heparin columns, we were able to correlate amino acid composition of insertions with heparin-binding phenotype and to propose a model to interpret the responsible molecular mechanisms. To prove that lack of heparin affinity column binding reflected lack of cellular HSPG binding, binding (B) and non-binding pools (NB) and AAV with wild-type capsid (RC) were produced as recombinant AAV vectors (rAAV) and heparin competition studies on the cervix carcinoma cell line HeLa were performed. The addition of heparin inhibited transduction by rAAV-RC and B-rAAV-pool while the effect on NB-rAAV-pool was negligible, demonstrating the initial hypothesis. Interestingly, in vivo studies allowed us to correlate the inability to bind to heparin with detargeting from liver and spleen in mice after systemic application, whereas AAV targeting mutants who retained the ability to bind to heparin showed an accumulation in liver and spleen comparable to AAV with unmodified capsid. These results provide a clear rationale to use the NB-AAV-pool for AAV Display selections of cell/tissue type specific AAV targeting vectors to avoid HSPG dependent retention in liver and spleen and increasing thereby the in-vivo-targeting ability of the respective vectors. Furthermore, our studies revealed different ways by which an inserted peptide is able to confer HSPG binding abilities to AAV targeting vectors.

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Potential of the oncolytic Adenovirus Delo3 RGD in the Treatment of Gliomas

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Oncolytic adenoviruses have attracted considerable interest, especially for the treatment of tumors refractory to current treatments such as glioblastoma. The E1A independent adenoviral replication of Ad dl520 is associated with nuclear localization of the transcription factor YB-1 (Cancer Research 64, 322-328, January 1, 2004). Ad dl520, which carries a deletion in E1A and does not express the large 289 amino acid long E1A protein, produces a strong antitumor effect in glioma and other carcinoma cell lines. To improve the cell killing capacity of Ad dl520, we introduced a deletion in the antiapoptotic E1B 19K gene and removed the E3 gp 19K gene in order to generate space for a therapeutic gene. Additionally we expanded the tropism of Ad5-Delo3 toward α v integrins by insertion of an Arg-Gly-Asp (RGD) motif into the fiber knob (Ad5-Delo3 RGD). We show that Ad5-Delo3 RGD had a stronger oncolytic effect than Ad dl520 and the wtE1B19K -expressing variant on a broad panel of tumor cells in preclinical studies. These results support further development of Delo3 RGD in combination with radiation therapy for treatment of these highly malignant tumors.