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# Optimised plasmids for sustained transgene expression in vivo

vorgelegt von

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## Erklärung

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

## Ehrenwörtliche Versicherung

Diese Dissertation wurde selbständig, ohne unerlaubte Hilfe erarbeitet.

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# 1. Introduction

## 1.1. Gene therapy

Gene therapy is a technique to introduce genetic material into patients for treating or preventing inherited or acquired disease, such as cancer and certain viral infections. The success of gene therapy is largely dependent on an efficient and safe gene transfer to the host cells or tissue and the appropriate expression of the introduced gene. For the gene transfer there basically exist 2 major branches; viral and non-viral vectors. The virally based vectors make use of the evolutionary adapted ability of virus particles to efficiently transduce their target cells. But evolution also led to powerful defence mechanism against the viral attack, leading to a fast immune answer from the hosts innate immune system and subsequent specific humoral and/or cell-mediated immune response directed against viral epitopes. This can not only hamper gene transfer but also lead to serious side effects, and in extreme cases causing death [1]. Another disadvantage of viral systems concerns the use of retroviruses, which integrate their DNA load into the host genome. This leads on the one hand to stable transgene retention, but is on the other hand connected with the dangers of insertional mutagenesis [2, 3], for example the introduction of a strong promoter upstream of an oncogene or disruption of the reading frames of vital genes.

Non-viral vectors, i.e. plasmid vectors, are generally safer, with less pronounced immunologic responses and low integrating potential, but suffer from a lower efficiency. Plasmid DNA (pDNA) can be delivered alone, for example by injection into muscle tissue or injection by hydrodynamic based methods, but is in most cases dependent on a carrier system which provide for shielding, targeting and cellular uptake of the DNA. Once released in the cytosol of the cell the plasmid must be able to fulfil its assignment of rendering a durable expression of the transgene at therapeutic levels. To implement this, the nature of the plasmid is decisive and the following passages will focus on the hurdles in transgene expression from pDNA and how an appropriate plasmid design can overcome them.

## 1.2. Non-viral vectors for gene therapy

### 1.2.1. Cis-acting transcriptional regulators

With the ambition of designing an optimised expression plasmid for gene therapy there are several aspects to consider. The choice of appropriate cis-acting transcriptional regulators, i.e. enhancers and promoters, is of immense importance, since they form the basis of a functional mammalian transgene expression, dictating the frequency of transcriptional initiation and therefore expression strength. Promoters can be divided into core and proximal promoters [4-6], depending on their position and function. The core promoter encompasses the transcriptional start site and contains the minimal sequences for correct initiation of RNA polymerase II (RNAPol II), i.e. binding sites for RNAPol II and basal transcription factors [4, 6]. It therefore is found in the absolute vicinity of the transcriptional start site (approx. +/-34 - 40bp) [4-6], whereas the proximal promoter can be located within a couple of hundred base pairs upstream or downstream of the transcriptional start site (approx. +/-250bp) [4] and contains only binding sites for transcription factors [4, 6]. The core promoter can contain motifs such as the TATA-box, TFIIB recognition element (BRE), initiator (Inr) and down-stream promoter elements (DPE) [4-6]. Each of these elements has specific functions related to transcription and is found in some, but not in all, core promoters [4]. There is no universal core promoter and its structural diversity together with its cognate cis-acting regulatory sequences, such as proximal promoters, enhancers and insulators, enables a differentiated expression as a response to changes in the nuclear environment.

The enhancer element differs from the promoter by not being orientation dependent and can act from large distances, up to many kb, away from the transcription start [6, 7]. In the event of transvection the enhancer even functions in trans to activate an allelic promoter on the other chromosome [7, 8]. The enhancer influences transcription from the distance by looping of the DNA, which brings the enhancer and core promoter in close proximity [7, 9, 10], enabling an orchestrated and interacting assembly of the enhanceosome (enhancer and associating transcription factors) and the transcriptional pre-initiation complex [11, 12]. Evidence show that the interaction is based on protein-protein interactions between transcription factors bound to the enhancer and promoter [7, 10], which could be the reason for the, in some cases, observed enhancer-promoter specificity**.** In these cases the enhancer increases

transcription from certain types of core promoters better [13, 14], or exclusively from certain promoters [15-18]. This probably serves to limit enhancer action to its designated promoters, since it is able to regulate gene transcription over large distances, hence, could otherwise also influence other promoters. Another way of regulating enhancer action are insulator regions, which limit the enhancer area of action within the insulator boundaries [6].

In plasmid design, viral promoters and enhancers are common features and the most extensively used originates from the cytomegalovirus (CMV). The genome of CMV is divided into 3 domains, depending on the temporal expression of the genes therein: immediate early, early and late. The immediate early genes are transcribed immediately after transduction, i.e. in acute infection, without the need for virally encoded proteins -but are silent in latency [19]. The major immediate early enhancer-containing promoter sequence (CMV-IEP) consist of a core promoter and a partially overlapping enhancer element, which together switch transcription on and off dependent on the status of the host immune system and cellular transduction processes [19]. The enhancer is a complex modular multicomponent region containing numerous binding sites for transcription factors, many of them repeated numerously [20]. Examples thereof are the 19-nucleotide repeats which contain binding sites for CRE (cyclic AMP response element) [21] and the 18-nucleotide repeats which specifically bind NFκB (nuclear factor κB)[22]. The sequence of the enhancer has been optimized to the respective host, hence, there exists a common basic blueprint with host specific variances. Many of the regulatory elements are conserved amongst the species strains of CMV, but the arrangement and number of transcription factor binding sites differ between them [23, 24]. Nevertheless, there also exist unique transcription factor binding sites, such as Elk-1, serum response factor, CBP and gamma-interferon activating site, which are only found in human CMV (hCMV) [23]. The close interrelation and still distinct functionality of the CMV enhancers has been demonstrated by replacing the enhancer of the murine CMV (mCMV) with the hCMV enhancer *in vitro* without changing the wild-type characteristic growth of mCMV in murine cells [24]. But the other way around, replacing the enhancer in hCMV with the murine analogue resulted in less efficient replication in human cells [25].

The CMV promoter might still be the most commonly used promoter since it renders strong and ubiquitous expression. But because of the reported fast inactivation by

promoter methylation [26, 27] other ubiquitous promoters are being investigated, for example the promoter of the elongation factor 1α (EF-1) or β-actin gene [28]. There are also other reasons for exchanging the CMV promoter. Depending on the gene therapy approach, it can be advantageous to limit the transgene expression. This can be achieved both in a temporal and tissue-specific manner by choosing a functional promoter and contingent auxiliary cis-acting modules. A temporal expression limitation is obtained by using an inducible expression system. It is based on a promoter which is activated by the presence or absence of biotic or abiotic factors, such as arabinose [29-31], antibiotics [32, 33] or heat [34-38]. A spatial expression limitation is acquired by using a tissue specific promoter which is active only in specific cellular environments. This so called transcriptional targeting can be a part of the overall targeting mechanism of the gene therapy complex, complementing a cellular targeting function of the plasmid carrier system, or act on its own. The field of transcriptional targeting was pioneered by Vile and Hart in 1993: They used 5´-control regions of genes related to melanin production (tyrosinase and tyrosinase related protein 1) to achieve gene expression explicitly in melanocytes, which are the natural melanin producing cells [39]. Since then the number of promoters used for transcriptional targeting has risen substantially. Particularly in the field of cancer gene therapy, this technique is of special interest. It aims to specifically kill the cancer cells and avoid off-target expression of, for example, a cytotoxic compound or a suicide gene, in normal tissue, which otherwise could have a devastating effect. Some promoters currently used for cancer gene therapy -in addition to the aforementioned tyrosinase promoter for melanocytes/melanoma, are prostate specific antigen (PSA), prostate specific membrane antigen (PSMA), probasin and human glandular kallikrein (hK2) promoter for prostate targeting, glial fibrillary acidic protein (GFAP), myelin basic protein (MBP) and myelin proteolipid protein promoter for glial/glioma targeting [40], just to mention a few. The disadvantage of tissue specific promoters is in general that they do not distinguish between healthy and cancerous tissue. Because of this, their use in cancer treatment is limited to cancer types originating from non-vital organs, for example melanoma, prostate or ovarian cancer. Alternatively, a promoter specific for tumour cells has to be used. For example, the promoter of albumin has been used for specific expression in hepatocytes [40, 41], but would be inappropriate for gene therapy of hepatomas with a direct or indirect cytotoxic gene, since normal hepatocytes would also be affected. One alternative to

the albumin promoter in liver cancer treatment is the tumour specific promoter derived from the α-fetoprotein (AFP) gene, which is normally only transcribed in foetal hepatocytes, but is reactivated in most hepatocellular carcinomas (HCC) [42]. The AFP promoter has been used with viral vectors, for example by promoting the expression of NIS (sodium iodide symporter) and consequent treatment with radioiodine [43-45], or HSV tk (herpes simplex virus thymidine kinase) and consequent treatment with the prodrug ganciclovir which is transformed into its toxic form by the TK [42, 46-49]. Also, the AFP promoter has been used for triggering the oncolytic activity of recombined adenovirus [50]. All approaches are showing promising results with a good transcriptional targeting with the AFP promoter, but so far no work has been done with non-viral vectors.

### 1.2.2. Bacterial DNA as trigger for immune responses

A prerequisite for a strong and enduring transgene expression are efficient promoter and enhancer elements, but they do not help if the transgene vector is silenced by the host cell, or even disposed of by the immune system. These events are common problems in gene therapy and they are caused by specific dinucleotides commonly found in bacterial DNA. The discovery of bacterial DNA as a trigger of immunological reactions was first done by the group around Tokunaga in 1984. They examined the bacille Calmette-Guérin (BCG)-mediated anti-tumour effect and by treatment with a DNA-rich BCG-fraction they observed regression and/or prevented metastasis of tumours in mice and guinea pigs [51]. *In vitro* they could show an augmentation of natural killer cell (NK) activity in peripheral blood lymphocytes by incubation with the same bacterial isolate [52] and the release of type I and type II interferon (IFN) [53]. The idea that bacterial DNA in general could be mitogenic was independently investigated by Pisetsky et al by observing the proliferation of murine lymphocytes after treatment with DNA from different bacterial strains, whereas no effect was seen after treatment with vertebrate DNA. They postulated that this effect must come from “non-conserved structural determinants”, which do not exist in vertebrate DNA [54]. Shortly thereafter, the group of Tokunaga also observed the differential mitogenic activity of bacterial and vertebrate DNA [55]. Krieg *et al* then discovered that specific CpG-containing motives in bacterial DNA trigger B-cell activation [56].

### 1.2.3. Difference between bacterial and vertebrate DNA

In comparison to bacteria and some viruses, which show the mathematically expected frequency of CpG-dinucleotides in the genome (1:16), vertebrate DNA is CpG suppressed, with a frequency of only one-fifth of the expected density [57]. Furthermore, in vertebrates about 80 % of the CpG´s are methylated at position 5 at the cytosine ring, which incapables the dinucleotides to an immunogenic stimulation, while bacterial DNA is largely unmethylated [56, 57]. This links the CpG-suppression of vertebrate DNA to the non-self pattern recognition by the immune system. The sparsity of CpG´s in the vertebrate genome is probably the result of the non-existence of an effective repair mechanism of deaminated 5-methylcytosin, which gives thymine [14, 57].

### 1.2.4. TLR-9

Bacterial DNA, with its higher frequency of non-methylated CpG-dinucleotides, and CpG-containing oligodeoxynucleotides (ODN´s), are highly stimulatory to leukocytes *in vitro*, inducing B-cell proliferation and immunoglobulin secretion [56], NK cell lytic activity [58] and dendritic cell (DC) [59] and macrophage [60] activation. The activation of these diverse events by unmethylated CpG´s has its triggering point at the toll-like receptor (TLR) 9, a member of the big evolutionary conserved TLR family. TLR´s recognize different pathogen-associated molecular patterns (PAMP´s) in mammals [61]. For example, TLR4 specifically binds to lipopolysaccharide (LPS), a component of the outer wall of gram negative bacteria, and the ligand of TLR8 is single stranded RNA of viruses [62]. TLR-9 was first recognized as the mediator of cellular response to unmethylated CpG in 2000 in the group of Akira [63]. They treated $TLR^{-/-}$ mice with bacterial DNA, which in this case were not able to elicit any of the characteristic immunologic events which are known to be triggered by unmethylated CpG-containing DNA.

TLR9 consist of, as every TLR in general, a leucin-rich repeat (LRR), a transmembrane component and a Toll/IL-1 receptor (TIR) homology domain (**fig.1**),

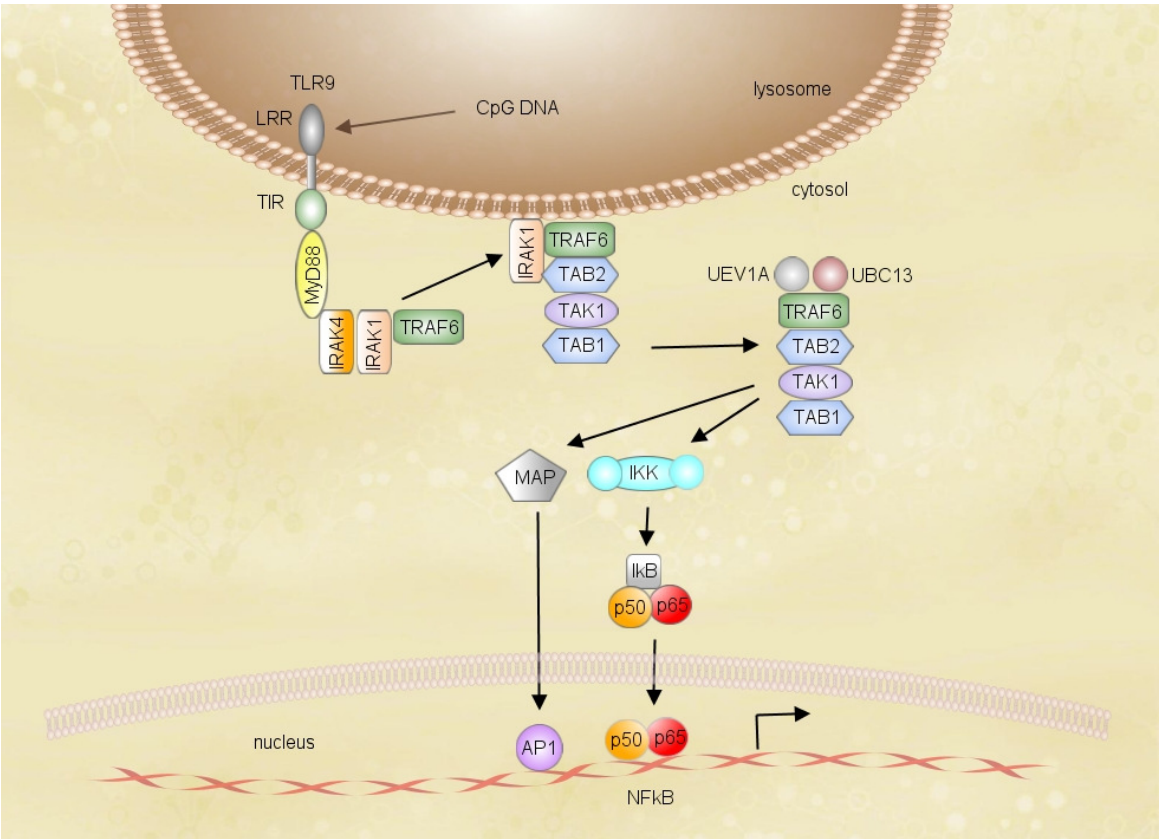


**Fig. 1: Transduction pathway of TLR9.** The transmembrane receptor TLR9 binds unmethylated CpG motifs of endocytosed DNA in the lysosomal lumen. The binding leads to formation of a multiprotein complex with MyD88, IRAK1, IRAK4 and TRAF6 on the cytosolic part of TLR9 (TIR). IRAK1 and TRAF6 dissociate and form a new complex with TAB1, TAB2 and TAK1 on the lysosomal membrane. IRAK1 is degraded and the complex binds UEV1a and UBC13, which activates TAK1 to phosphorylate MAP kinase and IKK complex. IKK in turn phosphorylates IκB, which is then ubiquitylated and degraded, thereby releasing NFκB to translocate to the nucleus and bind to NFκB binding motifs on the genome. AP-1 is activated by phosphorylation by MAP kinases and likewise binds to its corresponding genome sequences. As a result of NFκB and AP-1 binding, the expression of proinflammatory cytokines is triggered.

the latter receiving its name because of the 3 homologous regions (Box 1-3) with the IL-1 (interleukin 1) receptor [61-63]. TLR-9 is first located in the endoplasmic reticulum (ER) of macrophages and DC´s and then translocates to compartments containing exogenous DNA, which are early endosomes directly after endocytosis of bacteria and at a later stage motile lysosomal compartments. The TLR-9 directly binds to the ligand DNA [64] with the receptor LRR´s which is located in the

lysosomal lumen, whereas the TIR domain is protruding into the cytoplasm for consequent transduction signalling [65]. Upon receptor-ligand binding MyD88 (myeloid differentiation primary-response protein 88) is recruited to the TIR domain, where it in turn associates with IRAK4 (IL-1R associated kinase 4). IRAK4 in turn binds to IRAK1 and triggers its phosphorylation. The phosphorylated IRAK1 associate with TRAF6 (tumour necrosis factor receptor associated factor 6) and the IRAK1/TRAF6 heterodimer dissociate from the receptor and forms a new complex with TAK1 (transforming growth-factor-β-activated kinase), TAB1 (TAK1-binding protein 1) and TAB2 at the lysosomal membrane. IRAK1 is degraded and the remaining TRAF6/TAK1/TAB1/TAB2-complex binds to UBC13 (ubiquitin-conjugating enzyme 13) and UEV1A (ubiquitin-conjugating enzyme E2 variant 1) in the cytosol. This leads to the ubiquitylation of TRAF6 which, in turn, activates TAK1. The activated TAK1 phosphorylates MAP (mitogen-activated protein) kinases and the IKK-complex (IκB-kinase complex, consisting of IKK-α, IKK-β and IKK-γ). The activated IKK-complex subsequently phosphorylates IκB (inhibitor of NFκB), which leads to its ubiquitylation and degradation. The NFκB, released from IκB, then translocates to the nucleus and activates expression from NFκB-dependent genes [62]. Together with AP-1 (activating protein-1), which is activated by MAP-kinases, NFκB lead to the expression of pro-inflammatory cytokines, like TNF-α (tumour necrosis factor- α), IL-1 (interleukin-1), IL-6 and IL-12 [65].

## 1.2.5. CpG´s and their adverse effect on transgene expression

### *1.2.5.1. Immune response*

Unmethylated CpG dinucleotides can, as explained above, elicit a direct inflammatory response. This effect has already been harnessed to improve the immune response after vaccination by using CpG-motifs as adjuvants [65, 66] or for cancer immune therapy approaches [67]. In the context of gene therapy where a long-term transgene expression is desired, CpG-dinucleotides in the plasmid DNA have been shown to have a strong negative effect on the duration of transgene expression [68-72]. Even as few as one CpG dinucleotide in the plasmid DNA sequence can lead to increased levels of pro-inflammatory cytokines (TNF-α, IL-12, IFN-γ) and infiltrating neutrophils; additional CpG´s increased these levels

incrementally, whereas CpG-depleted plasmids showed no measurable elevation of the cytokine levels [69].

The mechanism by which unmethylated CpG-sequences reduces transgene expression is not entirely clear, but could derive from various factors. First, proinflammatory cytokines, such as Il-1, Il-6 and TNF-α, produced as a result of interaction of unmethylated CpG´s with TLR-9 in immune cells, can cause local tissue injury by inducing the production of nitric oxygen and reactive oxygen species intermediates. This injury can lead to further expression of chemokines and chemokine receptors, attraction of neutrophils and macrophages and activation of local endothelium and epithelium. This leads to elevated expression levels of adhesion molecules, chemokines and subsequent the attraction of antigen-dependent and –independent inflammatory cells to the site of inflammation [73]. The interaction of the unmethylated CpG DNA with the TLR-9 also activates DC´s and B cells and triggers the expression of costimulatory and class II MHC (major histocompatibility complex) molecules and chemokine receptors, preparing them for acting as a professional antigen presenting cell (APC) and migration to draining lymph nodes for interaction with T-cells. After internalization and processing of the antigen (in the case of plasmid vectors: the transgene product) B-cells can home with T helper ($T_H$) cells, thus being primed to become antibody producing plasma cells, and DC´s can activate naïve T cells, which once activated, will secrete cytokines to further strengthen the adaptive immune response [74]. The adaptive immune system needs three signals to develop an efficient immunological reaction against an antigen [74]: The first signal comes from the antigen itself, i.e. the transgene product. The second signal is derived from a co-stimulatory molecule, like CD80 or CD86, which itself is induced from the third signal, deriving from a PAMP. In the case of plasmid DNA vectors, the PAMP will be the unmethylated CpG sequences, which trigger TLR9 and subsequent gene expression of co-stimulatory molecules. That this third signal, the activation of the APC, can have a strong effect on the immune reaction against the transgene product was shown by Ertl and co-workers [75] in mice injected i.m. with plasmids, expressing an antigenic viral glycoprotein. The plasmid contained immunostimulatory CpG sequences and by methylation of the plasmid (containing a methylation-insensitive SV40 promoter) the longevity of the transgene expression was markedly increased, and also, the maturation and migration of DC´s to the lymph nodes were reduced. This result points to the importance of an

activated, mature professional APC in the initiation of an adaptive immune reaction directed against a transgene product. Hence, unmethylated CpG-motifs can cause a general ‘alert status’ of the innate immune system which in turn can activate transgene product specific B and T cells.

#### *1.2.5.2. Promoter attenuation:*

Another mechanism for CpG-mediated reduction of transgene expression is the attenuation of promoters, where the transfected cells are not killed, but the transgene expression itself is reduced. The effect seems to be on the level of mRNA and does neither alter the cell viability nor causes vector reduction [77]. It has for example been shown, that the CMV promoter can be attenuated by exposure to the cytokines IFN-γ [76-80] and IL-10 [79]. Other viral promoters which are sensitive to IFN-γ originate from the simian virus 40 (SV40) [76, 77], rous sarcoma virus (RSV) [77] and hepatitis B virus (HBV) [81]. In the case of mCMV, IFN-responsive elements were found scattered over the immediate early enhancer sequence, hence, IFN-γ probably reduced expression by transcription impairment [80]. Aside from diminution of expression there are also cases of augmentation by cytokines; the CMV promoter can be activated by TNF-α, IL-4 and IL-1 [79] and the MHC class I promoter is activated by IFN-γ [76].

#### *1.2.5.3. DNA-methylation:*

Another negative effect on transgene expression deriving from unmethylated CpG´s not correlated to the destruction of the host cell, is plasmid methylation. The cytosine in the context of a CpG dinucleotide can be methylated in position 5, thus losing its immunogenic character, but instead this modification can perturb transcription. It is generally accepted, that hypermethylated promoters lead to transcriptional silencing. About 60% of all genes, predominately house keeping genes and highly transcribed genes, contain in their 5´ end sequences dense stretches of CpG dinucleotides, the so called CpG islands [82],. These are normally unmethylated with hyperacetylated histones and are deficient in linker histones [83], whereas the vast majority of CpG´s scattered over the genome are methylated. Methylation of promoter CpG islands is seen as a mean for gene silencing [84], and plays a role in X-chromosome inactivation [85], genomic imprinting [86, 87], the silencing of intragenomic parasites [88], and carcinogenesis [89-91].

The most direct way of gene silencing by promoter methylation is steric shielding by methyl groups of the binding sites of the basal transcription machinery and transcription factors. Many transcription factors contain GC-rich sequences or CpG dinucleotides in their DNA binding site. The methyl group of methylated CpG´s protrudes into the major grove of DNA and, thus, binding of transcription factors to methylated DNA can be hampered or abrogated. Some examples for methylation sensitive transcription factors are AP-2, c-Myc, CREB and ATF [92]. The direct masking of promoter and enhancers by methyl groups is not the only and also not the most prominent way of transcription interference. There are transcription factors insensitive to methylation, for example AP-1 and CTF [92] and many have recognition sites without CpG´s. Further, Kass *et al* [93] has shown that methylation alone does not silence gene expression but leads to formation of a repressive complex which excludes the transcriptional machinery from the promoter. They demonstrated that transcription can be executed equally well from a methylated or unmethylated plasmid with a HSV *tk* (herpes simplex virus thymidine kinase) promoter directly after injection into *Xenopus* oocyte nuclei. At later time points the expression from the methylated plasmid was gradually reduced in comparison to the unmethylated plasmid. This coincided with a gradual loss in DNAse I hypersensitive sites in the methylated plasmid (which is generally thought of as the transcription factor binding sites where nucleosomes are excluded) and inclusion of the promoter into a nucleosomal array. It demonstrates how the methylation is the trigger for a time-dependent assembly of a repressive structure that includes a higher order nucleosomal complex.

The bridge between the methylated DNA and the repressive structure are methyl-CpG binding proteins, for example MeCP2 and MBD1-3, which attract corepressive proteins and histone deacetylases (HDAC´s) directing the remodelling of chromatin [82]. The MeCP2, for example, tethers a repression multiprotein complex that includes the corepressor protein mSin3A and the histone deacetylases HDAC1 and HDAC2 [94], and can operate over a distance of many hundred base pairs to induce repression [83]. The repressive effect could be abolished by treatment with histone deacetylases, indicating histone deacetylation as an essential component of the repression mechanism [94, 95].

The transcriptional repression mediated by methylation does not work as an on-off button, but can be graded according to various factors. Expression experiments with

patch-methylated plasmid vectors have led to deeper understanding of the relationship between reduced expression and methylation density and position. The effect of repression is transmissible in cis, which means that a methylation of the promoter is not required, but can spread from adjacent areas [93, 96-98]. The proximity of the methylated patch to the promoter seems in lesser extent to be decisive for repression than the number of methylated CpG´s [96-98]. The formation of a repressive chromatin complex is induced by methylated DNA and spreads onto unmodified flanking domains. Parameters such as methylation density could influence the stability of the complex, thus its ability to diffuse on the plasmid and exert its anti-transcriptional function. For example, a sparsely methylated transgene could repress expression completely, but this effect could be overridden by the introduction of an enhancer sequence. A densely methylated transgene, on the other hand, could not be saved from expressional repression by the enhancer. [99].

### 1.2.6. Reducing the number of CpG´s in plasmids

Because of the disadvantageous effects of CpG´s in the plasmid vector listed above, such as activation of the immune system or silencing of the transgene, there are great efforts done in reducing or eliminating these dinucleotides. As a normal CpG-replete plasmid contains several hundreds of CpG´s the method of site directed mutagenesis would be too labour intensive. Instead, synthetically manufactured DNA is used. The coding regions, such as transgene and antibiotic resistance genes, can be changed by using the degeneracy of the genetic code (codon optimization). Hence, the DNA sequence can be changed without perturbing the primary amino acid structure. Other functional DNA sequences, such as splice donor and acceptor and lariat branch point in introns must remain intact to excise its task. Promoters and enhancers can be CpG depleted, but with unforeseeable consequences of their functionality. Another regulatory sequence difficult to modify is the origin of replication (ori). Most commercially available plasmids have an ori based on the ColE1 ori [100], which transcribes for a short RNA primer initiating replication start [101]. About 60% of its CpG´s have successfully been substituted without extensive loss of activity, but a totally CpG free version is not possible [100]. As an alternative, the protein regulated replication of R6K is used. For this, the CpG-depleted γ-origin of replication

of R6K is incorporated into a plasmid, which is propagated in bacteria harbouring the *pir*-gene, over-expressing the π-protein, which initiates replication [102].

### 1.2.7. Mini-circles

One alternative to the CpG-reducing modifications mentioned above is to eliminate the plasmid backbone containing the bacterial sequences, such as ori and antibiotic resistance genes with accompanying promoter, which contain a big part of the CpG´s. Also, as an effect of reducing the plasmid size, investigators have demonstrated that vector size has a substantial inverse impact on transgene expression level when transfected into mouse muscle *in vivo* by electroporation [103] or intra-arterial delivery, or delivered to HeLa cells *in vitro* [104], probably by facilitating the vector delivery.

Linear DNA containing only the mammalian expression cassette produced by PCR was able to direct similar long-term transgene expression *in vivo* as the original plasmid vector (equimolar amounts), with the difference in inducing less TNF-α and IL-12 production [105]. A similar approach, but by producing a linear DNA expression cassette by endonuclease digestion of parental plasmid and subsequent purification, resulted in elevated transgene expression levels in mouse liver from the linear DNA compared to the circular plasmid [106].

The disadvantage with linear DNA is that the ends of the DNA molecule are exposed and can be targeted by exonucleases. For this reason, methods have been developed to manufacture a closed circular mammalian expression cassette by removing the bacterial backbone by intramolecular site specific recombination. Thus, two circular molecules are formed: one miniplasmid harbouring the bacterial sequences, such as bacterial promoter, antibiotic resistance gene and ori, and one with the mammalian expression cassette, the “minicircle”. In general, higher transgene levels are reported *in vitro* [29, 35] and *in vivo* [30, 34] from the minicircle compared to the full-length plasmid. For production by recombination it is crucial that the process is inducible, allowing a programmed start only after the growth phase in the bacteria, and a proper purification step, to separate the minicircle from the side product (miniplasmid and residual parental plasmid). Several types of recombinases have been tested. Darquet and co-workers [34-36]) have utilised the heat-inducible phage λ Int integrase to form minicircles which were isolated by restriction enzyme

digestion of the miniplasmid and subsequent caesium chloride centrifugation. Another recombinase system derived from the phage P1 Cre recombinase was used by Bigger and co-workers [29], putting the recombinases under the control of an arabinose operon. Also here, minicircle was isolated from side products by enzyme digestion and caesium chloride centrifugation. Nehlsen et al [37] used an agarose gel for separation of minicircle from the restriction enzyme digested side products after recombination with a heat-inducible Flp recombinase. An integrase from the phage ΦC31 was used for recombination by Chen et al [30, 31]. The recombinase and an endonuclease gene was put under the control of the arabinose inducible BAD promoter in the parental plasmid, allowing for recombination reaction at 32°C and subsequent digestion of side product plasmids at 37°C after adding arabinose to the bacteria medium. The minicircle could then be extracted by conventional plasmid isolation procedure from the bacteria. This direct *in vivo* separation of minicircle from side product plasmids has the advantage of being more efficient and less labour intensive than the previously described methods. Another efficient system of direct isolation of minicircle DNA was reported by Jechlinger *et al.* [38] using an arabinose induced expression of ParA resolvase and a minicircle-specific membrane anchor, which executes the formation of minicircles and the membrane anchorage thereof, respectively. After a heat-induced lysis of the bacteria the minicircle is found in the cell pellet whereas side product plasmids are in the supernatant. Of all recombinases the ParA resolvase is the most efficient in minicircle production with 100% recombination rate [38], which is also needed when using an affinity-based purification of the minicircle, since otherwise also residual parental plasmid would be co-purified.

### 1.2.8. S/MAR´s

For the design of an efficient plasmid for gene therapy approaches the choice of transcriptional regulators is crucial, as they dictate the basic transcriptional activity. Also, the CpG content should be minimized to circumvent transgene silencing and the loss of plasmid DNA by immunologic reactions. To further reduce the loss of plasmids it is also important that the plasmid is replicated properly in every cell cycle and segregated evenly onto the daughter cells in mitosis. Reports have shown that a scaffold/matrix attachment (S/MAR) sequence can support this [107, 108]. S/MAR´s

are naturally occurring AT-rich sequences which organizes the genome by binding DNA loops to the nuclear matrix. S/MAR´s do not have a shared consensus, but the physical property of being able to relax base-pairing under torsional stress [109], and they are often found in association with origins of bidirectional replication [108]. The first episomal plasmids -plasmids able to stably remain as a autonomously replicating, extrachromosal entity in the nucleus- were dependent on virally encoded proteins, such as the large T-antigen from SV (simian virus) 40 or EBNA-1 from EBV (Epstein-Barr virus). These proteins were supporting replication by interacting with the ori of the plasmid but could lead to transformation of the transfected cells and tumour formation *in vivo* [109]. By exchanging the large T-antigen with an S/MAR sequence the first episomal plasmid devoid of virally encoded proteins was created, pEPI-1 [108]. pEPI-1 was stably remained in transfected cells without selection pressure over more than 100 cell generations, without integrating into the genome [108]. The minimal requirement for efficient plasmid retention was shown to be an S/MAR sequence downstream of the transgene, allowing for transcription into the S/MAR [107]. Also, it was shown that the S/MAR bound pEPI-1 to the nuclear matrix, by association to nuclear proteins such as SAF-A [107, 110, 111] and non-covalently to mitotic chromosomes [107]. By binding to the nuclear matrix replication, which is tightly associated with the matrix, can be facilitated, and by binding to mitotic chromosomes, a successful transmission into the daughter cell nuclei can be achieved. Both processes are possible contributors to the high retention potential of pEPI-1 (>0,98) [107]. The efficiency of pEPI-1 is only hampered by a low episomal establishment rate, and establishment *in vitro* is only achieved after an initial selection period [108].

## 1.3. Aim of the thesis

The aim of this thesis was to develop and explore new and better plasmid vectors for the purpose of ameliorated nonviral gene therapy. So far gene therapy in general is hampered by low safety in the case of viral delivery and low efficiency in the case of the nonviral vectors. By concentrating on the nonviral approach, the most imminent safety issue is solved, but the nonviral vectors still need improvements to meet the requirements posed on a gene therapy product. Such a requirement is a high and prolonged gene expression, thus delivering the gene product at therapeutic levels and circumventing repeated applications and the discomforts and costs associated with them. In this thesis this was aimed at by evaluating a combination of the ubiquitous mammalian promoter EF-1 with a murine or human CMV enhancer *in vivo*. Also, a promising novel synthetic promoter, SCEP, was investigated. The transgene expression was measured over an extended period of time to follow the long-term stability and allow a better understanding to why transgene signal is lost, in order to further improve plasmid design. Additionally to the EF-1 and SCEP promoter, liver specific promoters were examined for transcriptional targeting of the liver, which can be useful in avoiding off-target expression, thus reducing side-effects of a liver specific gene therapy.

A further aim of this thesis is the testing of a minicircle, produced by a novel and highly efficient production and purification process. By comparison with its parental plasmid *in vivo* conclusion were drawn about its functionality and advantages of avoiding the bacterial backbone.

# 2. Materials and methods

## 2.1. Cloning and propagation of plasmids

The plasmid pCpG-mCMV/EF1-LucSH was cloned by inserting the *BglII-NehI*-digested *Luc::Sh* fusion gene from pMOD-LucSh (Cayla-Invivogen, Toulose, France) in the *BglII-NehI*-digested backbone of pCpG-mcs (Cayla-Invivogen). The resulting plasmid is CpG-depleted and driven by an EF-1 promoter. For the pCpG-hCMV/EF1-LucSH the murine CMV enhancer of pCpG-mCMV/EF1-LucSHSH was excised by digestion with *SpeI* and *SfbI* and exchanged for the human CMV enhancer from pGZCUBI [167], which was amplified by PCR with primers containing *SpeI* and *SfbI* sites, respectively. The pCpG-2xhCMV/EF1-LucSH was constructed by inserting a further PCR-amplified hCMV enhancer element, featuring *PstI* sites at both ends, into the *SbfI* site of pCpG-hCMV/EF1-LucSH. The orientation of this further enhancer element was verified by sequencing (AGOWA, Berlin, Germany). pCpG-hCMV/EF1-eFLuc was constructed by excision of Luc::Sh from pCpG-hCMV/EF1-LucSH by *BglII-NehI*-digestion and ligation with the *BglII-AvrII*-digested eFLuc sequence from pV2011-oFL, a kind gift from Rabinovich, M.D. Anderson Cancer Center, Houston, [112]. pCpG-hCMV/CMV-eFLuc was cloned by inserting the CMV promoter, cut out from pCMV-GLuc (New England BioLabs (NEB), Frankfurt am Main, Germany) with *SnaBI* and *HindIII*, into the equally digested pCpG-hCMV/EF1-eFLuc, resulting in an exchange of the EF-1 promoter for the CMV promoter. The synthetic promoter SCEP sequence was produced by Geneart (Regensburg, Germany) and cloned into the SpeI/HindIII digested pCpG-hCMV/EF1-eFLuc backbone, thus exchanging the EF-1 promoter and forming the plasmid pCpG-hCMV/SCEP-eFLuc.

The construction of the CpG-replete pEPI-1 and pEPI1ΔS/MAR vector backbones, with the transgene EGFP under the control of the CMV-IEP, has been described previously [108]. The EGFP gene was exchanged for the fusion gene EGFP::Luc by digestion of the backbones with *NehI/BglII* and insertion of the PCR amplified EGFP::Luc sequence from pEGFPLuc (Clontech, Saint-Germaine-en-Laye, France), thereby using 5´ *NehI* and 3´ *BglII* primers. The resulting plasmids, pEPI1-CMV-EGFPLuc and pEPI1ΔS/MAR-CMV-EGFPLuc, were digested with *PciI/NehI* to remove the CMV-IEP and the HPGL and APOE promoter were inserted instead. All liver specific promoters were generated by PCR of human genomic DNA with *PciI*

and *NehI* primers. The resulting plasmids were: pEPI1-HPGL-EGFPLuc, pEPI1-APOE-EGFPLuc, pEPI1ΔS/MAR-HPGL-EGFPLuc and pEPI1ΔS/MAR-APOE-EGFPLuc.

The cloning of the shuttle vector pHulk, which served as a template for all pEPito and pEPItoΔS/MAR based plasmids, has been described already [113]. pHulk is CpG-depleted with a R6K ori, a zeosine resistance and β-lactamase gene, and a MCS (multiple cloning site). To add the EGFP::Luc gene it was amplified by PCR, as described above, and inserted into the *NehI/BglII* digested pHulk, resulting in pEPItoΔS/MAR-EGFPLuc. By inserting the CMV-IEP, isolated as a *PciI/NehI* fragment from pEGFP-C1 (Clontech), pEPItoΔS/MAR-CMV-EGFPLuc was created. The S/MAR sequence originates from pTZ-E20, from where it is cut out and ligated into the pEPItoΔS/MAR-CMV-EGFPLuc by *EcoRI/BglII* digestion, thus forming pEPIto-CMV-EGFPLuc. By inserting liver specific promoters, generated as described above, instead of the CMV-IPE (excised by *PciI/NehI* digestion), the plasmids pEPIto-AFP-EGFPLuc, pEPIto-HPGL-EGFPLuc, pEPIto-APOE-EGFPLuc and pEPIto-FMO-EGFPLuc were created. The human CMV enhancer from pGZCUBI was PCR amplified with *NcoI* and *PciI* primers and inserted into the *PciI* digested pEPIto-AFP-EGFPLuc, pEPIto-HPGL-EGFPLuc and pEPIto-APOE-EGFPLuc plasmids to generate pEPIto-AFP/CMV-EGFPLuc, pEPIto-HPGL/CMV-EGFPLuc and pEPIto-APOE/CMV-EGFPLuc. pEPIto-CMV-GLuc was cloned by digesting pEPIto-CMV-EGFPLuc with *NehI/BglII*, thereby excising the EGFPLuc, and in its place inserting the GLuc sequence from pCMV-GLuc (NEB), amplified with *NehI* and *BglII* primers. All cloning work of plasmids was conducted by Rudolf Haase (Ludwig-Maximilians University, Munich). All plasmids were amplified in *E. coli* GT115 or DB3.1λpir, except for pEGFPLuc and pCMV-GLuc, which were amplified in DH5α, and isolated using a Qiagen (Hilden, Germany) Plasmid Giga or Maxi Prep.

## 2.2. In vitro studies

### 2.2.1. Cell lines and cell culture conditions

Primary porcine smooth muscle cells (PSMC) were grown in Ham´s F12 (Biochrom, Berlin, Germany)/ Waymouth (Gibco, Darmstadt, Germany) medium 1:1, 37,5 ‰ sodium bicarbonate (Gibco), 0,01M HEPES, 10% bovine calf serum (FCS) (Gibco),

40mM stable glutamine (Biochrom), 100U/ml penicillin and 100µg/ml streptomycin (Biochrom), 0,25 µg/ml amphotericin B (Sigma Aldrich, Steinheim, Germany).

Murine neuroblastoma cell line Neuro 2A (N2A, ATCC CCL-131), murine melanoma cell line B16F10 (kindly provided by I. J. Fidler, Texas Medical Center, Houston, TX, USA), human glioblastoma cell line U87MG (ATCC HTB 14), human colon carcinoma C26 (kindly provided by A. Anz, Klinikum der LMU, München, Germany) and the human cervix carcinoma cell line HeLA (ATCC CCL-2) were cultured in Dulbecco's modified Eagle's medium (DMEM, 1g glucose/l (Biochrom)) completed with 10% FCS. U87MG was grown on collagen G (Biochrom) coated plates.

Human hepatocellular carcinoma cell lines HuH7 (JCRB 0403, Tokyo, Japan) and HepG2 (kindly provided by Prof. Spitzweg, Klinikum der LMU, München) and human melanoma cell line MDA MB-435 (kindly provided by A. Ulrich, Max-Planck Institute, Munich, Germany) were cultivated in DMEM/Ham's F-12 medium 1:1 supplemented with 10% FCS and 2mM stable glutamine.

BT459, a human breast cancer cell line (CLS, #300132), Du145 (ATCC HTB-81), a human prostate cancer cell line, and the murine fibroblast cell line L929 (ATCC CCL-1) were cultivated in RPMI 1640 (Biochrom) medium supplemented with 10% FCS. The human breast adenocarcinoma cell line MCF-7 (kindly provided by A. Ulrich, Max-Planck Institute, Munich, Germany) was cultured in DMEM medium (5g glucose/l) supplemented with 20% FCS.

All cultured cells were grown at 37 °C in 5% $CO_2$ humidified atmosphere.

### 2.2.2. Isolation of primary porcine smooth muscle cells (PSMC´s)

Porcine aortas where transported cold in PBS with antibiotics (500 U/ml penicillin, 500 µg/ml streptomycin and an antifungal (1,25 µg/ml amphotericin B) until isolation. One organ was divided into 2 parts and the endothelial side was washed once with PBS. 5 ml of enzyme solution (0,1%, collagenase II (Sigma Aldrich) and 0,1% trypsin (Biochrom) diluted in PBS) was added to the aorta piece and incubated for 20 minutes at 37°C. The enzyme solution was carefully exchanged for 5 ml M199 medium (Biochrom). By pipetting up and down and careful scraping with the pipette the endothelial cells (EC´s) were detached. The medium containing the EC´s were collected and the scraping process was repeated several times. The collected EC´s were processed further for other purposes and the remaining aorta were cut into

small pieces (2-5 mm) for the isolation of PSMC´s. 75 cm² Corning cell culture flasks were filled with 10 ml PSMC isolation medium (Ham´s F12 and Waymouth medium 1:1, 37,5 ‰ sodium bicarbonate, 0,01M HEPES, 10% FCS , 40mM L-Glutamine, 500U/ml penicillin, 500µg/ml streptomycin, 1,25 µg/ml Amphotericin B) and the medium was evenly distributed in the flask. The flask was then tilted and the small pieces of aortas were put on the upper part of the bottle bottom, which was bare of medium, to allow them to stick to the bottom over night. On the following day the flasks were laid horizontal again, flooding the cells. The medium was exchanged every 2-3 days and after 2-3 weeks, when PSMC´s were growing out, the aorta pieces were removed and the cells splitted. After a confluence of 70-90% was reached, the cells were aliquoted in bovine calf serum with 10% DMSO and frozen in liquid nitrogen for storage.

### 2.2.3. In vitro transfections

*General procedure (if otherwise not noted):*

Cells were seeded 24h before transfection and the cell medium was exchanged for fresh growth medium (with serum) directly prior transfection. Polyplexes were formed by rapid mixing of pDNA and the transfection reagent H DO (synthesized by Verena Russ, Ludwig-Maximilians University, Germany [168]) solution at a c/p (conjugate to plasmid) ratio of 2 in HEPES-buffered saline (HBS, 20 mM HEPES, 150 mM NaCl, pH 7.1) and incubated for 20 minutes at room temperature. After 4h of incubation of the cells with the polyplexes the medium was changed for fresh cell medium. For measurements the cells were trypsinised and a part thereof was lysed in 0,5x lysis buffer (Promega, Mannheim, Germany) and analysed by luciferase-assay.

*Transfection of MDA MB-435:*

125 000 cells were seeded in duplicates in 24-wells and were transfected with 800ng pDNA (pEGFPLuc, pCpG-mCMV/EF1-LucSH, pCpG-hCMV/EF1-LucSH, pCpG-2xhCMV/EF1-LucSH).

*Transfection of PSMC´s with GLuc-plasmids:*

Cells were transfected in 2$^{nd}$ passage.

Quiescent cells: PSMC´s were seeded in 6-wells (n=4) and were grown to a confluency of about 70 %. Then the normal growth medium was exchanged for medium with additional 300 µg Heparin/ ml to stop cell growth. After an incubation of

24h in heparin the cells were transfected with 4 µg of pDNA (pEPIto-CMV-GLuc, pCMV-Gluc). During transfection, medium without heparin was used, which was exchanged for heparin containing medium after 4h, which was then used throughout the experiment. Every second day medium was collected and measured for GLuc activity.

Cycling cells: 100 000 cells were seeded in triplicates in 24-wells and let to grow until 40-50% confluency before transfection with 4µg of pDNA (pEPIto-CMV-EGFPLuc, pEGFPLuc).

*Transfection with eFLuc-plasmids:*

10 000 HuH7, MCF-7, N2A and MDA MB-435 cells were seeded in 96-wells (n=5) and transfected with 200ng of pDNA (pCpG-hCMV/EF1-LucSH, pCpG-hCMV-eFLuc, pEPIto-CMV/AFP-EGFPLuc, pEPIto-CMV/AFP-eFLuc, pEPIto-CMV-EGFPLuc, pEPIto-CMV-eFLuc) and LPEI (linear polyethylenimine, synthesized in the working group of Prof. Wagner, Ludwig-Maximilians University, Germany) at an N/P (nitrogen to phosphate) ratio of 6 in HBS. 24h after transfection all cells were lysed and luciferase activity was measured by luciferase assay.

*Transfection with pCMV-Luc-MC and pCMV-Luc::*

50 000 PSMC and N2A cells were seeded in triplicates in 24-wells. For transfection 750ng pCMV-Luc-MC for PSMC´s and 500ng for N2A were used. pCMV-Luc was applied either with an equal pDNA weight or in equimolar amounts (PSMC:1200ng and N2a:795ng) compared to pCMV-Luc-MC.

*Transfection of HuH7 cells with plasmids containing liver specific promoters:*

50 000 HuH7 cells (n=2-3) were seeded into 24-wells and transfected with 1,5 µg pDNA.

*Transfection of pEPIto-AFP/CMV-EGFPLuc and pEPIto-CMV-EGFPLuc into different cell lines:*

10 000 (HuH7, Du147, N2a, U87MG, L292, HepG2) or 5000 (B16F10, BT459, C26, HeLa) cells were seeded in 96-wells (n=5) and transfected with 200 ng pDNA complexed with LPEI at an N/P ratio of 6 in HBS. 24h after transfection all cells were lysed and luciferase activity was measured by luciferase assay.

## 2.3. In vivo studies

### 2.3.1. Hydrodynamic delivery

6-7 weeks old female Balb/C (Elevage Janvier, Le Genest de Saint Isle, France) or immune deficient, hairless SHO-SCID ( Crl:SHO-Prkdc$^{scid}$Hr$^{hr}$ male and female, Charles River, Borchen, Germany) were rapidly injected via the tail vein with a volume corresponding to 10% of the body weight of Ringer-Lactate (B. Braun, Melsungen, Germany) solution containing 12,5 µg/ml of pDNA using a 27-gauge needle. Mice were kept anaesthetised by inhalation of isoflurane (Forene, Abbott, Wiebaden-Delkenheim., Germany) by a gas manifold with a flow rate of 2% during treatment.

All animals were given adequate care in compliance with institutional and state guidelines.

### 2.3.2. Xenograft implantation and electroporation

3 million MDA MB-435 cells in 100µl PBS were injected s.c. into each flank of SCID ( CB17/Icr-Prkdc$^{scid}$/IcrCrl, Charles River) or hairless SCO-SCID mice. The growth of tumours was followed by measurements with a vernier caliper in three orthogonal dimensions ($L \times W \times H$, mm). When the tumours had reached an average volume of about 100 mm² electroporation was performed: Mice were anaesthetised by inhalation of isoflurane. Plasmid solution, containing 25µg pDNA diluted in 25µl Ringer-Lactate solution were injected i.t. Subsequently, two parallel plate electrodes of stainless steel (9,5 x 9,5mm), fixed on a vernier caliper, were placed on both sides of the tumour and four rectangular-shaped electrical pulses with amplitude of 300V, pulse duration of 25 ms and time interval of 100ms were delivered using the BTX ECM-830 Electro Square Porator (BTX Harvard Apparatus, Holliston, MA, USA). Good contact between the electrodes and skin was ensured by conductive gel (Ultraschallgel, TMP Tüshaus Medical Produkte).

### 2.3.3. In vivo imaging

For live examination of luciferase expression *in vivo* mice were anaesthetised by administration of isoflurane and injected i.p. with 100µl D-luciferin (60mg/ml,

Promega) diluted in PBS. After 10 minutes the mice were imaged for bioluminescence in an IVIS Lumina device (Caliper Life Sciences, Mainz, Germany) in a light-tight chamber on an adjustable, heat-controlled stage. Images were acquired at a small (hydrodynamic delivery and electroporation) or large (systemic administration) binning level. The acquisition time was set depending on the bioluminescence intensity. Data were analysed using the Xenogen Living Image 3.0 software.

### 2.3.4. Isolation of tumour cells

The mice were sacrificed by cervical dislocation and the tumours resected. Tumor tissue was homogenised in 0,7ml PBS with the Medimachine system (DakoCytomation, Glostrup, Denmark) for 20 seconds. The cell suspension was sieved through a 40 µm strainer (BD Biosciences, Heidelberg, Germany) and divided into 2 parts. One part was lysed immediately in 1x lysis buffer and analysed by protein measurement and luciferase assay. The other part was taken into cell culture in isolation medium, containing DMEM/Ham´s F12 1:1, 10% FCS, 200 U/ml penicillin, 200 µg/ml streptomycin, 0,25µg/ml amphotericin B and 2mM stable glutamine. The cells were analysed by luciferase assay continuously thereafter.

The protein measurement was done with a BCA-kit (Thermo Scientific, Schwerte, Germany) according to the manufacturer's recommendations. First, 25µl of the cell lysate was mixed with 5µl 0,1M iodacetamide and incubated for 30 minutes at 37°C to inactivate the DDT (dithiothreitol) of the lysis buffer. Then the solution was mixed with 200µl of working solution from the kit and incubated a second time for 30 minutes at 37°C before the absorbance was measured at 590 nm in a plate reader (Spectrafluor plus, Tecan Austria GmbH, Grödig, Austria). For quantification purposes a standard curve of serially diluted BSA was included.

## 2.4. Luciferase-assay

### 2.4.1. Firefly luciferase

Cells were trypsinised, a part thereof collected by centrifugation at 1000 rpm for 10 minutes and consequently lysed in 0,5x lysis buffer (Promega) for 20-30 minutes at room temperature. Alternatively, all cells were lysed directly in the wells after removal of the cell medium. The lysate was measured in a tube luminometer (Lumat LB 9507,

Berthold Technologies, Bad Wildbad, Germany) with an integration time of 10 seconds following a 2 seconds lag time after the injection of luciferase assay reagent (LAR; 20mM glycylglycine, 1 mM $MgCl_2$, 0,1mM EDTA, 3mM DTT (Sigma-Aldrich), 0,55mM ATP (Roche, Mannheim, Germany), 0,3mM CoA (Sigma-Aldrich), 0,5 mM D-luciferin (Promega), pH 8,5).

Small changes in the protocol were made for the *in vivo* electroporated and passaged tumour cells. They were lysed in 1x lysis buffer and the measurement time was increased to 3 minutes.

As a control of background autofluoresence level, untransfected cells were measured.

### 2.4.2. Gaussia luciferase

Cell medium was spun at 300 g for 5 minutes to remove detached cells. 5µl was measured using the tube luminometer after injection of the coelenterazine solution (50mM Tris-Cl, 0,5M NaCl, 20µM coelenterazine (Synchem, Felsberg, Germany), pH 7,8). Measurement parameter was identical as for the firefly luciferase assay (2.4.1)

## 2.5. Q-PCR

Livers were pulverised in liquid nitrogen using a mortar and pestle and from this total liver DNA was extracted using a QIAmp DNA Mini Kit (Qiagen) according to the manufacturer's protocol. The DNA was concentrated by ethanol precipitation and the final DNA concentration was determined by photometric measurements at 260nm.

The plasmid copy number was determined by absolute quantification using primers and probes complementary to the Luciferase::Sh gene (forward: 5´-tcatgtctggctatgtgacaac-3´, reverse: 5-agccatccttgtcaatcagg-3´, probe #49 of Universal Probe Library, Roche). One amplification reaction of 20µl contained template DNA, 1x LightCycler 480 Probes Master (Roche), 200nM primers and 100nM probe. As template 5µg of liver DNA was used. For quantification purposes a standard curve of serial dilutions of pDNA mixed with 5µg of liver DNA from untreated animals was generated. All samples were done in duplicates.

Q-PCR was carried out with a LightCycler 480 (Roche) device with an initial heating at 95°C for 10min, then 40 cycles of 60°C for 30s, 72°C for 1s and 40°C for 10s.

Primer sequences were designed using the Universal ProbeLibrary Assay Design Center (https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp?id=uplct_030000) and the corresponding oligonucleotides were synthesized by Eurofins MWG Operon (Ebersberg, Germany).

# 3. Results

## 3.1. Comparison of human versus murine CMV enhancer and their effect on transgene expression in vivo

Many factors can influence the stability of transgene expression *in vivo*, such as the CpG-content, the choice of promoter and enhancer, as well as the transgene itself. Here we wanted to investigate the effect of the enhancer sequence on the level of transgene expression. For this purpose we used the plasmids pCpG-hCMV/EF1-LucSH and pCpG-mCMV/EF1-LucSH (**fig. 2**), which are both CpG-reduced to minimize immune reactions. They are carrying the transgene luciferase in the form of a fusion gene (LucSh), where the luciferase is fused to the bla-gene to stabilize the CpG-reduced luciferase. The transgene is driven by the strong, ubiquitous EF-1 promoter. The plasmids also contain two naturally CpG-deplete S/MAR-sequences from the IFN-β and β-globulin gene, which are situated between the bacterial and mammalian expression cassette for insulation. The only differing sequence in these 2 plasmids is the about 300 bp long enhancer, which is originating from either the human or murine CMV, and is located upstream of the promoter. By injecting the two plasmids into mice by hydrodynamic delivery and measuring the luciferase expression in the liver by *in vivo* imaging, a direct comparison of transgene expression duration and strength could be made between the plasmids. The method of hydrodynamic delivery was chosen, since it is a fast procedure to achieve well measurable transgene signals without possible disturbing effects from a carrier system.

The luciferase signals from the two plasmids are showing approximately the same level of activity after injection and remain relatively stable the first two weeks (except for a minor drop in signal at day 5 after injection in the case of pCpG-mCMV/EF1-LucSH) (**fig. 3**). After this 14-day plateau in signal strength, an abrupt drop in luciferase expression is observed. After another week the signals are somewhat stabilizing in both cases, leading to a persistent and still strong signal from pCpG-hCMV/EF1-LucSH, which remains for over 100 days post administration, whereas the signal of pCpG-mCMV/EF1-LucSH is gradually decreasing, resulting in a total loss of transgene signal at day 43 after plasmid injection

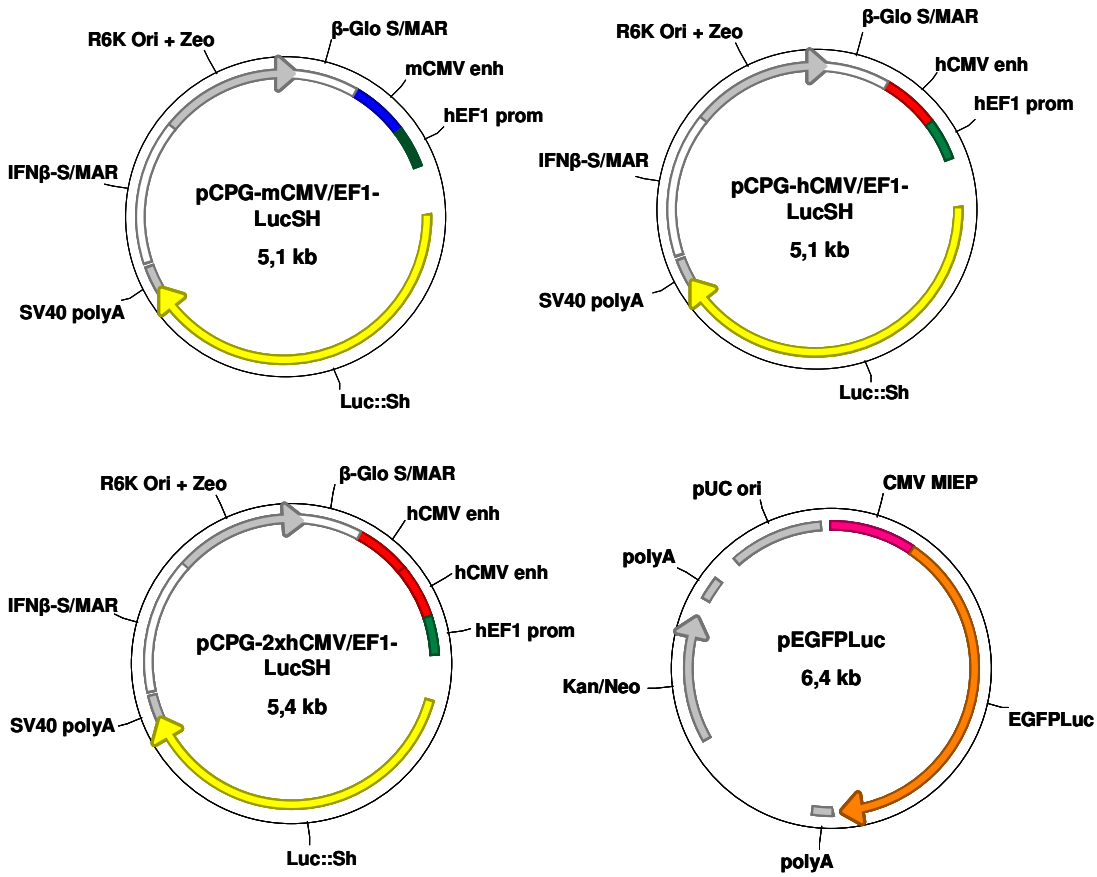


**Fig. 2: Plasmid map of the CpG-free pCpG-hCMV/EF1-LucSH, pCpG-mCMV/EF1-LucSH and pCpG-2xhCMV/EF1-LucSH and CpG-replete pEGFPLuc plasmid**. Scaffold matrix attachment region of 5´-region of the human IFN- β (IFN-β S/MAR) and β-globulin (β–Glo S/MAR) genes, which are naturally CpG-free sequences; R6K ori from *E. coli* for propagation in bacteria having the *pir* gene, expressing the initiator protein *π* ; Zeo, zeocin resistance gene for antibiotic selection, driven by the E2MK promoter (CpG-free version of bacterial EM7 promoter); hCMV enh and mCMV enh, enhancer from either human or murine cytomegalovirus; hEF1 prom, core promoter and 5´UTR of human elongation factor 1 alpha gene; Luc::SH, fusion gene of CpG-free firefly luciferase and Sh bla gene, the latter conferring zeocin resistance; SV40 polyA, late polyadenylation signal from simian virus 40; CMV-IEP, human cytomegalovirus immediate early promoter and enhancer; EGFP::Luc, fusion gene of enhanced fluorescent protein and firefly luciferase; Kan/Neo, neomycin phosphotransferase gene, rendering kanamycin and neomycin resistance, driven by SV40 promoter and bacterial promoter; HSV-tk polyA, herpes simplex virus thymidine kinase polyadenylation signal; pUC ori, pUC plasmid replication ori

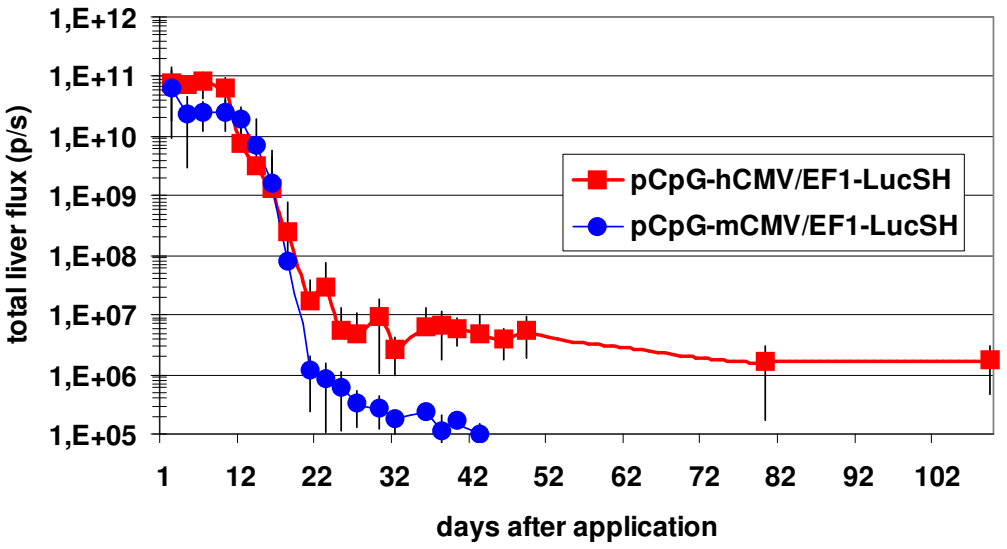


**Fig. 3: Transgene expression in liver of Balb/C mice treated with naked plasmid DNA by hydrodynamic delivery**. Luciferase activity was measured by in vivo imaging**.** pCpG-hCMV/EF1-LucSH:SH n=8, pCpG-mCMV/EF1-LucSHSH: n=10. At day 37 half of the mice were sacrificed for DNA-isolation of liver cells. p/s: photons/second**.**

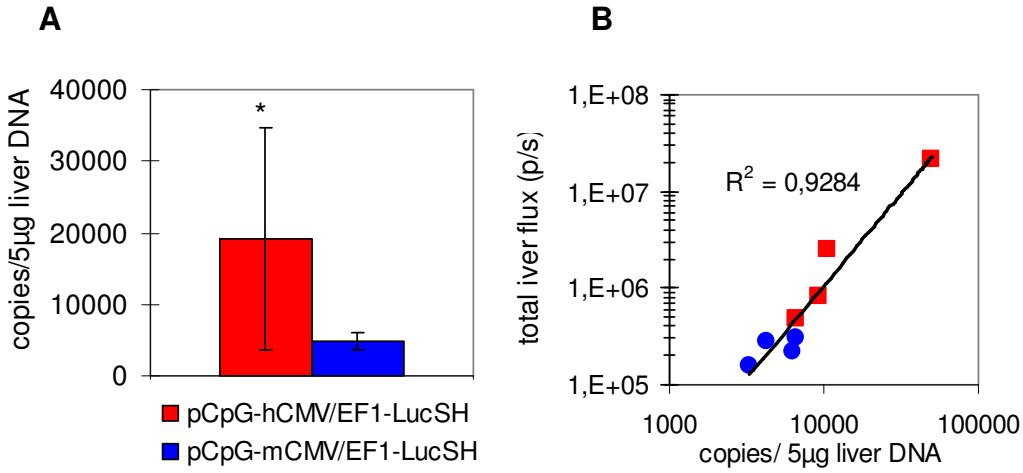


**Fig. 4: Plasmid copy number in liver tissue 37 days after hydrodynamic delivery.** Total DNA was investigated by QPCR. A standard curve of plasmid DNA allowed for quantification of the plasmid number in the liver (4A, left). The plasmid number was plotted against the transgene signal (4B, right). p/s: photons/second. *p<0,05, t-test

To determine if this difference in transgene signal level and endurance also was manifesting itself in the plasmid copy number, an absolute quantification by QPCR. was made from total DNA isolated from liver tissue at day 37 after hydrodynamic injection. The result in **fig. 4A** shows a 4-fold higher mean plasmid copy number in the group treated with pCpG-hCMV/EF1-LucSH compared with the group of pCpG-mCMV/EF1-LucSH. When the plasmid copy number was plotted against the luciferase signal (**fig. 4B**) an almost linear correlation is produced. Hence, there is a connection between high transgene expression and plasmid retention, and they are both positively influenced by the human CMV-enhancer, when compared with the murine counterpart.

The human and murine enhancers have evolutionary been optimized to fit to their respective host cells, resulting in different design. They are both containing numerous binding sites for transcription factors in different compositions. The human CMV-enhancer is in total having more transcription factor binding sites than the murine, leading to the assumption that this could be contributing to the differences in expression between pCpG-hCMV/EF1-LucSHSH and pCpG-mCMV/EF1-LucSHSH. To examine this, a second human CMV-enhancer was inserted into pCpG-

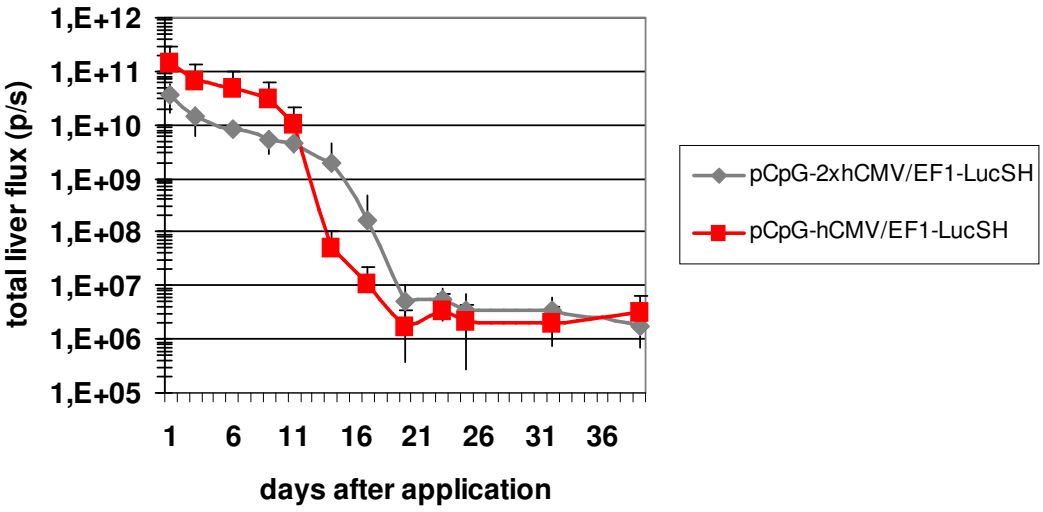


**Fig. 5: Effect of tandem hCMV enhancer sequence (pCpG-2xhCMV/EF1-LucSH) on transgene expression *in vivo,* compared to single sequence (pCpG-hCMV/EF1-LucSH).** Luciferase activity in liver was measured by *in vivo* imaging after hydrodynamic delivery of naked pDNA in Balb/C mice. pCpG-2xhCMV/EF1-LucSH; n=6, pCpG-hCMV/EF1-LucSH; n=4, p/s= photons per second.

hCMV/EF1-LucSHSH, resulting in a tandem repeat of the sequence. The final construct, pCpG-2xhCMV/EF1-LucSHSH, (**fig. 2**) was then compared with pCpG-hCMV/EF1-LucSH by hydrodynamic injection into mice and consequent measurement of liver expression.

The first two weeks after injection the pCpG-hCMV/EF1-LucSH is showing a higher expression when compared to pCpG-2xhCMV/EF1-LucSH, but the signal from both plasmids is rather stable (**fig. 5**). Then CpG-hCMV-Luc is experiencing the strong decrease in signal strength, as already observed (**fig. 3**), whereas the pCpG-2xhCMV/EF1-LucSH is having a somewhat delayed drop. 3 weeks after injection, the transgene expression from both plasmids is equal strong and stays stable at this level for an extended period of time. So the introduction of a second human CMV-enhancer is extending the signal but does not lead to a stronger transgene expression.

## 3.2. Influence of immune status on transgene expression *in vivo*

The rather stable luciferase signal in the beginning, followed by the rapid decrease over several log-units of pCpG-mCMV/EF1-LucSH and pCpG-hCMV/EF1-LucSH in **fig. 3** is greatly differing from the expression profile of the commercially available plasmid pEGFPLuc in **fig. 6**, where it was compared with the pCpG-mCMV/EF1-LucSH by injection into mice by the hydrodynamic method. This led us to the question, as to what caused the characteristic expression-over-time profile of pCpG-hCMV/EF1-LucSH and pCpG-mCMV/EF1-LucSH. To investigate if the sudden drop around day 14 after plasmid injection could be due to immunological reactions, the plasmids pCpG-hCMV/EF1-LucSH, pCpG-mCMV/EF1-LucSH, pCpG-2xhCMV/EF1-LucSH and pEGFPLuc were injected by hydrodynamic delivery into immunodeficient SCID mice, lacking functional B- and T-cells. The plasmid pEGFPLuc (**fig. 2**) is, in comparison to the other plasmids used, CpG-replete and its transgene, a fusion of luciferase and EGFP, is driven by a CMV-promoter.

The results show that the expression profile of pEGFPLuc is very similar in immunocompetent (**fig. 6**) and –deficient (**fig. 7**) mice, where in both cases the signal is drastically reduced within 2 weeks. This reduction could in both cases be due to

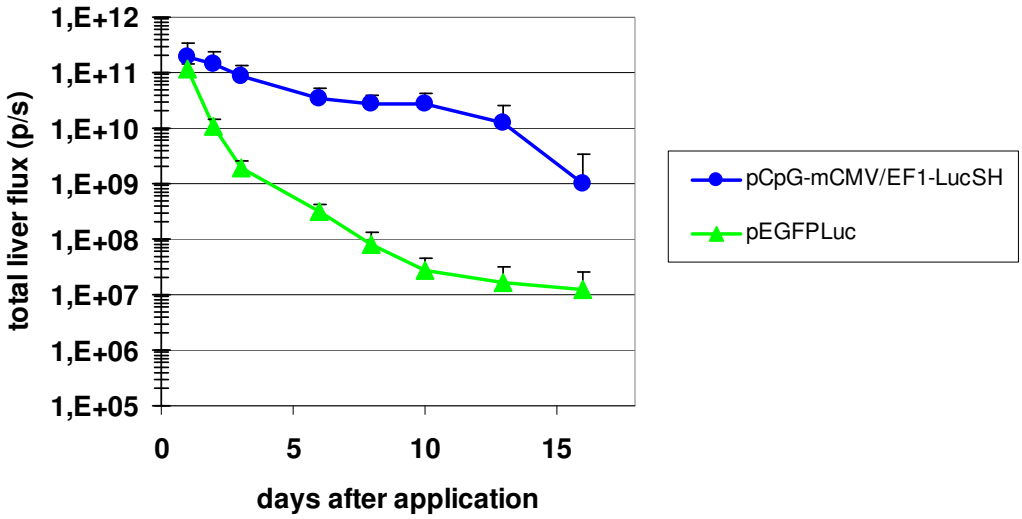


**Fig. 6: Comparison of CpG-replete, CMV-promoter driven (pEGFPLuc) with CpG-deplete, EF1-promoter driven (pCpG-mCMV/EF1-LucSH) luciferase expression in liver of Balb/C mice.** Plasmids were injected by hydrodynamic delivery and luciferase signal was measured by in vivo imaging. pEGFPLuc; n=4, pCpG-mCMV/EF1-LucSH; n=6. Data was generated by Nicole Tietze.

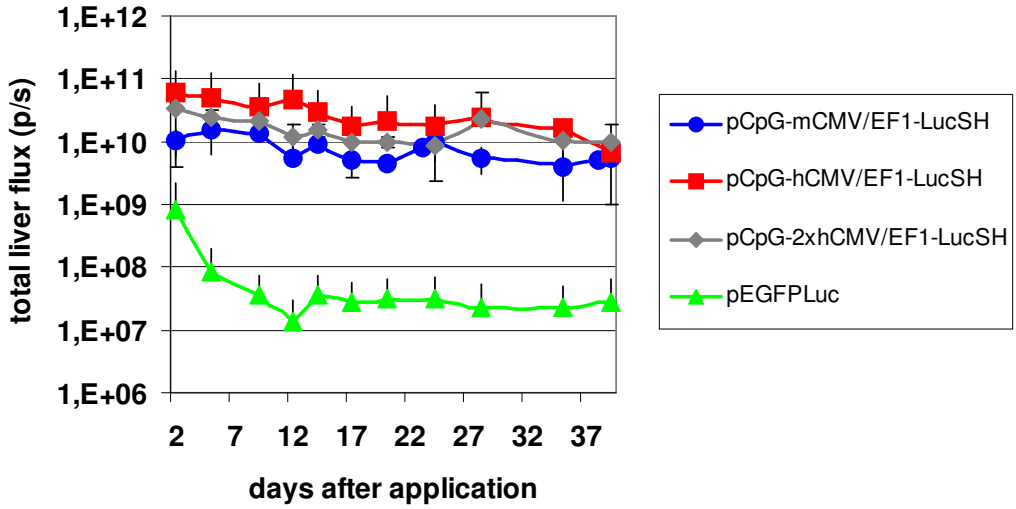


**Fig. 7: Comparison of CpG-deplete, EF1-promoter driven plasmids (pCpG-mCMV/EF1-LucSH, pCpG-hCMV/EF1-LucSH and pCpG-2xhCMV/EF1-LucSH) and CpG-replete, CMV-promoter driven plasmid (pEGFPLuc) in immune deficient mice.** Plasmids were injected by hydrodynamic delivery into SCID mice and the transgene expression was followed by in vivo bioluminescence measurements. n=4 (pCpG-mCMV/EF1-LucSH, pCpG-2xhCMV/EF1-LucSH), n=5 (pCpG-hCMV/EF1-LucSH, pEGFPLuc)

he already reported fast CMV-promoter inactivation by methylation [26, 27]- The EF-1 driven plasmids, pCpG-hCMV/EF1-LucSH, pCpG-mCMV/EF1-LucSH and pCpG-2xhCMV/EF1-LucSH, rendered very stable and high expression in the immunodeficient SCID mice (**fig. 7**), and there was no sudden reduction in transgene signal, as in the case of the immunocompetent Balb/C mice (**fig. 3** and **5**). Therefore, one can assume, that the host adaptive immune system is causing the fast reduction in transgene signal two weeks after injection.

## 3.3. Plasmid retention in a tumour model

After having seen the advantages of the hCMV versus the mCMV enhancer on the transgene expression in the liver, we were investigating if this is also the case in a xenograft model of human tumor in SCID mice.The human melanoma cell line MDA MB-435, which we know from previous work is forming slow growing tumours, was selected to allow monitoring of the transgene expression over a sufficiently long time period.

After inoculation of the tumour cells s.c. into immune deprived SCID mice the plasmids pCpG-hCMV/EF1-LucSH, pCpG-mCMV/EF1-LucSH, pCpG-2xhCMV/EF1-LucSH and pEGFPLuc were electroporated i.t. when tumours had reached a palpable size. Afterwards, the luciferase expression of the plasmids was measured continuously by *in vivo* bioluminescence measurements.

As seen in **fig. 8** the expression in the tumour does not change considerably over time and is 25 days after electroporation at more or less the same level as at day one. This matches the expression in the liver of immune deficient mice (**fig. 7**). In contrast to liver transfection, the plasmid pEGFPLuc also gives a stable signal in tumor tissue, although its signal in the liver is decreasing over time (**fig. 6** and **7**).

To see if the persistent gene expression would remain when the tumour cells were taken out of the *in vivo* surroundings the experiment was repeated, this time only with pCpG-hCMV/EF1-LucSH. At day one and day 27 after electroporation tumours were resected and the tumour cells taken into cell culture. A luciferase gene expression assay was performed at the day of isolation and again after 10 and 15 days.

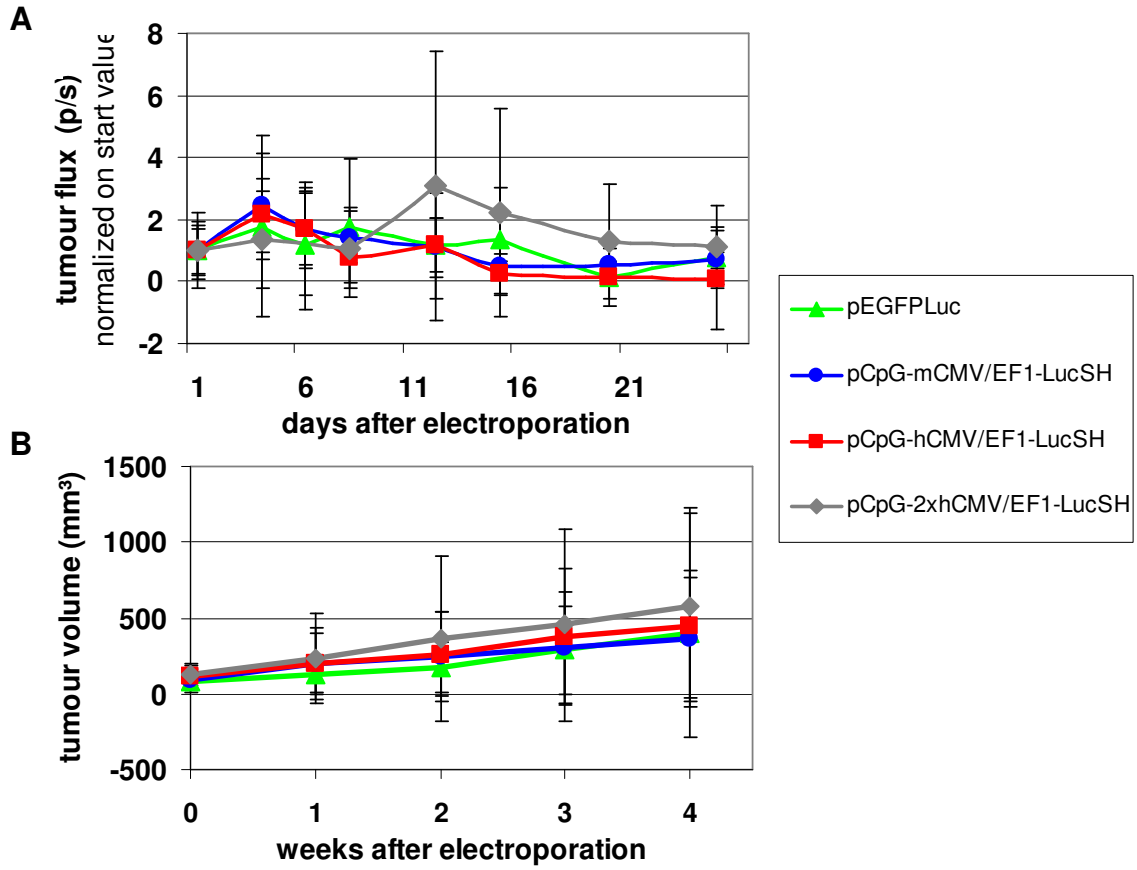


**Fig. 8: Transgene expression in a subcutaneous tumour model.** Naked plasmid DNA was delivered into subcutaneous MDA MB-435 tumours in SCID mice by electroporation. The luciferase signal was monitored thereafter by in vivo imaging (A) and the tumour growth by calliper measurements (B). n=8 tumours per group.

Again, the luciferase expression stayed relatively stable over time in the subcutaneous tumour (**fig. 9A**). When the cells were removed from the animal, the luciferase expression in cell culture was reduced to background levels within 2 weeks, no matter if the cells were isolated from the mouse on day 1 (**fig. 9B**) or day 27 (**fig. 9C**) after electroporation. MDA MB-435 cells were also transfected *in vitro* with a polyplex formulation. The luciferase activity was continuously measured by luciferase assays and the result is displayed in **fig. 10.** Similar as in the reisolated cells, the transgene signal is lost 2 weeks post transfection. This fast loss of transgene signal *in vitro* and the stable signal *in vivo* could be explained with the higher cell growth in

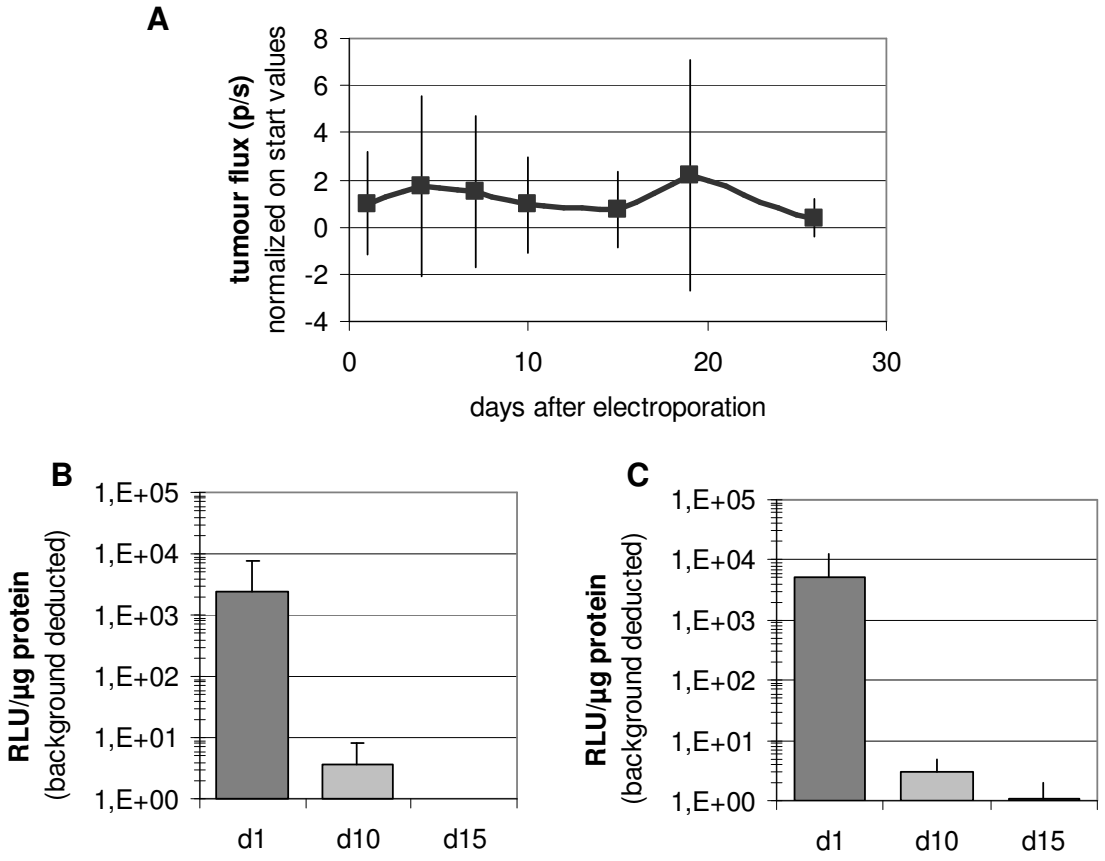


**Fig. 9: Transgene expression in vivo and in vitro. (A)** pCpG-hCMV/EF1-LucSH was injected into subcutaneous MDA MB-435 tumours and subsequently electroporated. Luciferase expression in the tumours was measured by in vivo imaging and the values were normalised on the day one value. On the first day after electroporation half of the mice were sacrificed and tumour cells were isolated and taken into cell culture. A luciferase assay was performed on the day of cell isolation (d1), as well as on day 10 and 15. **(B).** On day 27 after electroporation the rest of the mice were sacrificed and the procedure was repeated **(C).** n=10 tumours in total. RLU: relative light units.

the cell culture. Growing freely in the flask the cells would have undergone approximately 10-14 cell divisions in the 2 weeks of culture (doubling time~24h, but the reisolated cells were growing slower in the time directly after isolation). The tumour cells were growing slower *in vivo;* tumour volume increased from 125mm³ to about 500mm³ from day 1 to day 27 after electroporation (**fig. 8B**). This could correlate to two cell divisions or slightly more, since MDA MB-435 forms compact, hard tumours.

So loss of pDNA seems to be tightly associated with cell division. This was also investigated in an experiment where cells were transfected with

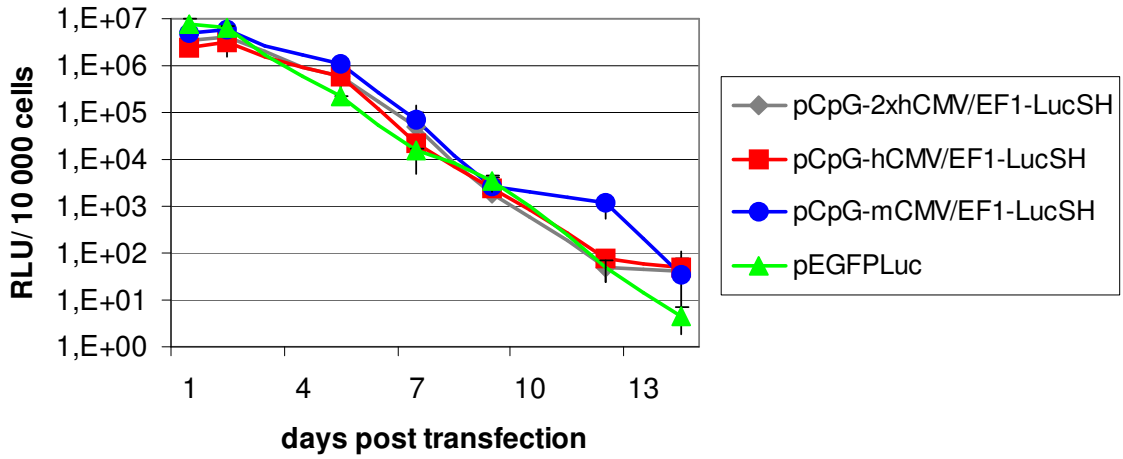


**Fig. 10: In vitro transfection of MDA MB-435 cells** with pDNA complexed with H DO. Luciferase assays were done continuously thereafter to monitor transgene expression. Background level was deducted from signals. RLU: relative light units.

luciferase expressing plasmids and then either let to grow without restrains or stopped in their proliferation. For this, primary porcine smooth muscle cells (PSMC´s) were used because they are known to be inhibited by treatment with heparin [114, 115]. For the growth inhibition assay the cells were transfected with the plasmids pEPIto-CMV-GLuc and pCMV-GLuc (**fig. 11A, C**), both carrying the gene for the naturally secreted luciferase from the marine copepod *Gaussia princeps* (GLuc), enabling a luciferase measurement of the secreted luciferase in the medium without disturbing the cells. The transgene is in both plasmids driven by a CMV-promoter and in the pEPIto-CMV-Gluc the luciferase gene is followed by an S/MAR-sequence. Directly after transfection heparin was added to the cell medium to stop cell growth and cell medium was continuously collected to be analysed by luciferase assay. The result is seen in **fig. 11D**: The expression stays relatively stable over 1 month time.

As a comparison to the growth arrest assay PSMC´s were let to grow with no restrictions. They were transfected with the plasmids pEPIto-CMV-EGFPLuc (**fig. 11B**) and pEGFPLuc (**fig. 2**), both expressing an EGFP-Luc fusion protein. Also here, the expression is driven by a CMV-promoter and an S/MAR-sequence had been introduced behind the transgene in the pEPIto-CMV-EGFPLuc. The expression

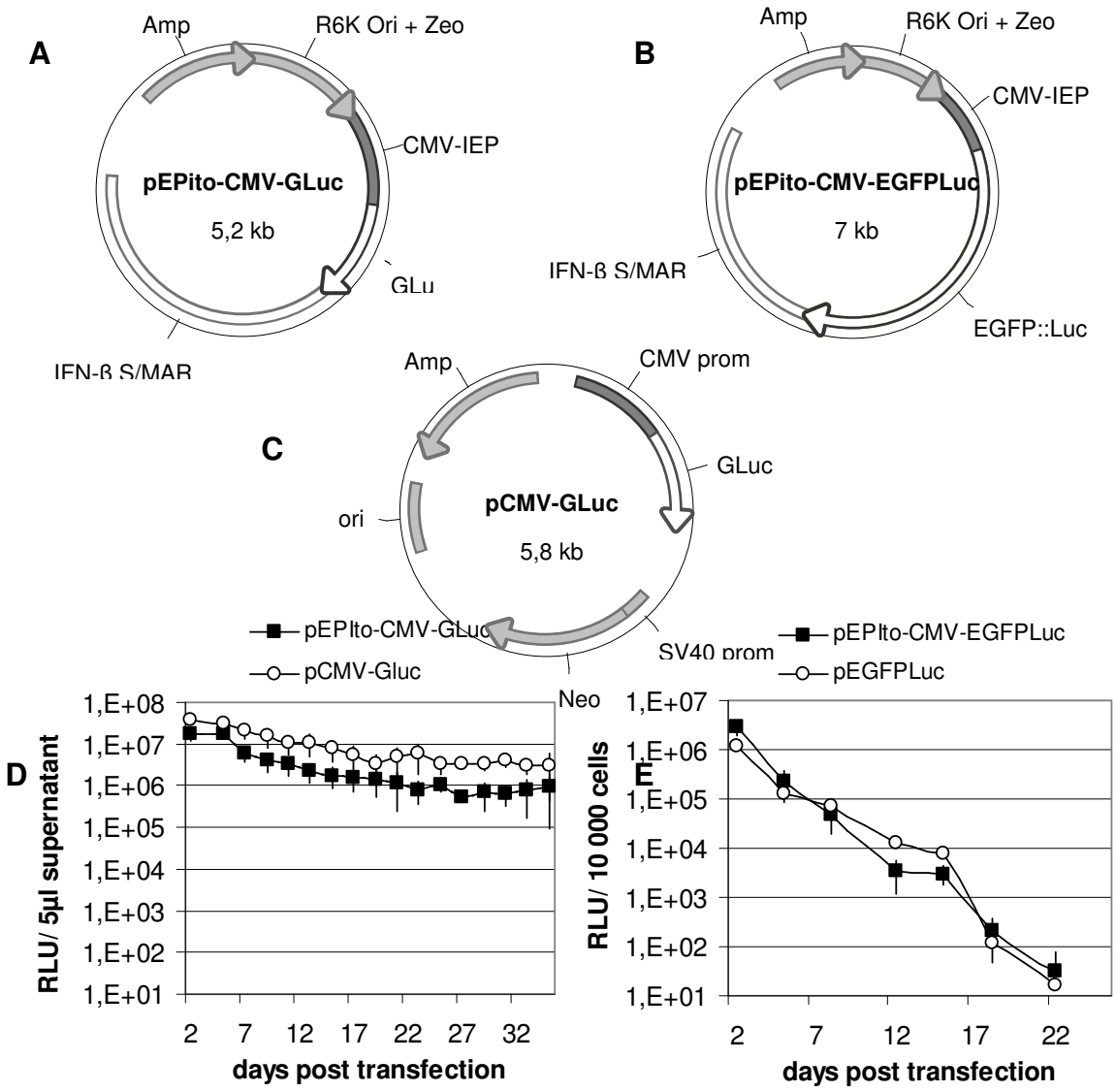


**Fig. 11: Transgene expression in primary PSMC´s after transfection with plasmids pEPIto-CMV-Gluc (A), pEPIto-CMV-EGFPLuc (B), pCMV-Luc (C) and pEGFPLuc, with (D) or without (E) growth inhibition.** (A)-(C): R6K ori from *E. coli* for propagation in bacteria containing the *pir* gene, expressing the initiator protein π; Zeo, zeocin resistance gene; CMV-IEP, human cytomegalovirus immediate early promoter and enhancer region; GLuc; *Gaussia* luciferase; IFN-β S/MAR, scaffold matrix attachment region of the human interferon-β gene, Amp, ampicillin resistance gene, Neo, neomycin resistance gene, SV40 prom, simian virus 40 promoter, CMV prom, cytomegalovirus promoter; Ori, origin of replication; EGFP::Luc fusion gene of enhanced green fluorescent protein and *Firefly* luciferase. (D): PSMC`s were transfected and then treated with heparin to stop proliferation. Luciferase activity was continuously measured in the supernatant. n=4 (E): Transfection of PSMC under normal growth conditions (without heparin). n=3. Background signal is deducted from measured values.

over time was observed by lysing a part of the cell population and performing luciferase assays on the lysate. In this case the expression is rapidly decreasing (**fig. 11E**) and has reached the background level within 3 weeks. Hence, the plasmids are lost when the cells are dividing freely, as shown with the PSMC and MDAMB-435 cells in culture, and are retained when the cells are in a slow growing-status, as in the MDA MB-435 tumour or the heparin treated PSMC´s.

## 3.4. Evaluation of a novel synthetic promoter, SCEP, in vivo

The two promoters used so far, the CMV-IEP and EF-1 both render ubiquitous transgene expression, but they are not optimal and have both pros as well as cons: the CMV-IEP is generally stronger than the EF-1, but is of viral origin, whereas the EF-1 is derived from mammalian DNA. CMV-IEP undergoes promoter methylation shortly after nuclear entry, a phenomenon which does not occur in the case of EF-1. Our idea was to combine these two promoters to take advantage of the transcriptional strength of the CMV-IEP and the transcriptional stability of the EF-1 to create a novel, ameliorated promoter. By identifying homologous regions between the 2 sequences and combining these to a new optimized promoter for strong expression and binding of transcriptionally strong transcription factors, as tested by various programs, the CpG-free promoter SCEP was created. SCEP stands for shuffled CMV EF-1 promoter. For *in vivo* testing of the novel promoter, plasmids were cloned on the basis of the CpG-reduced pCpG-hCMV/EF1-LucSH (**fig. 2**). First, the transgene *Luc::Sh* was exchanged for an enhanced firefly luciferase (eFLuc) gene, which has previously been created by codon optimisation, removal of cryptic splicing sites and retroviral optimisation of the firefly luciferase [112]. By introducing the novel transgene, forming the plasmid pCpG-hCMV/EF1-eFLuc, the resulting luciferase signals were clearly enhanced when comparing to the original plasmid pCpG-hCMV/EF1-LucSH in vitro (**fig. 12**). The second step was to replace the EF-1 promoter with the SCEP or CMV promoter to create pCpG-hCMV/SCEP-efFLuc and pCpG-hCMV/CMV-eFLuc, respectively. As a result, all 3 plasmids have the same CpG-depleted backbone with the enhanced firefly luciferase as a transgene. They were introduced by hydrodynamic delivery into Balb/C mice and the transgene expression in the liver was measured by in vivo imaging. The result is shown in **fig.**

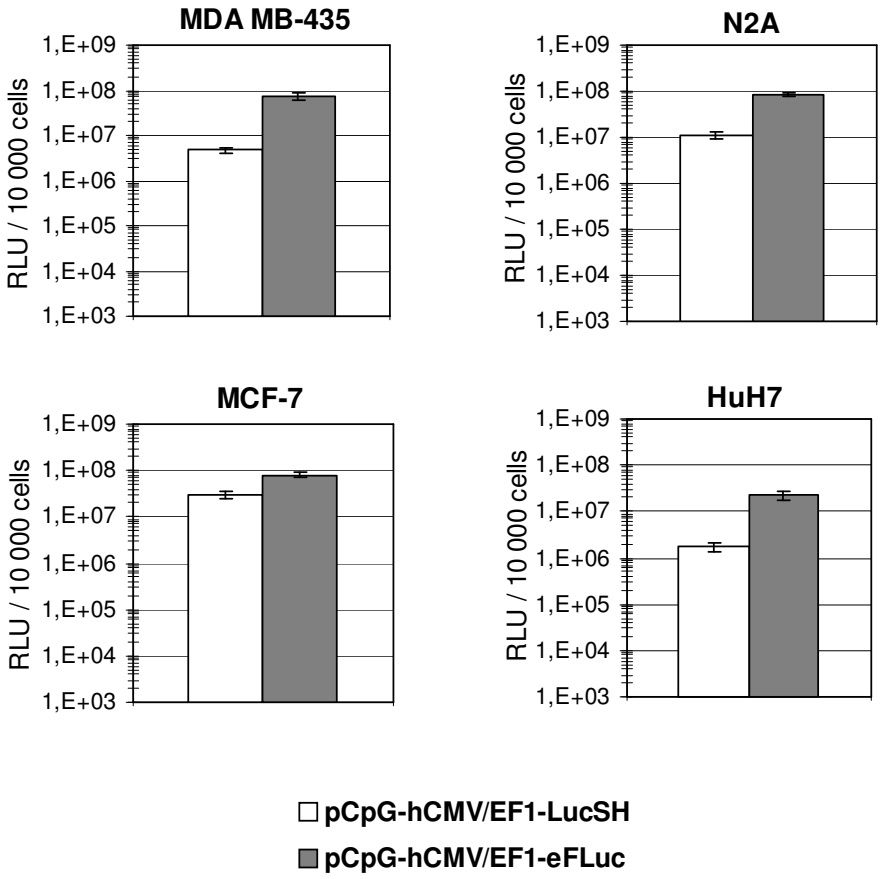


**Fig. 12: In vitro comparison of enhanced firefly luciferase, eFLuc, with fusion protein LucSH in different cell lines.** The cell lines MDA MB-435 (human melanoma), N2A (murine neuroblastoma), MCF-7 (human breast cancer) and HuH7 (human hepatoma) were transfected with polyplexes formed by condensation of pDNA and LPEI. Transgene expression was measured by luciferase assay 24h post transfection. The plasmids used are both CpG-reduced and express its transgene under the control of an EF-1 promoter and a human CMV enhancer. n=5, RLU: relative light units.

**13.** The new promoter SCEP renders a higher transgene expression than the EF-1 promoter, but their expression profiles follows the same pattern, with a drop of signal strength at around 2 weeks after treatment, which previously could be seen with the EF-1 promoter containing plasmids pCpG-mCMV/EF1-LucSH and pCpG-hCMV/EF1-LucSH (**fig. 3**) This time also the SCEP promoter is affected; hence, this delayed effect is not specific for the EF-1 promoter.

The signal from pCpG-hCMV/CMV-eFLuc is rapidly decreased, in compliance with the observed silencing of the CMV-IEP [26, 27] and is then stabilized. After the drop

of signal from the pCpG-hCMV/EF1-eFLuc and pCpG-hCMV/SCEP-eFLuc plasmid, the signal of pCpG-hCMV/CMV-eFLuc is clearly superior. Thus, the drop in signal caused by the immune system in the case of the EF-1 and, probably also, the SCEP promoter had a greater impact on transgene signal than the silencing by methylation in the case of the CMV promoter.

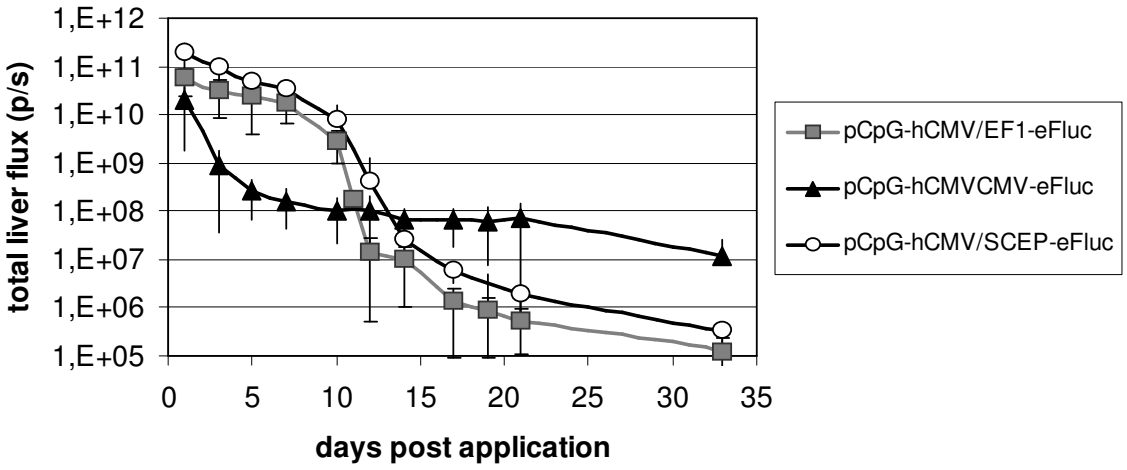


**Fig. 13**: **Comparison of CMV, EF-1 and synthetic SCEP promoter *in vivo***. All plasmids are CpG-reduced with an hCMV enhancer and the enhanced firefly luciferase (eFLuc) as a reporter gene. Luciferase expression was measured by in vivo imaging after hydrodynamic delivery of the naked plasmids into Balb/C mice. n=6. p/s: photons per second.

## 3.5. Comparison of minicircles with full-length plasmid

In the search for an optimal vector for gene therapy results have shown that the covalent binding of bacterial DNA to a mammalian expression cassette can significantly reduce transgene expression [116]. Since the bacterial backbone is indispensable for the propagation in bacteria it was sought to eliminate this sequence post propagation, thus forming a minicircle devoid of bacterial sequences [35]. Much work has been investigated on developing methods to improve the efficiency of minicircle production [29-31, 34-38, 117], which was the major bottleneck so far. Another general problem occurring when isolating plasmids from bacteria is the relatively high amount of co-isolated bacterial DNA, which can elicit an immunologic response. To circumvent this, and also to increase the minicircle production efficiency for large-scale production, a new method was generated by the company

PlasmidFactory (Bielefeld, Germany), our minicircle provider: Recombination was performed by the highly efficient ParA resolvase (recombination efficiency >99,5 %), inducible by arabinose, followed by an affinity chromatography purification step of the minicircle [117]. For the purification serially arranged lactose operator (*lacOs*) sites were incorporated in the minicircle part of the parental plasmid. After arabinose induced recombination, the *lacOs* will reversible bind the minicircle to repressor of the lactose operon (lacI), which has been covalently bound to the solid phase of the chromatography. This allows for the separation of minicircle from unwanted side products, such as miniplasmid, parental plasmid, bacterial DNA and endotoxins, resulting in a purity of >98,5%.

For biological testing one model minicircle was generated, pCMV-Luc-MC. It contains the CMV promoter, the luciferase gene as a transgene and is devoid of the bacterial expression cassette. To evaluate the minicircle *in vitro*, it was transfected into the murine tumor cell line N2A as well as into primary cells (PSMC). As a control, the full-length paternal plasmid, pCMV-Luc, was used. The transfection was conducted with equal weights of plasmid DNA as well as equimolar amounts, to adjust for the lower molecular weight of pCMV-Luc-MC. The result is depicted in **fig. 14**. The long-term expression profile from minicircle and plasmid are not differing in N2A cells. Only in the PSMC´s there is an advantage of the minicircle compared to its parental plasmid. These results were in both cell types independent of the amount of used

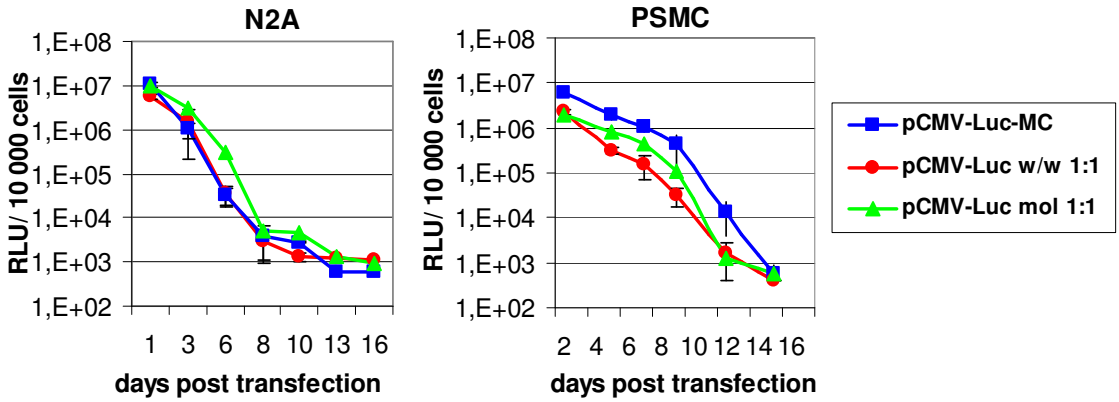


**Fig. 14: *In vitro* testing of the minicircle, pCMV-Luc-MC, and its corresponding plasmid, pCMV-Luc**. N2A and PSMC cells were transfected with pDNA complexed with H DO, either in equal weight to weight ratio (w/w 1:1) or in equimolar amounts (mol 1:1). Luciferase expression was monitored by continuous luciferase assays. RLU: relative light units. n=3.

pCMV-Luc. This indicates that the positive effect of removing the bacterial backbone is, *in vitro* at least, cell type dependent.

Since the effect of the bacterial backbone has been associated primarily to its unmethylated CpG´s and their interaction with the immune system, we tested the plasmids in immunocompetent Balb/C mice.

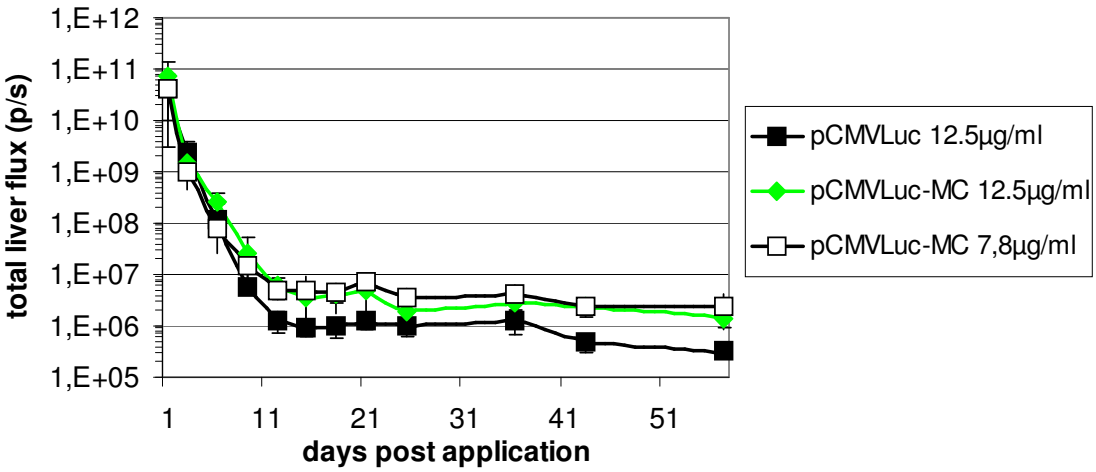


**Fig. 15: Expression in liver from minicircle pCMVLuc-MC and its corresponding plasmid pCMVLuc.** Plasmid DNA was injected into Balb/C mice by hydrodynamic delivery and transgene expression was assayed by in vivo bioluminescence measurement. pCMVLuc-MC was applied either in a w/w ratio of 1 (12,5µ g/ml) or in equimolar ratio (7,8µg/ml) compared to the pCMVLuc. n=4. p/s: photons per second.

The resulting expression-over-time graph is shown in **fig. 15.** The transgene expression from pCMV-Luc is slightly lower than from pCMV-Luc-MC, pointing to the negative effect of the bacterial backbone in pCMV-Luc. Like in the *in vitro* data, the amount of injected pCMV-Luc-MC compared to pCMV-Luc, equimolar or equal weight ratio, was not important for the relative signal strength. In this case it probably depends on the injection method: With the hydrodynamic delivery a plateau of maximal expression with high amounts of plasmids is reached, which can not be increased further with more pDNA [118]. Because of this we decreased the amounts of injected plasmid amount to reach a state of non-saturation, where the differences in transgene signal between minicircle and plasmid might be better detectable. For this experiment only equimolar amounts of plasmids were used.

By decreasing the amount of plasmid (**fig. 16**) the transgene signals also generally decrease, but so does also the difference between mini- and plasmid. The highest difference is still seen with the first tested, highest plasmid concentration.

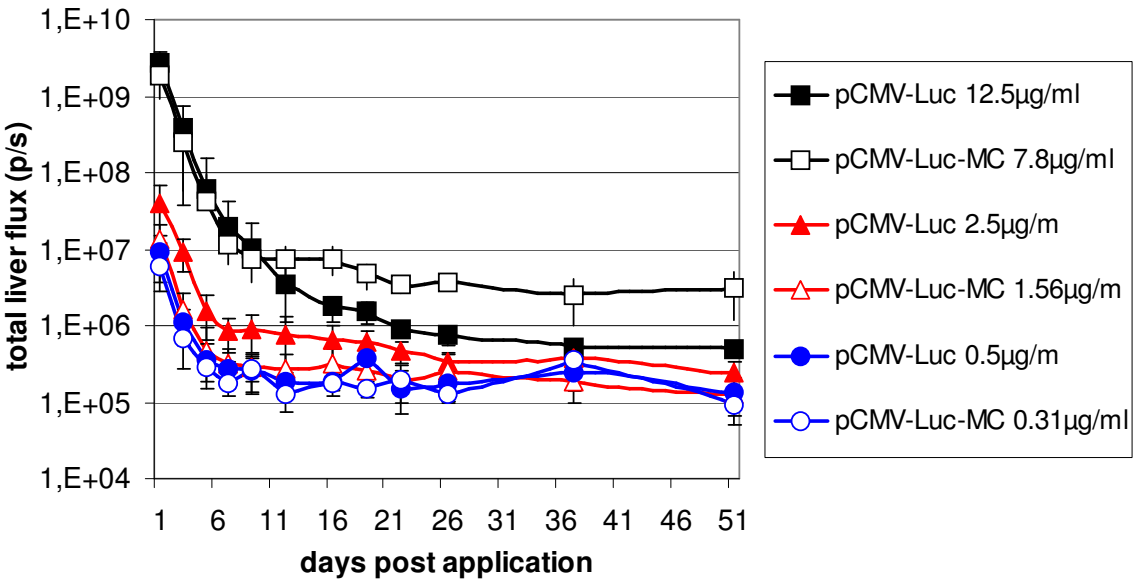


**Fig. 16: Expression in liver from minicircle (pCMV-Luc-MC) and full plasmid (pCMV-Luc).** pDNA was injected by hydrodynamic method into mice in 3 different concentrations (high concentration: black, middle: red, low: blue) with paired equimolar amounts of pCMV-Luc (filled symbols) and pCMV-Luc-MC (outlined symbols). Luciferase activity in the livers was measured by in vivo imaging. n=5. p/s: photons per second.

## 3.6. Transcriptional targeting with liver specific promoters

In order to keep gene therapy side effects low, it is in many cases important to achieve a locally restricted transgene expression. This restriction can be achieved on the level of gene delivery, where the gene delivery vehicle, provided with a targeting function, will direct the gene cargo to the target cells, avoiding off-target tissue. Additionally, or alternatively, the transgene expression itself can be restricted to specific organs by means of transcriptional targeting by using a tissue specific promoter. To investigate promoters for transcriptional targeting of the liver, different plasmids were designed (**fig. 17**): The core promoters from the liver specific genes FMO (flavin-containing monooxygenase), AFP (alpha-fetoprotein), APOE (apolipoprotein E) and HPGL (haptoglobin) were incorporated into a S/MAR-

containing CpG-reduced backbone (pEPIto) containing a fusion of luciferase and EGFP as a transgene, directly followed by an S/MAR sequence (pEPIto-x-EGFPLuc; x=promoter) (**fig. 17**). As a control, the CMV-promoter was used, with the plasmid

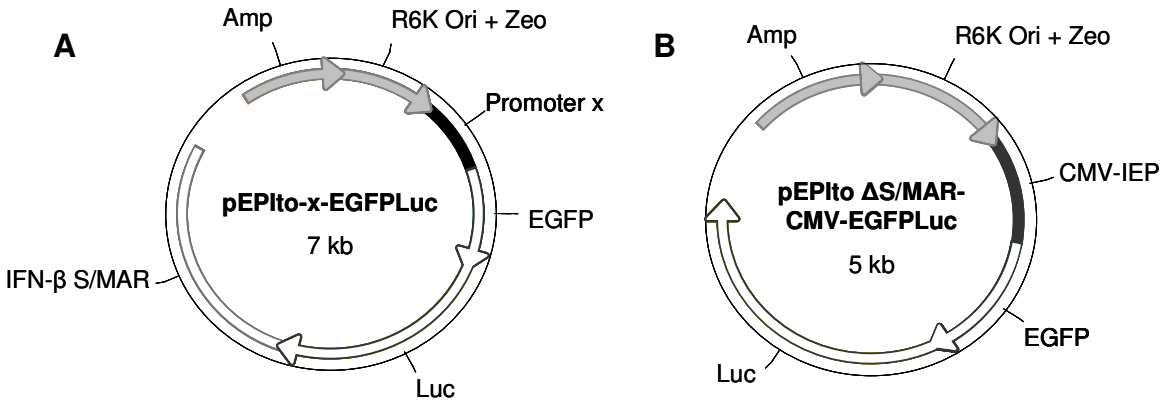


**Fig. 17: Plasmid maps of CpG-reduced vectors (pEPIto).** For selection purposes the gene for ampicillin resistance, Amp, in bacteria and zeocin resistance, Zeo, in mammalian cells were inserted. The R6K ori from *E. coli* serves the propagation in bacteria containing the *pir* gene, expressing the initiator protein *π*. The transgene is a fusion gene of the firefly luciferase and the EGFP gene, EGFP::Luc, which is driven by an promoter x; either the ubiquitously expressing CMV-IEP (human cytomegalovirus immediate early promoter and enhancer region) or the liver specific promoters HPGL (haptoglobin), AFP (alpha-fetoprotein), FMO (flavin-containing monooxygenase) or APOE (apolipoprotein). (A) The transgene is followed by a 2 kb scaffold matrix attachment region (S/MAR) from the 5´-region of the human IFN- β. (B) A CMV-promoter driven plasmid without the S/MAR sequence; pEPItoΔS/MAR-CMV-EGFPLuc.

backbone either containing the S/MAR (pEPIto-CMV-EGFPLuc) or lacking it (pEPItoΔS/MAR-CMV-EGFPLuc).

For comparing the liver specific promoters with the CMV-promoter the above mentioned plasmids were transfected into the hepatocellular carcinoma (HCC) cell line HuH7 and the transgene expression strength and duration was monitored by luciferase assays. As expected (the CMV promoter is one of the strongest promoters used commercially), the plasmids with the tissue specific promoters showed a lower transgene expression than the pEPIto-CMV-EGFPLuc and pEPItoΔS/MAR-CMV-EGFPLuc plasmids (**fig. 18**). The promoter of the FMO gene was the weakest promoter, with a 10-fold lower transgene expression compared to the other tissue

specific promoters. After only 5 days after transfection, the luciferase signal of the cells transfected with pEPIto-FMO-EGFPLuc was undetectable,

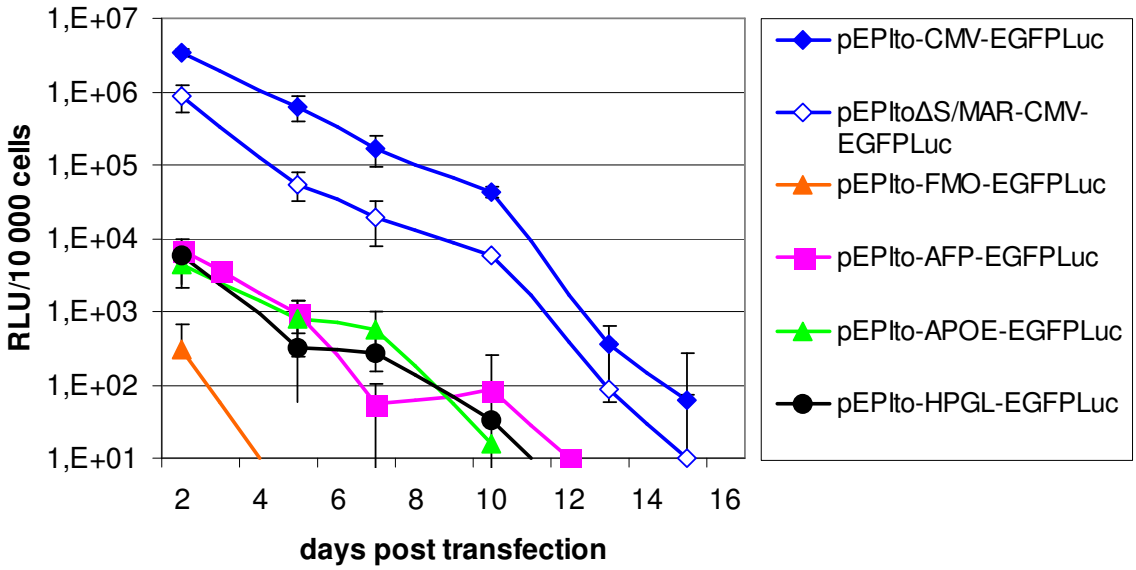


**Fig. 18: Luciferase activity of transfected HuH7 cells.** Transfection was performed with pDNA complexed with H DO and the transgene expression was continuously examined by luciferase assays. Auto-fluorescence of untransfected cells (background) was deducted. RLU: relative light units. n=2.

whereas the signal from the other plasmids lasted about 2 weeks. The signal loss is presumably primarily due to the fast cell growth *in vitro*, as discussed in 4.4. To test the persistence of the plasmids we therefore decided to use the hydrodynamic delivery method to measure the expression in mouse livers, since the proliferation rate in the liver is very low (only 1 of 20 000 hepatocytes in the adult liver is cycling [119]).

Due to its low signal we excluded the pEPIto-FMO-EGFPLuc from further examinations. Also the pEPIto-AFP-EGFPLuc is not suitable for this set-up, since AFP is mainly expressed in foetal liver or liver carcinomas. The remaining plasmids with liver specific promoters, the pEPIto-HPGL-EGFPLuc and pEPIto-APOE-EGFPLuc, had similar expression levels *in vitro*. To decide which promoter to use for the *in vivo* experiment, a small pilot study with 4 animals comparing the HPGL and APOE promoter was conducted,. For this purpose a CpG-replete plasmid series (pEPI-1) with control plasmids without S/MAR´s was utilised. The plasmids pEPI-1-HPGL-EGFPLuc and pEPI-1-APOE-EGFPLuc (with S/MAR) and pEPI1ΔS/MAR-

HPGL-EGFPLuc and pEPI-1ΔS/MAR-APOE-EGFPLuc (without S/MAR) were injected into mice by the hydrodynamic method.

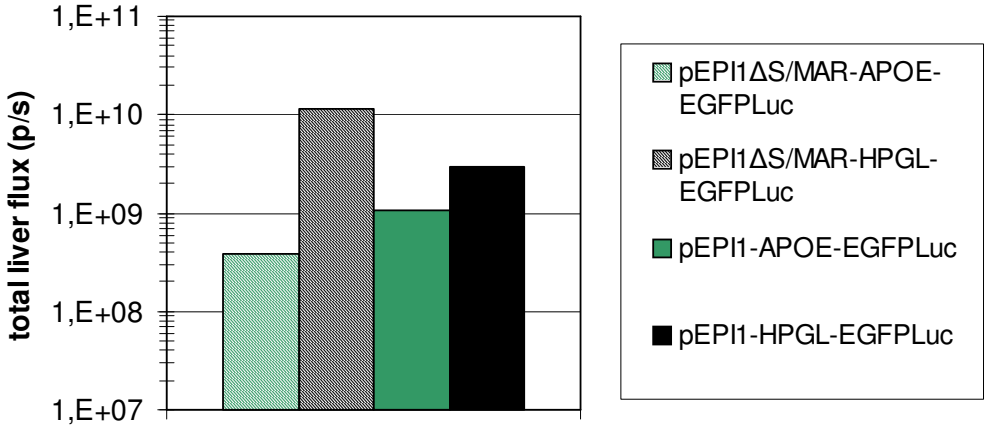


**Fig. 19: Comparison of plasmids with the liver specific promoters HPGL and APOE in vivo.** The plasmids pEPI1-HPGL-EGFPLuc and pEPI1-APOE-EGFPLuc (with S/MAR) and pEPI1ΔS/MAR-HPGL-EGFPLuc and pEPI1ΔS/MAR-APOE-EGFPLuc (without S/MAR), all CpG-replete, were injected by hydrodynamic delivery into Balb/C mice. 24h after treatment the luciferase activity was measured by in vivo bioluminescence imaging. n=1. p/s: photons per second.

As seen in **fig. 19** the plasmids with the HPGL promoter are superior to the vectors with the APOE promoter, independent on the presence of the S/MAR. Based on this we decided to proceed with the HPGL promoter. Aside from pEPIto-HPGL-EGFPLuc also pEPI1-HPGL-EGFPLuc and pEPI1ΔS/MAR-HPGL-EGFPLuc were included to examine the long-term effect of a CpG-containing backbone and additional S/MAR-depletion on the transgene expression. As a control pEPIto-CMV-EGFPLuc was used. The pDNA was injected into mice by hydrodynamic delivery and monitored over time by live bioluminescence measurements.

The result from the comparison of the HPGL and CMV promoter is shown in **fig. 20.** Similar to the data presented in **fig. 6** and **7,** the signal from the CMV-driven plasmid decreases rapidly and thereafter stabilised. The signals from the plasmids with the HPGL promoter are following a similar pattern, but are generally one log-unit weaker than the signal from pEPIto-CMV-EGFPLuc. The CpG´s in the plasmid sequence of pEPI-1-HPGL-EGFPLuc and the additional removal of the S/MAR sequence in pEPI1ΔS/MAR-HPGL-EGFPLuc tend to decrease expression incrementally, but the

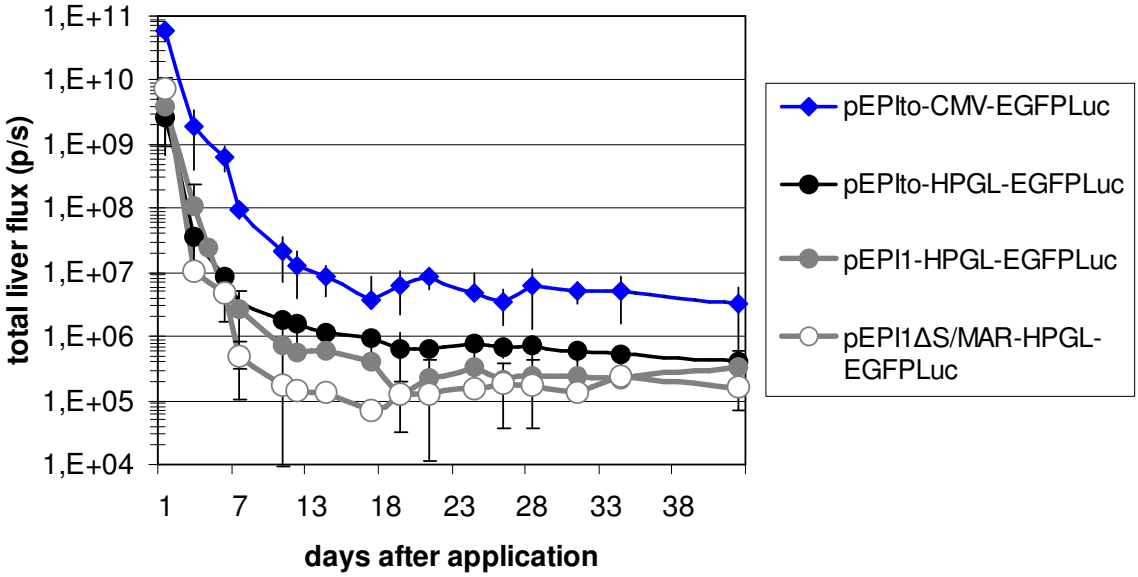


**Fig. 20: Comparative *in vivo* study of plasmids with liver specific HPGL promoter or ubiquitous CMV-promoter.** Plasmid DNA was injected into Balb/C mice by hydrodynamic delivery method and luciferase expression was monitored by in vivo imaging. pEPI1: CpG-replete plasmid series, pEPIto: CpG-deplete plasmid series. n=5 **(**pEPI1ΔS/MAR-HPGL-EGFPLuc), n=4 (pEPIto-HPGL-EGFPLuc, pEPI1-HPGL-EGFPLuc, pEPIto-CMV-EGFPLuc). p/s: photons per second.

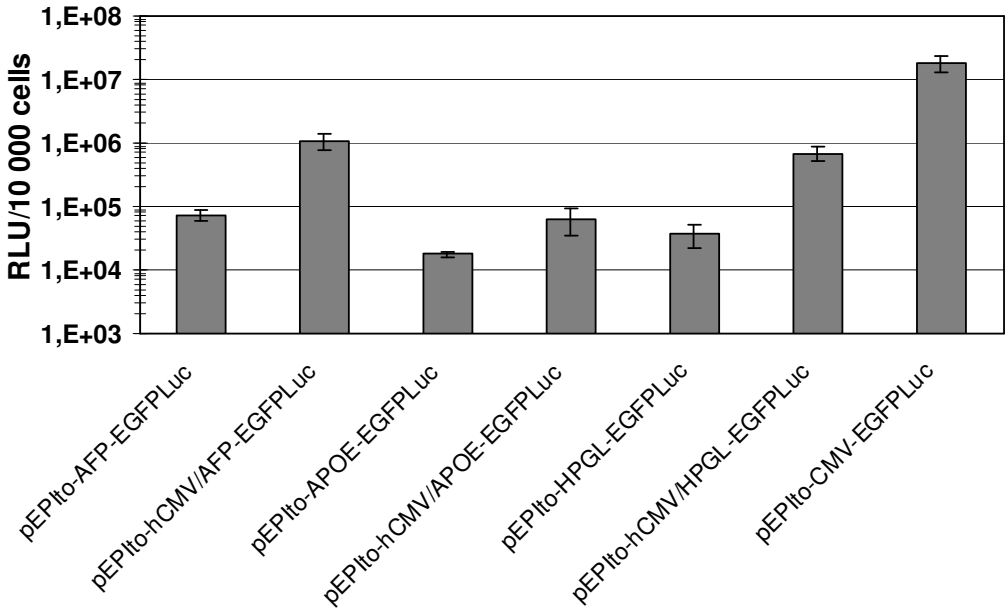


**Fig. 21: Introduction of hCMV enhancer into plasmids bearing liver specific promoters enhances expression.** HuH7 cells were transfected with pDNA complexed with H DO and transgene expression was evaluated by luciferase assay 24h post transfection. RLU: relative light units.

differences are small. With the aim to increase the expression level from the plasmids with liver specific promoters an enhancer from the hCMV was inserted upstream of the promoter. The resulting plasmids, pEPIto-hCMV/HPGL-EGFPLuc, pEPIto-hCMV/AFP-EGFPLuc and pEPIto-hCMV/APOE-EGFPLuc were tested by *in vitro* transfection of HuH7 cells (**fig. 21**). In all cases the CMV enhancer was able to increase the transgene expression and the highest expression was achieved with the plasmid pEPIto-hCMV/AFP-EGFPLuc, rendering a luciferase activity about 1 log-unit below the signal from pEPIto-CMV-EGFPLuc. Hence, the signal from pEPIto-hCMV/AFP-EGFPLuc is still lower, but in the context of a gene therapy vector, this could be counterbalanced with a tissue specificity, which the pEPIto-CMV-EGFPLuc does not exhibit. To test how well defined this tissue specific expression, e.g. the "leakiness", of the plasmids is, the plasmids were transfected into different cell lines (**fig. 22**). In all cases, except for in the HCC cell lines, HuH7, HepG2, and the AFP-producing MCF-7 cell line, the signal from the pEPIto-hCMV/AFP-EGFPLuc was close to the background, showing a low off-target expression, whereas the pEPIto-CMV-EGFPLuc exhibited a ubiquitous expression.

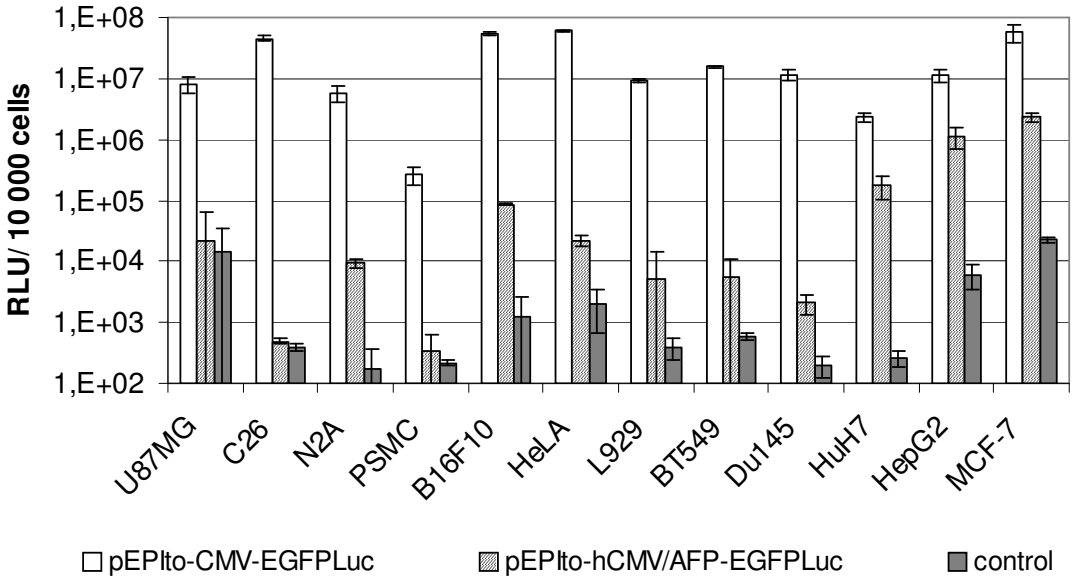


**Fig. 22: Transfection of various cell lines with the plasmids pEPIto-CMV-EGFPLuc and pPEIto-hCMV/AFP-EGFPLuc, driven by the CMV-IEP and AFP-promoter/hCMV-enhancer hybrid, respectively.** pDNA was condensed with LPEI in HBS for polyplex formation. Transgene expression was assayed 24h after transfection by luciferase activity measurements. RLU: relative light units. Control: untransfected cells.

# 4. Discussion

## 4.1. Human CMV enhancer leads to higher expression and better plasmid retention than murine CMV enhancer

For an optimal gene therapy treatment the duration and strength of gene expression is of major importance. It dictates how often the treatment has to be performed and how much from the therapeutics you need to apply. Ideally, only a single treatment would be necessary, thus avoiding repetitive applications. This is especially desirable if the therapy is complicated, associated with physical discomfort or expensive. It is also worthwhile to reduce the dosis of the used therapeutics, to lower costs and possible side effects.

There are many ways to increase the stability and strength of transgene expression. Amongst others, the choice of a good enhancer is crucial, since it can influence the transcription from the core promoter in a decisive fashion. It has been reported, for example, that the deletion of the enhancer in the otherwise intact murine CMV caused severe deficiencies in virus production [120]. In this study we have compared the enhancer from murine and human CMV and their ability to drive efficient expression from a luciferase expressing plasmid.

The sequence of the CMV enhancer is only about 300 bp long in pCpG-hCMV/EF1-LucSH and pCpG-mCMV/EF1-LucSH, containing the human and murine CMV enhancer, respectively, and comprises therefore only 6 % of the total plasmid DNA mass. It is the only differing sequence between the two plasmids, but despite its size, it has a great impact on the transgene expression. We could show that the expression from pCpG-hCMV/EF1-LucSH is clearly enhanced and prolonged in mouse liver when compared with the pCpG-mCMV/EF1-LucSH (**fig. 3**). In agreement with this Hyde *et al* [69] could show an extended transgene expression when replacing the murine CMV enhancer with the human counterpart in an EF1-driven plasmid and applying the plasmid DNA in a lipid formulation nebulised to the lung. Why the human CMV enhancer is working better in mouse cells is not clear yet, but one possible explanation could be a better enhancer-promoter interaction between the hCMV enhancer and the EF-1 promoter compared with the mCMV enhancer.

Since experimenting with *Drosophila* gene expression starting in 1994 the concept of enhancer-promoter specificity is known [17, 18], and by now it is clear that some enhancers exhibit a strong preference [13, 14], or even specificity [14, 16] when it comes to activation of the core promoters. The activation of the promoter by the enhancer can occur over several kb distance [121] and it is postulated that a loop is formed to bring the promoter and enhancer in close proximity [9, 10, 122], where a protein-protein interaction between bound transcription factors on the enhancer interacts with the promoter/transcription initiation complex to facilitate its assembly and transcription initiation [123]. In this scenario, it can be easily imagined that the nature of the enhancer bound transcription factors and the resulting enhanceosome complex should have a decisive role in the activation of the transcription from the EF-1 promoter. The murine and human CMV enhancer sequences in the plasmids are akin with 38% sequence similarity and they are sharing about 50% of the transcription factor binding site types. The remaining binding sites are differing between the 2 enhancers, and with binding of different transcription factors the EF-1 promoter could be activated in a variable way. Looking at the different expression profile caused by the murine and human CMV enhancers, there is possibly a greater enhancer-promoter preference between the EF-1 promoter and the hCMV enhancer than the mCMV enhancer.

The difference between the pCpG-hCMV/EF1-LucSH and pCpG-mCMV/EF1-LucSH is also reflected in the plasmid copy number (**fig. 4**), where a higher copy number is coinciding with a higher transgene expression. There is basically no reason why the pCpG-hCMV/EF1-LucSH should have a better retention potential when just looking at the plasmid sequence, since they are almost the same, except for the enhancer. Therefore, the better retention potential must derive from the enhancer itself; or more specifically, by its ability to enhance the expression of the transgene. An active transcription might influence the replication, which is an indispensable prerequisite for a successful segregation of plasmid DNA onto the two daughter cells in mitosis, i.e. plasmid retention. A clear connection between transcription and replication was found scanning a Drosophila chromosome, where transcriptional activity was shown to correlate with the replication timing, i.e. a densely transcribed domain replicated earlier in S-phase [124]. This phenomenon has also been reported in other work [125, 126]. One explanation to this interrelation of the two processes could be found in their similarities. Both transcription and replication are highly organized -temporally

and spatially- and demand the assembly of high order protein complexes as well as chromatin decondensation. They are locally restricted in transcription factories [127, 128] and replication foci [129-133], areas where the needed enzymes and factors are concentrated to facilitate the initiation and onset of these processes. Depending on transcriptional activity gene domains are localized in different subnuclear positions, pending between the nuclear periphery and heterochromatin for transcriptionally inactive genes to the euchromatin for active gene loci [134, 135]. One hypothesis why an active transcription leads to an early replication in S-phase is that the earliest replication complexes form at the location where they can assemble easiest, which would be proximal to active genes in the open euchromatin [136]. This would lead to the assumption that transcription and replication factories are also spatially connected to each other, preventing the need of transport of the genes from transcriptional sites to replication foci. Indeed, there has been evidence for a global colocalization in the nucleus at the onset of S-phase [137], but there is hardly any evidence for a direct over-lapping of the two functional foci [138]. Judging from the distribution pattern of the factories there is likely a discontinuous dynamical zonal change from transcription factory areas into replication factories areas, where transcription factories in a distinct zone are decommissioned and replication factories are established instead. When replication is finished, replication factories are disintegrated and transcription starts again. Thereafter, the next zone is turned into a replication factories area for the restricted time of replication [139, 140]. In any case, there seems to exist a temporal replication advantage for actively transcribed genes since these will automatically be in the area of early replication. In addition, it can be speculated that the binding of the transcription factors and transcriptional machinery units to their cognate sites could facilitate replication by preparing DNA for the unwinding process, since both events demand the separation and unwinding of the two DNA strands. We therefore hypothesise that by increasing the transcription and subsequent expression of the transgene indirectly also the replication and retention of the plasmid DNA, two important factors for achieving a prolonged and strong gene expression for gene therapy, will improve.

Seeing the clear advantage of the human CMV-enhancer over the murine we decided to examine if a second copy of the human CMV-enhancer could enhance the transgene expression even more, as in previous cases; [141-143]. As seen in **fig 5,** the second enhancer does not lead to better expression. On the contrary, it

decreases the expression level in the beginning, is however delaying the drop in signal and reaches the same level as the plasmid with one enhancer after the signal drop. Since the assembly of the enhanceosome and the interaction with the core promoter and associated factors seem to be a complex process [144-149] it is likely that a second enhancer is not coercively increasing the transcription rate. In this case, in the two first weeks of the experiment, it seems like the second enhancer is rather delaying the transcription initiation than promoting it. Most probably it is depending on the nature of the enhancer if a second sequence is additive -or even synergistic- or if it is merely hindering the process. It has been shown that the hCMV enhancer is modularly built, with several different repeated sequences. A single copy of the 18bp or 19bp repetitive sequence alone was able to drive expression from the minimal core promoter, with the 19 bp repeat, containing the binding site for ATF, being the strongest one. By repeating these sequences the expression was increased, even though the activation by the natural full length enhancer always was superior [150]. This shows the modular nature of the enhancer, where the single modules (enhansons) can act separately to initiate transcription. By interaction with other modules and their associating activators the enhancing effect on the transcription can be incremented accordingly. The enhancer sequence in pCpG-hCMV/EF1-LucSH is not the full length of the naturally occurring enhancer, but is containing 15 consensus sequences of binding sites for ATF and numerous other cis-acting factors. It has presumably conserved some of its integrative modular function, and even though the multiplication of the 19bp repeat was leading to increased expression, this must not be the case of the longer and, probably, much more complex enhancer of pCpG-hCMV/EF1-LucSH.

## 4.2. Adaptive immune system is involved in transgene regulation in vivo

A loss of transgene signal can have many causes, but generally depends on either: i) silencing of the transgene, i.e. by DNA methylation, ii) loss of plasmid during, for example, mitosis or iii) elimination of transfected cells by apoptosis or immune cells. So what could be the cause of the rapid decrease of transgene signal of pCpG-mCMV/EF1-LucSH and pCpG-hCMV/EF1-LucSH after hydrodynamic delivery (**fig. 3**)? One option would be that it is the result of a transgene silencing effect induced by

methylation. This is not very likely since the plasmids pCpG-mCMV/EF1-LucSH and pCpG-hCMV/EF1-LucSH are CpG-reduced. They contain 16 CpG´s in the hCMV enhancer and none in the mCMV enhancer, and only 3 CpG´s in the rest of the plasmid. The three CpG´s in the backbone of pCpG-mCMV/EF1-LucSH are most likely not enough for triggering a formation of heterochromatin [98] and, in addition, a strong promoter, such as the EF-1, could be able to override a weak repression [96]. In pCpG-hCMV/EF1-LucSH, in theory, 16 methylated CpG´s in the enhancer could trigger transcriptional repression, as shown by Kass et al [96], where a similar number (9-21) of methylated CpG´s could decrease the transgene expression, independent of their location in the plasmid. But this decrease was maximum 2-fold and stands in no comparison to the decrease in expression observed with pCpG-mCMV/EF1-LucSH and pCpG-hCMV/EF1-LucSH (10 000-fold decrease). For a repression over the human CMV enhancer it would probably also necessitate a complete methylation of all its CpG´s, since the length of the methylated CpG-island is decisive for the repression [96, 97]. In comparison to the CpG-reduced plasmids, pEGFPLuc contains several hundreds of CpG´s evenly spread over the whole sequence and its CMV-IEP is known from previous work to be methylated fast [26, 27]. This is probably also occurring, since the signal from pEGFPLuc is plummeting from the first day on (**fig. 6).** In contrary to this, the signal from pCpG-mCMV/EF1-LucSH and pCpG-hCMV/EF1-LucSH is decreased only 2 weeks after injection. This points to that the signal from pCpG-mCMV/EF1-LucSH and pCpG-hCMV/EF1-LucSH is decreased by the same mean and probably not by methylation.

The decrease in transgene signal does also not seem to be the result of a loss of plasmid during cell cycling, since this would have been expected to occur mainly directly after the hydrodynamic injection, when the regeneration of the liver after the hydrodynamic delivery largely occurs. Instead, the signal is relatively stable the first 2 weeks after treatment.

The last alternative, elimination of the transfected cells, could be the result of direct apoptosis of cells severely damaged from the hydrodynamic treatment or by apoptosis or destruction of the transfected cells influenced by the immune system. The apoptosis induced by cell damage is not likely, since this would also have happened primarily directly after injection, so the most probable cause for the signal drop is by influence of the immune system.

To test if the immune defence is actually the leading factor, the plasmids pCpG-mCMV/EF1-LucSH, pCpG-hCMV/EF1-LucSH, pCpG-2xhCMV/EF1-LucSH and pEGFPLuc were injected into SCID mice, lacking mature T- and B-cells and therefore a functional adaptive immune system. The resulting expression profile of pCpG-mCMV/EF1-LucSH, pCpG-hCMV/EF1-LucSH and pCpG-2xhCMV/EF1-LucSH in the immunodeficient mice (**fig. 7**) is clearly deviating from the profile in the immunocompetent Balb/C mice (**fig. 3** and **5**). In the immunodeficient mice the transgene signal is stable over time, without any loss of strength, whereas it is reduced in the immunocompetent mice. Only in the case of pEGFPLuc the expression-over-time pattern is the same in both immunocompetent and immunodeficient mice (**fig. 6** and **7**). This clearly shows that the signal from pEGFPLuc is silenced without the influence of the T- and B-cells, probably by DNA methylation, and that the adaptive immune defence is playing a crucial role in the regulation of the transgene signal of pCpG-mCMV/EF1-LucSH, pCpG-hCMV/EF1-LucSH and pCpG-2xhCMV/EF1-LucSH.

Most probably, the gene product of the luciferase::Sh gene is immunogenic, acting as an antigen to trigger the cellular and/or humoral host response. This would correspond to the finding of the group of Davis *et al* where muscle fibres were destroyed around 10 days after injection of an antigen-expressing plasmid, and when co-injecting with a luciferase-expressing plasmid, they could see strong luciferase expression still at day 5 and a >99% loss by day 20 [151]. They noted an activation of $CD8^+$ cytotoxic T lymphocytes 3-6 days after injection, which reached a maximum at 6-12 days [152]. This temporal pattern of immune cell activation and elimination of transfected cells and consequent decrease of transgene expression fits to the delayed drop of luciferase signal of pCpG-mCMV/EF1-LucSH, pCpG-hCMV/EF1-LucSH and pCpG-2xhCMV/EF1-LucSH. It must be noted, though, that the gene product of the luciferase-expression plasmid Davis *et al* used was on its own not causing a decrease in signal, i.e. immune reaction. On the other hand, the luciferase used in Davis work and the LucSH used here are not the same, since the luciferase gene in LucSH is fused to another gene and is additionally modified to remove CpG-dinucleotides.

## 4.3. Plasmid retention is dependent on cell growth activity

The human enhancer was clearly advantageous compared to the murine enhancer in driving the expression in the liver and we wanted to investigate this effect also in a tumour model. For this purpose, SCID mice carrying subcutaneous human tumours were electroporated with the plasmids pCpG-mCMV/EF1-LucSH, pCpG-hCMV/EF1-LucSH, pCpG-2xhCMV/EF1-LucSH and pEGFPLuc and the consequent luciferase expression was followed by *in vivo* imaging. The cell line we used for the subcutaneous model was MDA MB-435, which lately attracted some attention because of its rather uncertain identity. It was used for a long time as a standard cell line for research on breast cancer. After examination of its expression profile doubts about its origin came up, since it resembled more the melanocytic lineage than the mammary cancer cell lines [153, 154]. But later investigations revealed that it did show most of the characteristics of the epithelial breast cancer lineage, but has probably with time delineated and is now expressing also melanocyte-specific proteins [155]. Indifferent of its exact cell type, we decided to use this cell line since it forms slow growing subcutaneous tumours, giving us time to follow the plasmid faith in a tumour environment.

The result shows a relatively stable luciferase signal in the tumours over time, with none of the plasmids exhibiting a significantly better retention potential than the others (**fig. 8A**). In the case of the plasmids pCpG-hCMV/EF1-LucSH, pCpG-mCMV/EF1-LucSH and pCpG-2xhCMV/EF1-LucSH this coincides with the expression pattern in the liver of the immunodeficient mice (**fig. 7**), where the luciferase signal, due to the lack of an adaptive immune system, is also not changing over a long period of time. What was surprisingly different when comparing the transgene expression in the liver and tumour is the expression from the plasmid pEGFPLuc. In the tumour its transgene signal is stable, whereas the signal is abruptly decreased in the liver. The reason for this might be found in its promoter, CMV-IEP, and its interaction with the tumour cells.

The MDA MB-435 cells have been shown to have a constitutively active NFκB, which is probably partly the result of decreased levels of the IκB-α and -β [156] and a lack of oestrogen receptor (ER), which can reduce the NFκB in various ways [156]. It is said that this active transcription factor allow ER-negative breast cancer cells to grow in a non-oestrogen dependent manner and also survive radiation and chemotherapy

treatment since it prevents apoptosis. Other target genes of NFκB influence angiogenesis, inflammation, degradation of extracellular matrix and cell adhesion, which could be why ER-negative breast cancer is often associated with invasiveness and metastasis, accompanied with a poor prognosis. NFκB is activated by various stimuli, such as cellular or environmental stress, cytokines, DNA-damage, radiation or pathogens [157]. To the last category also CMV infection counts, where glycoproteins of the virus envelop or immediate-early gene products trigger the nuclear translocation of the transcription factor [158-160]. This NFκB activation, which leads to an inflammatory response aiming at fighting the virus, has been taken advantage by the CMV by incorporating several functional NFκB binding sites into its immediate-early gene promoter/enhancer. Thus, the binding of NFκB to these sites leads to a priming of expression of the immediate-early genes [22, 161]. NFκB is also able to reactivate latent CMV. This was shown by treating transgenic mice expressing the β-galactosidase under the control of the CMV-promoter with adenovirus. The adenovirus induced the formation of active NFκB and the reactivation of the β-galactosidase expression, both coinciding temporally with each other [162]. Taking together that MDA MB-435 cells harbour a constitutively active NFκB and that this transcription factor is able to increase the level of gene expression by binding to the hCMV-IEP, it is possible that this is the reason to why the CMV promoter is not silenced in the MDA MB-435 tumours.

In contrary to the stable signals from all the plasmids in the tumour, the transgene signal was not measurable anymore after about 2 weeks when the *in vivo* electroporated and passaged MDA MB-435 tumour cells were taken into cell culture (**fig. 9**) or when the cells where transfected directly *in vitro* (**fig. 10**). This is probably due to the faster cell growth *in vitro* compared to the tumour. A phenomenon underlining this was observed when transfecting cycling or quiescent primary smooth muscle cells. While the transgene signal from the quiescent cells was stable for more than one month, the signal from the freely growing cells was lost after 3 weeks (**fig. 11**). Apparently, cell growth can lead to a loss of plasmid material. This can happen during mitosis when the nuclear membrane is disrupted, enabling the plasmid DNA to distribute into the cellular compartment. When the nuclear membrane is re-established in telophase, the plasmids which are not in close proximity to the chromosomes will be excluded from the newly formed nucleus. Alternatively, or additionally to this, over-growth of transfected cells by untransfected cells (which

could have a metabolic advantage not having to express a large transgene), can lead to a loss of transgene signal in cycling cells.

For future gene therapy approaches these results are important to bear in mind when trying to reach a persistent transgene expression. If the target cells are slow dividing or quiescent the problems with loss of plasmid DNA are minor, such as in the case of muscle fibre cells, where plasmids can be retained in an extrachromosomal actively transcribing state over several months [163]. If the target cells, on the other hand, are constantly cycling, like for example skin cells, one will need a plasmid vector which is stably retained also during mitosis.

## 4.4. Efficient immune response requires minimal amount of transgene

Nature has developed many strong and ubiquitous expression systems with time and the succession to even stronger or better controllable systems are now investigated by gene technology and synthetic production in an accelerating pace. By combining already existing enhancers and promoters hybrid control elements are created, some more efficient in gene expression than the cognate enhancer-promoter, for example the promoter CAG, which is composed of the CMV enhancer and chicken β-actin promoter [164]. Aside shuffling of known enhancers and promoters, the construction of new synthetic regulatory elements open up totally new combinatorial possibilities. By combining the homologous regions of 2 strong ubiquitous promoters, the EF-1 and CMV-IEP, our aim was to bring together the advantages of both promoters. The CMV-IEP is one of the strongest promoters commercially used, but suffers from silencing events, whereas the EF-1, being of human origin, lacks the pronounced silencing effect, but gives short-term weaker transgene expression than the CMV-IEP. The promoters show innate high homology (44%) and by combining and optimizing these homologous regions the synthetic promoter SCEP was formed. SCEP shows a high similarity with the 2 parental promoters (74% and 60% to EF-1 and CMV respectively) but exhibits some interesting new features. It renders higher transgene expression values than both CMV and EF-1 promoter and additionally escapes the initial silencing, the characteristic of CMV-IEP.

Interestingly, the signal from pCpG-hMCV/SCEP-efFLuc undergoes the same decrease in strength 2 weeks after injection as pCpG-hMCV/EF-1-efFLuc. This implies that pCpG-hMCV/SCEP-efFLuc also is subjected to an immune response by the host cell defence. The signal from pCpG-hCMV/CMV-eFLuc, on the other hand, does not exhibit this signal drop, even though it expresses the same transgene product, which could be recognized by an APC as an antigen, thus triggering a specific immune response against transfected cells. We hypothesise that the amount of produced luciferase is the key point and that a certain threshold level of luciferase must be produced to trigger an immunologic reaction strong enough to affect transgene expression. A similar observation was made when Labarriere *et al* [165] examined the mRNA levels of tumour antigens in melanoma cell lines and their ability to elicit an antigen specific cytotoxic T lymphocyte (CTL) response. The result showed that a critical lower level of antigen mRNA was necessary to trigger IL-2 expression (a strong autocrine activator for $T_H$-cells) and that CTL lytic activity was grossly proportional to antigen transcription. This indicates that the amount of MHC I bound antigen presented on the tumour cell surface is decisive for the activation of T cells; a conclusion also found by Rawson et al [166]. They hypothesise that multiple protein epitopes are necessary to elicit an effective antigen specific immune reaction, something which is promoted by higher transgene expression and subsequent digestion and presenting on the cell surface.

## 4.5. Minicircles lead to enhanced transgene expression in vivo

A highly efficient process for minicircle production has been developed using the ParA resolvase recombinase, with a recombination efficiency of >99,5 %, combined with an affinity chromatography approach for purification, leading to a purity of >98,5% [117]. For the biological testing of a minicircle manufactured according to this procedure, pCMV-Luc-MC, it was compared with its parental plasmid, pCMV-Luc, *in vitro* and *in vivo*. In general, the pCMV-Luc-MC was performing equally well independent if it was applied in equimolar or equal weight ratios to pCMV-Luc. *In vitro* the transfection with the minicircle led to a higher transgene expression in PSMC cells (**fig. 14**). The better performance is probably due to facilitated uptake and trafficking of the smaller minicircle, but this feature seems to be cell type specific, since the expression from the minicircle and parental plasmid was similar in N2A cells.

By injection of the two plasmids in equimolar amounts by hydrodynamic delivery in mice the transgene expression was monitored in the liver over time (**fig. 15, 16**). The resulting data show a difference in luciferase activity between the plasmids only in the highest applied concentration of pDNA, with the signals from the minicircle being superior. In the lower pDNA concentrations no difference between minicircle and plasmid was seen.

By reducing the number of unmethylated CpG´s in the plasmid sequence the negative effect of DNA methylation and inactivation of plasmid, promoter attenuation and immune reactions can theoretically be reduced. The CMV promoter is known to be inactivated fast by promoter methylation [26, 27], and here it probably also is, judging from the fast decrease in transgene signal from both plasmids directly after injection. Additionally to this, it has been shown that also methylation of other sequences in the plasmid leads to decreased gene expression [93, 96-98]. This could cause higher repression of the parental plasmid in comparison to the minicircle. But the effect of silencing by DNA methylation is not plasmid dose-dependent; hence, if DNA methylation would be the driving force to the reduced transgene signal, there should be a difference in transgene expression between minicircle and plasmid in all pDNA concentrations. But there is only a difference in the highest plasmid concentration, therefore this mechanism can largely be ruled out.

The other possible explanations to the different expression level between parental plasmid and minicircle are promoter attenuation and immune reactions. These alternatives are more likely, since they are both plasmid dose-dependent: The more unmethylated CpG´s there are in the cells, the more pro-inflammatory cytokines are produced [69], which can bind to and reduce the expression from the promoter [76-80] or activate the immune system to trigger a transgene specific immune reaction more efficiently [73,74].

Minicircle production as an alternative to reduce CpG´s, and also to reduce plasmid size, is one option to take into consideration when producing a vector for gene therapeutic purposes. Especially with the improved production process, promising high efficiency and facilitated purification, the production can be scaled-up for commercial sales.

## 4.6. Transcriptional targeting with AFP promoter/CMV enhancer

By using transcriptional targeting the aim was to increase safety of liver gene therapy by reducing side effects associated with gene expression in extrahepatic cells. The main problem with the tested liver specific promoters was a relative low expression level *in vitro* and *in vivo* relative to the CMV promoter (**fig. 18** and **20**). This could be problematic when trying to reach therapeutic levels of a transgene. By introducing the enhancer sequence from the hCMV an increase in transgene expression was observed for all liver-specific promoters tested, most prominent in the case of the AFP promoter **(fig. 21)**. Since the CMV-IEP is not liver-specific but renders ubiquitous gene expression, the risk by combining the hCMV enhancer with the AFP promoter could have been a loss of the tissue-specificity. As seen in **fig. 22** only low expression in non-HCC cell lines was observed, hence the chimeric enhancer-promoter is still activated mainly in HCC. The special characteristic of the natural AFP promoter compared to the other investigated liver specific promoters is its specificity for HCC cells, since it is silenced in adult liver tissue [42]. Therefore, the AFP promoter is suitable for targeted HCC gene therapy.

# 5. Summary

Gene therapy poses a relatively new approach for treatment of various diseases (first gene therapy clinical trial in 1990). It is still on an experimental basis and there will be still research needed before the first gene therapy product will be accepted for commercial sale. The major hurdles for reaching this so far have been safety and efficiency, two problems generally associated with viral and nonviral gene therapy, respectively. To avoid the safety issues arising from viral delivery, such as reported insertional mutagenesis and severe immune responses, lately the focus has been turned to nonviral vectors. To deal with the low efficiency of these vectors much work has been done on the gene transfer vehicle of the DNA cargo, i.e. liposome and polyplex formulations, for example. But also the construction of the DNA itself, the plasmid, can influence the gene therapy potential considerably, as reported in this thesis.

By choosing and combining the modules of the plasmids in an optimised way, transgene expression can both be enhanced and prolonged. This has been shown by combining a ubiquitous EF-1 promoter with a CMV enhancer, the latter originating either from murine or human CMV. The plasmid with the human CMV enhancer showed clearly a higher and longer-lasting transgene expression *in vivo* in comparison with the murine analogue, probably due to a better promoter-enhancer interaction. This advantage in expression was also paired with a higher plasmid copy number, showing that a high expression of a plasmid leads to a better retention thereof in the host cell, an important factor for gene therapy. In all cases with plasmids containing mCMV or hCMV enhancers, the transgene signal is first constant, but drops abruptly about 2 weeks after application. To clarify the reason for this sudden reduction of signal the experiment was repeated in immunodeficient mice. The result showed, in contrast to previous measurements, stable expression signals, indicating that the immune system was responsible for the loss of transgene signal. We could also show that a high level of transgene expression was necessary to trigger such an immune response: A plasmid with a CMV promoter showed similar expression profiles in both immunocompetent and –deficient mice; a sharp decrease from day one after application, which within a few days after application stabilised on much lower levels, but showed no further decrease 2 weeks after application. The early signal drop is probably due to previously reported methylation of the CMV

promoter and leads to lower transgene signals, which do not elicit a strong immune response.

The strong, viral CMV promoter suffers from early silencing processes, whereas the signal from the mammalian EF-1 promoter is more stable. With the idea of taking advantage of the best features of the two, a new synthetic promoter was designed by combining the shared homologous regions. The novel promoter, SCEP, showed enhanced transgene signals *in vivo* compared to both the CMV and EF-1 promoter, but elicited, because of the high levels of transgene product, in the same fashion as the EF-1 containing promoter, an immune response, which led to a drop in signal two weeks after plasmid injection. The two luciferase variants used here as reporter genes are apparently mitogenic, thus, reduces the longevity of their expression. Therefore, it would be of great interest to investigate other transgenes, i.e. endogenous therapeutic genes with less or no immunological effect, to evaluate if the stability of the gene expression can be increased and to which extent.

The transgene signal durability is also connected with the cycling frequency of the transfected cells, as shown in a slow-growing tumour model. The plasmids were introduced into the tumours by electroporation and the tumour cells were isolated and taken into cell culture directly or two weeks after electroporation. Measurements of the transgene signal revealed constant levels in the tumours over one month time, whereas the signal in the cell culture, where the cell doubling time is considerably shorter, was lost within 2 weeks. This result points to the event of mitosis as a decisive time point when plasmids easily are lost, and should be taken into consideration when choosing the target cells for gene therapy.

One way of reducing plasmid loss caused by the host immune defence is to minimise the amount of unmethylated CpG´s in the plasmid sequence. Reduction of the CpG´s can be done either by an overall elimination at the step of plasmid design, or later in the plasmid production process by removing the bacterial backbone, containing most of the unmethylated CpG´s. Thus, a minicircle is formed, containing only the mammalian expression cassette. By a highly efficient, novel production process one model minicircle was produced and in this work tested biologically. The minicircle showed an advantage against the corresponding full-length plasmid in higher application concentrations *in vivo*. The approach of minicircles is therefore a promising part of the puzzle to complete an efficient therapeutic vector, especially since its production now is efficient enough to be scaled up.

One last area of plasmid regulation design evaluated in this work concerned the transcriptional control using liver specific promoters. Compared to the CMV promoter these liver specific promoters were generally weaker, but the transcriptional strength could be enhanced by combination with a hCMV enhancer. The strongest of the tested hybrid liver promoter/ hCMV enhancer was based on the AFP promoter, which is silent in adult liver tissue and reactivated in most HCC. It was tested *in vitro* and the result showed a low transgene expression in off-target cell lines and high in HCC cell lines, a feature which can be used for transcriptional targeting of cancer cells in the treatment of HCC.

# 6. Appendix

## 6.1. Abbreviations

| | |
|---|---|
| AFP | α-fetoprotein |
| AMP | adenosine monophosphate |
| Amp | ampicillin resistance gene |
| AP-1, AP-2 | activating protein 1, 2 |
| APC | antigen presenting cell |
| ATF | activating transcription factor |
| BCG | bacille Calmette Guérin |
| bp | base pair |
| BRE | TFIIB recognition element |
| BSA | bovine serum albumin |
| c/p-ratio | Weight ratio of conjugate to plasmid |
| cAMP | cyclic adenine monophosphate |
| cDNA | complementary DNA |
| CMV | Cytomegalovirus |
| CMV-IEP | cytomegalovirus immediate early promoter |
| CpG | cytosine-guanine dinucleotide |
| CRE | cyclic AMP response element |
| CREB | cAMP response element binding |
| CTF | CAAT (controlled amino acid treatment) transcription factor |
| DC | dendritic cell |
| DMEM | Dulbecco´s modified Eagle´s medium |
| DMSO | Dimethyl sulfoxide |
| DNA | deoxyribonucleic acid |
| DPE | down-stream promoter elements |
| DTT | Dithiothreitol |
| *E. coli* | *Escherichia coli* |
| EBV | Epstein-Barr virus |
| EC | endothelial cell |

| | |
|---|---|
| EDTA | ethylenediaminetetraacetic acid |
| EF-1 | elongation factor-1α |
| eFLuc | enhanced firefly luciferase |
| EGFP | enhanced green fluorescent protein |
| ER | endoplasmic reticulum |
| EtBr | Ethidium bromide |
| FCS | Fetal calf serum |
| Fig. | figure |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| GFAP | glial fibrillary acidic protein |
| GLuc | *Gaussia princeps* luciferase |
| HBS | HEPES-buffered saline |
| HBV | hepatitis B virus |
| HCC | hepatocellular carcinoma |
| hCMV, mCMV | human, murine cytomegalovirus |
| HDAC | histone deacetylase |
| HEPES | N-(2-hydroxyethyl) piperazine-N'-(2-ethansulfonic acid) |
| hK2 | human glandular kallikrein 2 |
| HSV tk | herpes simplex virus thymidine kinase gene |
| i.p. | intraperitoneal |
| i.t. | intratumoral |
| i.v. | intravenous |
| IFN | interferon |
| IKK | IκB-kinase complex |
| IL | interleukin |
| Inr | initiator |
| IRAK1, IRAK4 | IL-1 receptor associated kinase 1, 4 |
| IκB | inhibitor of NFκB (nuclear factor κB) |
| Kan/Neo | neomycin phosphotransferase gene |
| lacI | repressor of lactose operon |
| lacO | lactose operator |
| LPEI | linear polyethyleneimine of a Mw of 22 kDa |

| | |
|---|---|
| LPS | lipopolysaccharide |
| LRR | leucin-rich repeat |
| Luc | luciferase |
| MAP | mitogen-activated protein |
| MBD 1-3 | methyl CpG binding domain protein 1-3 |
| MBP | myelin basic protein |
| MCS | multiple cloning site |
| MeCP2 | methyl CpG binding protein 2 |
| MHC | major histocompatibility complex |
| mRNA | messenger RNA |
| MyD88 | myeloid differentiation primary-response protein 88 |
| N/P-ratio | Molar ratio of PEI nitrogen to DNA phosphate |
| NFκB | nuclear factor 'kappa-light-chain-enhancer' of activated B-cells |
| NIS | sodium iodide symporter |
| NK | natural killer cell |
| ODN | oligodeoxynucleotide |
| ori | origin of replication |
| p/s | photons per second |
| PAMP | pathogen-associated molecular pattern |
| PBS | phosphate-buffered saline |
| PCR | polymerase chain reaction |
| pDNA | plasmid DNA |
| polyA | polyadenylation signal |
| PSMA | prostate specific membrane antigen |
| PSMC | porcine smooth muscle cell |
| QPCR | real-time PCR |
| RLU | Relative light units |
| RNA | ribonucleic acid |
| RNAPol | RNA polymerase |
| RSV | rous sarcoma virus |
| S/MAR | scaffold matrix attachment region |
| SAF-A | scaffold attachment factor A |

| | |
|---|---|
| SV40 | simian virus 40 |
| TAB1, TAB2 | TAK1-binding protein 1, 2 |
| TAK1 | transforming growth-factor-β-activated kinase 1 |
| $T_H$ | T helper cell |
| TIR | Toll/IL-1 receptor |
| TLR | toll-like receptor |
| TNF-α | tumour necrosis factor α |
| TRAF6 | TNF receptor associated factor 6 |
| UBC13 | ubiquitin-conjugating enzyme 13 |
| UEV1A | ubiquitin-conjugating enzyme E2 variant 1 |
| UTR | untranslated region |
| w/w | weight to weight ratio |
| Zeo | zeosine resistance gene |
| β-Glo | β-globuline |

## 6.2. Publications

### 6.2.1. Poster presentations

- Terese Magnusson, Verena Russ, Isabella Stapff, Rudolf Haase, Ernst Wagner and Manfred Ogris: *Optimising synthetic ex vivo transferrin mediated transfection of the erythroleukemia cell line K562 with episomal plasmids,* DGGT, 2008, Berlin, Germany

- Terese Magnusson, Rudolf Haase, Armin Baiker, Ernst Wagner and Manfred Ogris: *Sustained, high transgene expression in liver with CpG-free plasmids using optimised promoter-enhancer combinations*, combined ESGCT and DGGT, 2009, Hannover, Germany.

- Rudolf Haase, Terese Magnusson, Ernst Wagner and Manfred Ogris: Generation of the novel, synthetic hybrid SCE promoter for gene-therapeutical applications, DGGT, 2010, Munich, Germany.

### 6.2.2. Publications

- Rudolf Haase, Orestis Argyros, Suet-Ping Wong, Richard P Harbottle, Hans J Lipps, Manfred Ogris, Terese Magnusson, Maria Vizoso Pinto, Jürgen Haas, Armin Baiker: *pEPito: a significantly improved non-viral episomal expression vector for mammalian cells*, BMC Biotechnol., 2010, 10-20.

- Sina Rupprecht, Claudia Hagedorn, Davide Seruggia, Terese Magnusson, Ernst Wagner, Manfred Ogris, Hans J. Lipps: *Controlled removal of nonviral episomal vector from transfected cells,* Gene, 2010, 466 (1-2): 36-42

- Philipp Dussmann, Judith I. Pagel, Sabina Vogel, Terese Magnusson, Rene Zimmermann, Ernst Wagner, Wolfgang Schaper, Manfred Ogris, Elisabeth Deindl: *Live in vivo imaging of Egr-1 promoter activity during neonatal development, liver regeneration and wound healing.* Submitted.

- Terese Magnusson, Rudolf Haase, Ernst Wagner and Manfred Ogris: *Sustained, high transgene expression in liver with CpG-free plasmids using optimized promoter-enhancer combinations*. Submitted.

# 7. References


1. Lehrmann, S., *Virus treatment questioned after gene therapy death.* Nature, 1999. **401**: p. 517-518.
2. Marshall, E., *Gene therapy a suspect in leukemia-like desease.* Science, 2002. **298**: p. 34-35.
3. Marshall, E., *Second child in french trial is found to have leukemia.* Science, 2003. **299**: p. 320.
4. Jennifer I.F. Butler, J.T.K., *The RNA polymerase II core promoter: a key component in the regulation of gene expression.* Genes & Development, 2002. **16**: p. 2583-2592.
5. Smale, S.T. and J.T. Kadonaga, *The Rna Polymerase II Core Promoter.* Annual Review of Biochemistry, 2003. **72**(1): p. 449-479.
6. Blackwood, E.M. and J.T. Kadonaga, *Going the distance: A current view of enhancer action.* Science, 1998. **281**: p. 60-63.
7. Nolis, I.K., et al., *Transcription factors mediate long-range enhancer-promoter interactions.* Proceedings of the National Academy of Sciences, 2009. **106**(48): p. 20222-20227.
8. Geyer, P.K., M.M. Green, and V.G. Corces, *Tissue-specific transcriptional enhancers may act in trans on the gene located in the homologous chromosome: the molecular basis for transvection in Drosophila* EMBO, 1990. **9**(7): p. 2247-2256.
9. Müller, H.-P., J.M. Sogo, and W. Schaffner, *An enhancer stimulates transcritpion in trans when attached to the promoter via a promoter bridge.* Cell, 1989. **58**: p. 767-777.
10. Su, W., et al., *DNA looping between sites for transcriptional activation: self-association of DNA-bound Sp1.* Genes & Development, 1991. **5**(5): p. 820-826.
11. Yie, Y., K. Senger, and D. Thanos, *Mechanism by which the IFN-beta enhanceosome activates transcription.* PNAS, 1999. **96**(23): p. 13108-13113.
12. Merika, M. and D. Thanos, *Enhanceosomes.* Current Opinion in Genetics and Development, 2001. **11**: p. 205-208.
13. Ohtsuki, S., M. Levine, and H.N. Cai, *Different core promoters possess distinct regulatory activities in the Drosophila embryo.* Genes and Development, 1998. **12**: p. 547-556.
14. Butler, J.E.F., *Enhancer-promoter specificity mediated by DPE or TATA core promoter motifs.* Genes & Development, 2001. **15**(19): p. 2515-2519.
15. Butler, J.E.F. and J.T. Kadonaga, *Enhancer-promoter specificity mediated by DPE or TATA core promoter motifs.* Genes & Development, 2001. **15**(19): p. 2515-2519.
16. Gerig, J., et al., *Automated high-throughput mapping of promoter-enhancer interactions in zebrafish embryos.* Nature Methods, 2009. **6**(12): p. 911-918.
17. Li, X. and M. Noll, *Combatibility between enhancers and promoters determines the transcriptional specificity of goosberry and gooseberry neuro in Drosophila embryo* EMBO, 1994. **13**(2): p. 400-406.

18. Merli, C., et al., *Promoter specificity mediates the independent regulation of neighboring genes.* Genes & Development, 1996. **10**(10): p. 1260-1270.
19. F.Stinski, M. and H. Isomura, *Role of the cytomegalovirus major immediate early enhancer in acute infection and reactivation from latency.* Medical Microbiology and Immunology, 2007. **197**(2): p. 223-231.
20. Stinski, M.F. and T.J. Roehr, *Activation of the major immediate early gene of human cytomegalovirus by cis-acting elements in the promoter regulatory sequence and by virus-specific trans-acting components.* Journal of Virology, 1985. **55**(2): p. 431-441.
21. Hunninghake, G.W., et al., *The promoter-regulatory region of the major immediate early gene of human cytomegalovirus responds to T-lymphocyte stimulation and contains functional cyclic AMP-response elements.* Journal of Virology, 1989. **63**(7): p. 3026-3033.
22. Prösch, S., et al., *Stimulation of the human cytomegalovirus IE enhancer/promoter in HL60 cells by TNF-alpha is mediated via induction of NFKB.* Virology, 1995. **208**: p. 197-206.
23. Stinski, M.F. and H. Isomura, *Role of the cytomegalovirus major immediate early enhancer in acute infection and reactivation from latency.* Medical Microbiology and Immunology, 2007. **197**(2): p. 223-231.
24. Angulo, A., et al., *Enhancer requirement for murine cytomegalovirus growth and genetic complementation by the human cytomegalovirus enhancer.* Journal of Virology, 1998. **72**(11): p. 8502-8509.
25. Isomura, H. and M.F. Stinski, *The Human Cytomegalovirus Major Immediate-Early Enhancer Determines the Efficiency of Immediate-Early Gene Transcription and Viral Replication in Permissive Cells at Low Multiplicity of Infection.* Journal of Virology, 2003. **77**(6): p. 3602-3614.
26. Brooks, A.R., et al., *Transcriptional silencing is associated with extensive methylation of the CMV promoter following adenoviral gene delivery to muscle.* The Journal of Gene Medicine, 2004. **6**(4): p. 395-404.
27. Collas, P., *Modulation of plasmid DNA methylation and expression in zebrafish embryos.* Nucleic acids research, 1998. **26**(19): p. 4454-4461.
28. Alexopoulou, A.N., J.R. Couchman, and J.R. Whiteford, *The CMV early enhancer/chicken β-actin (CAG) promoter can be used to drive trasngene expression during the differentiation of murine embryonic stem cells into vascular progenitors.* BMC Cell Biology 2008 **9** (2 ).
29. Bigger, B.W., et al., *An araC-controlled bacterial cre expression system to produce DNA minicircle vectors for nuclear and mitochondrial gene therapy.* The Journal of Biological Chemistry, 2001. **276**(25): p. 23018-23027.
30. Chen, Z.-Y., et al., *Minicircle DNA vectors devoid of bacterial DNA result in persistent and high-level transgene expression in vivo* Molecular Therapy, 2003. **8**(3): p. 495-500.
31. Chen, Z.-Y., C.-Y. He, and M.A. Kay, *Improved production and purification of minicircle DNA vector free of plasmid bacterial sequences and capable of persistent transgene expression in vivo.* Human Gene Therapy, 2005. **16**: p. 126-131.

32. Mascher, T., et al., *Antibiotic-inducible promoter regulated by the cell envelope stress-sensing two-component system LiaRS of Bacillus subtilis.* Antimicrobial Agents and Chemotherapy, 2004. **48**(8): p. 2888-2896.
33. Ruengrairatanaroje, P., et al., *Development of a tetracyclin-inducible systrem for expression of Ca2+ permeable TRPL channel and killing of prostate cancer cells.* Cancer Therapy, 2004. **2**: p. 389-402.
34. Darquet, A.-M., et al., *Minicircle: an improved DNA molecule for in vitro and in vivo gene transfer.* Gene Therapy, 1999. **6**: p. 209-218.
35. Darquet, A.-M., et al., *A new DNA vehicle for nonviral gene delivery: supercoiled minicircle* Gene Therapy, 1997. **4**: p. 1341-1349.
36. Kreiss, P., et al., *Production of a new DNA vehicle for gene transfer using site-specific recombination.* Appl Microbiol Biotechnol, 1998. **49**: p. 560-567.
37. Nehlsen, K., S. Broll, and J. Bode, *Replicating minicircles: Generation of nonviral episomes for the efficient modification of dividing cells.* Gene Therapy and Molecular Biology, 2006. **10**: p. 233-244.
38. Jechlinger, W., et al., *Minicircle DNA immobilized in bacterial ghosts: In vivo production of safe nonviral DNA delivery vehicles.* Journal of Molecular Microbiology and Biotechnology, 2004. **8**: p. 222-231.
39. Vile, R.G. and I.R. Hart, *In vitro and in vivo targeting of gene expression to melanoma cells.* Cancer Research, 1993. **53**: p. 962-967.
40. Robson, T. and D.G. Hirst, *Transcritpional targeting in cancer gene therapy* Journal of Biomedicine and Biotechnology, 2003. **2**: p. 110-137.
41. Kuriyama, S., et al., *Complete cure of established murine hepatocellular carcinoma is achieved by repeated injection of retroviruses carrying the herpes simplex virus thymidine kinase gene.* Gene Therapy, 1999. **6**: p. 525-533.
42. Ido, A., et al., *Gene therapy for hepatoma cells using a retrovirus vector carrying herpes simplex virus thymidine kinase gene under the control of human alpha-fetoprotein gene promoter* Cancer Research, 1995. **55**: p. 3105-3109.
43. Liu, R.-S., et al., *Specific activation of sodium iodide symporter gene in hepatoma using alpha-fetoprotein promoter combined with hepatitis B virus enhancer (EIIAPA).* Anticancer Research, 2009. **29**: p. 211-222.
44. Jin, Y.N., et al., *Radioiodine gene therapy of hepatocellular carcinoma targeted human alpha fetoprotein.* Cancer Biotherapy and Radiopharmaceuticals, 2008. **23**(5): p. 551-560.
45. Ma, X.-J., R. Huang, and A.-R. Kuang, *AFP promoter enhancer increased specific expression of the human sodium iodide symporter (hNIS) for targeted radioiodine therapy of hepatocellular carcinoma.* Cancer Investigation, 2009. **27**(6): p. 673-681.
46. Hirano, T., et al., *HVJ-liposome-mediated transfection of HSVtk gene driven by AFP promoter inhibits hepatic tumor growth of hepatocellular carcinoma in SCID mice.* Gene Therapy, 2001. **8**: p. 80-83.
47. Ido, A., et al., *Gene therapy targeting for hepatocellular carcinoma: Selective and enhanced suicide gene expression regulated by a hypoxia-inducible enhancer linked to a human alpha-fetoprotein promoter.* Cancer Research, 2001. **61**: p. 3016-3021.

48. Gerolami, R., et al., *Evaluation of HSVtk-gene therapy in a rat model of chemically induced hepatocellular carcinoma by intrahepatic artery routes.* Cancer Research, 2000. **60**: p. 993-1001.
49. Wills, K.N., et al., *Gene therapy for hepatocellular carcinoma: chemosensitivity conferred by adenovirus-mediated trasnfer of the HSV-1 thymidine kinase gene.* Cancer Gene Therapy, 1995. **2**(3): p. 191-197.
50. Mao, C.-Y., et al., *Combined use of chemotherapeutics and oncolytic adenovirus in treatment of AFP-expressing hepatocellular carcinoma.* Hepatobiliary Pancreat Dis Int, 2009. **8**(3): p. 282-287.
51. Tokunaga, T., et al., *Antitumor activity of deoxyribonucleic acid fraction from Mycobacterium bovis BCG.I. isolation, physicochemical characterisation and antitumor activity.* J. Natl. Cancer Inst., 1984. **72**(4): p. 955-962.
52. Mashiba, H., et al., *In vitro augmentation of natural killer activity of peripheral blood cells from cancer patients by a DNA fraction from Mycobacterium bovis BCG.* Jpn. J. Med. Sci. Bio., 1988. **41**(5-6): p. 197-202.
53. Yamamoto, S., et al., *In vitro augmentation of natural killer cell activity and production of interferon-alpha/beta and -gamma with deoxyribonucleic acid fraction from Mycobacterium bovis BCG.* Jpn. J. Cancer Res. , 1988. **79**: p. 886-873.
54. Messina, J.P., G.S. Gilkeson, and D.S. Pisetsky, *Stimulation of in vitro murine lymphocyte proliferation by bacterial DNA.* The Journal of Immunology, 1991. **147**: p. 1759-1764.
55. Yamamoto, S., et al., *DNA from bacteria, but not from vertebrate, induces interferons, activates natural killer cells and inhibits tumour growth.* Microbiol. Immunol., 1992. **36**(9): p. 983-987.
56. Krieg, A.M., et al., *CpG motifs in bacterial DNA trigger direct B-cell activation.* Nature, 1995. **374**: p. 546-549.
57. Bird, A.P., *CpG-rich islands and the function of DNA methylation.* Nature, 1986. **321**: p. 209-213.
58. Ballas, Z.K., W.L. Rasmussen, and A.M. Krieg, *Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA* The Journal of Immunology, 1996. **157**: p. 1840-1845.
59. Hartmann, G., G.J. Weiner, and A.M. Krieg, *CpG DNA: A potent signal for growth, activation and maturation of human dendritic cells.* Proc. Natl. Acad. Sci. USA, 1999. **96**: p. 9305-9310.
60. Stacy, K.J., M.J. Sweet, and D.A. Hume, *Macrophages ingest and are activated by bacterial DNA.* The Journal of Immunology, 1996. **157**: p. 2116-2122.
61. Akira, S., K. Takeda, and T. Kaisho, *Toll-like receptors: critical proteins linking innate and aqcuired immunity.* Nature, 2001. **2**(8): p. 675-680.
62. Akira, S. and K. Takeda, *Toll-like receptor signalling.* Nature Reviews Immunology, 2004. **4**: p. 499-511.
63. Hemmi, H., et al., *A toll-like receptor recognises bacterial DNA.* Nature, 2000. **408**(740-745).
64. Latz, E., et al., *TLR9 signals after translocating from the ER to CpG DNA in the lysosome.* Nature Immunology, 2004. **5**(2): p. 190-198.

65. Kumagai, Y., O. Takeuchi, and S. Akira, *TLR9 as a key receptor for the recognition of DNA☆.* Advanced Drug Delivery Reviews, 2008. **60**(7): p. 795-804.
66. McCluskie, M.J., R.D. Weeratna, and H.L. Davis, *The role of CpG in DNA vaccines.* Springer Seminars in Immunopathology, 2000. **25**: p. 125-132.
67. Wooldridge, J.D. and G.J. Weiner, *CpG DNA and cancer immunotherapy.* Current Opinion in Oncology, 2003. **15**(6): p. 440-445.
68. Hodges, B., et al., *Long-term Transgene Expression from Plasmid DNA Gene Therapy Vectors Is Negatively Affected by CpG Dinucleotides.* Molecular Therapy, 2004. **10**(2): p. 269-278.
69. Hyde, S.C., et al., *CpG-free plasmids confer reduced inflammation and sustained pulmonary gene expression.* Nature Biotechnology, 2008. **26**(5): p. 549-551.
70. Yew, N.S., et al., *Reduced Inflammatory Response to Plasmid DNA Vectors by Elimination and Inhibition of Immunostimulatory CpG Motifs.* Molecular Therapy, 2000. **1**(3): p. 255-262.
71. Mitsui, M., et al., *Effect of the content of unmethylated CpG dinucleotides in plasmid DNA on the sustainability of transgene expression.* The Journal of Gene Medicine, 2009. **11**(5): p. 435-443.
72. Yew, N.S., et al., *CpG-Depleted Plasmid DNA Vectors with Enhanced Safety and Long-Term Gene Expression in Vivo.* Molecular Therapy, 2002. **5**(6): p. 731-738.
73. Chen, D., et al., *Adaptive and innate immune responses to gene transfer vectors: role of cytokines and chemokines in vector function.* Gene Therapy, 2003. **10**(11): p. 991-998.
74. Pascuini, S., et al., *The effect of CpG sequences on the B cell response to a viral glycoprotein encoded by a plasmid vector.* Gene Therapy, 1999. **6**: p. 1448-1455.
75. Reyes-Sandoval, A. and H.C.J. Ertl, *CpG Methylation of a Plasmid Vector Results in Extended Transgene Product Expression by Circumventing Induction of Immune Responses.* Molecular Therapy, 2004. **9**(2): p. 249-261.
76. Harms, J.S. and G.A. Splitter, *Interferon-γ inhibits transgene expression driven by SV40 or CMV promoters but augments expression driven by the mammalian MHC I promoter.* Human Gene Therapy, 1995. **6**: p. 1291-1297.
77. Qin, L., et al., *Promoter attenuation in gene therapy: Interferon-gamma and tumor necrosis factor-alpha inhibit transgene expression.* Human Gene Therapy, 1997. **8**: p. 2019-2029.
78. Wai Yan Lui, V., L. Domenic Falo Jr, and L. Huang, *Systemic production of IL-12 by naked DNA mediated gene transfer: toxicity and attenuation of transgene expressionin vivo.* The Journal of Gene Medicine, 2001. **3**(4): p. 384-393.
79. Ritter, T., et al., *Stimulatory and inhibitory actions of cytokines on the regulation of hCMV-IE promoter activity in human endothelial cells.* Cytokine, 2000. **12**(8): p. 1163-1170.
80. Gribaudo, G., et al., *Interferons inhibit onset of murine cytomegalovirus immediate-early gene transcription.* Virology, 1993. **197**: p. 303-311.

81. Guidotti, L.G., et al., *Intracellular inactivation of the hepatitis B virus by cototoxic T lymphocytes.* Immunity, 1996. **4**(25-36).
82. Newell-Price, J., A.J.L. Clark, and P. King, *DNA methylation and silencing of gene expression.* TEM, 2000. **11**(4): p. 142-148.
83. Bird, A.P. and A.P.-. Wolfe, *Methylation-induced repression -belts, braces and chromatin.* Cell, 1999. **99**: p. 451-454.
84. Bird, A.P., *DNA methylation patterns and epigenetic memory* genes & Development 2002 **16**: p. 6-12.
85. Heard, E., P. Clerc, and P. Avner, *<B121) x-chromosome inactivationin mammals, Heard,1997.pdf>.* Annual Review of Genetics 1997. **31**: p. 571-610.
86. Feil, R. and S. Khosla, *Genomic imprinting in mammals: an interplay between chromatin and DNA methylation* TIG, 1999. **15**(11): p. 431-435.
87. Li, E., C. Beard, and R. Jaenisch, *Role for DNA methylation in genomic imprinting.* Nature, 1993. **366**: p. 362-365.
88. Yoder, J.A., C.P. Walsch, and T.H. Bestor, *Cytosine methylation and the ecology of intragenomic parasites.* TIG, 1997. **13**(8): p. 335-340.
89. Jones, P.A. and P.W. Laird, *Cancer epigenetics comes of age.* Nature, 1999. **21**: p. 163-167.
90. Rashid, A. and J.P.J. Isa, *CpG island methylation in gastroenterologic neoplasia: A maturing field.* Gastroenterology, 2004. **127**: p. 1578-1588.
91. Herman, J.G. and S.B. Baylin, *Gene silencing in cancer in association with promoter hypermethylation.* The New England Journal of Medicine, 2003. **349**: p. 2042-2054.
92. Tate, P.H. and A.P. Bird, *Effects of DNA methylation on DNA-binding proteins and gene expression.* Current Opinion in Genetics and Development, 1993. **3**: p. 226-231.
93. Kass, S.U., N. Landsberger, and A.P. Wolffe, *DNA methylation directs a time-dependent repression of transcription initation.* Current Biology, 1997. **7**(3): p. 157-165.
94. Nan, X., et al., *Trasncriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex* Nature, 1998. **393**: p. 386-389.
95. Jones, P.L., et al., *Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription.* Nature, 1998. **19** p. 187-191.
96. Kass, S.U., J.P. Goddard, and R.L. Adams, *Inactive chromatin spreads from a focus of methylation.* Molecular and Cellular Biology, 1993. **13**(12): p. 7372-7379.
97. Hsieh, C.-L., *Stability of path methylation and its impact in in regons of trasncriptional initiation and elongation* Molecular and Cellular Biology, 1997. **17**(10): p. 5897-5904.
98. Curradi, M., et al., *Molecular mechanism of gene silencing mediated by DNA methylation.* Molecular and Cellular Biology, 2002. **22**(9): p. 3157-3173.
99. Boyes, J. and A. Bird, *Repression of genes by DNA methylation depends on CpG density and promoter strength: evidence for envolvement of a methyl-CpG binding protein.* EMBO, 1992. **11**(1): p. 327-333.

100. Yew, N.S. and S.H. Cheng, *Reducing the immunostimulatory activity of CpG-containing plasmid DNA vectors for non-viral gene therapy.* Expert Opinion Drug Delivery, 2004. **1**(1): p. 115-125.
101. Davison, J., *Mechanism of control of DNA replication and incompability in ColE1-type plasmids - a review.* Gene, 1984. **28**: p. 1-15.
102. Wu, F., I. Levchenko, and M. Filutowicz, *A DNA segment conferring stable maintanance of R6K gamma-origin core replicons.* Journal of Baceriology, 1995. **177**(22): p. 6338-6345.
103. Molnar, M.J., et al., *Factors influencing the efficacy, longevity and safety of electroporation-assisted plasmid-based gene transfer into mouse muscles.* Molecular Theraphy, 2004 **10**(3): p. 447-455.
104. Zhang, G., et al., *Intraarterial delivery of naked plasmid DNA expressing full-lenght mouse dystrophin in the mdx mouse model of Duchenne muscular dystrophy.* Human Gene Therapy, 2004. **15**: p. 770-782.
105. Hofman, C., et al., *Efficient in vivo gene transfer with PCR amplified fragment with reduced inflammatory activity.* Gene Therapy, 2001. **8**: p. 71-74.
106. Chen, Z.-Y., et al., *Linear DNAs concatemirize in vivo and result in sustained transgene expression in mouse liver.* Molecular Theraphy, 2001. **3**(3): p. 2001.
107. Jenke, A.C.W., et al., *Nuclear scaffold/attached region modules linked to a transcription unit are sufficient for replication and maintenance of a mammalian episome* PNAS, 2004. **101**(31): p. 11322-11327.
108. Piechaszek, C., et al., *A vector based on the SV40 origin of replication and chromosomal S/MARs replicates episomally in CHO cells.* Nucleic acids research, 1999. **27**(2): p. 426-428.
109. Berezney, R. and K.W. Jeon, *Nuclear Matrix: Structural and functional organization*. 1995: Elsevier. 471.
110. Baiker, A., et al., *Mitotic stability of an episomal vector containing a human scaffold/matrix attached region is provided by association with nuclear matrix.* nature Cell Biology, 2000. **2**: p. 182-184.
111. Jenke, B.H.C., et al., *An episomal replicating vector binds to the nuclear matrix protein SAF-A in vivo.* EMBO, 2002. **3**(4): p. 349-354.
112. Rabinovich, B.A., et al., *Visualizing fewer than 10 mouse T cells with an enhanced firefly luciferasein immunecompetent mouse models of cancer.* PNAS, 2008. **105**(38): p. 14342-14346.
113. Haase, R., et al., *pEPito: a significantly improved non-viral episomal expression vector for mammalian cells.* BMC Biotechnology, 2010. **10**(20).
114. Reilly, C.R., et al., *Heparin prevents vascular smooth muscle cell progression through the G1 phase of the cell cycle.* The Journal of Biological Chemistry, 1989. **264**(12): p. 6990-6995.
115. Antonio, J.S., et al., *Isolarion of heparin-insensitive smooth muscle cells. Growth and differentiation.* Arteriosclerosis, theombosis and vascular biology, 1993. **13**: p. 748-757.
116. Chen, Z., et al., *Silencing of episomal transgene expression by plasmid bacterial DNA elements in vivo.* Gene Therapy, 2004. **11**: p. 856-864.
117. Mayrhofer, P., et al., *Minicircle-DNA production by site-specific recombination and protein-DNA interaction chromatography.* The Journal of Gene Medicine, 2008. **10**: p. 1253-1269.

118. Kobayashi, N., et al., *Hydrodynamics-based procedure involves transient hyperpermeability in the hepatic cellular membrane: implication of a nonspecific process in efficient intracellular gene delivery* The Journal of Gene Medicine, 2004. **6**(584-592).
119. Steer, C.J., *Liver regeneration.* FASEB, 1995. **9**(14): p. 13961400.
120. Angulo, A., et al., *Enhancer requirements for murine cytomegalovirus growth and genetic complementation by the human cytomegalovirus enhancer.* Journal of Virology, 1998. **72**(11): p. 8502-8509.
121. Serfling, E., M. Jasin, and W. Schaffner, *Enhancers and eukaryotic transcription.* TIG, 1985: p. 224-230.
122. Pirrotta, V., *Transvection and long-distance gene regulation.* Bioessays, 1990. **12**(9): p. 409-414.
123. Carey, M., *The enhanceosome and transcriptional synergy.* Cell, 1998. **92**: p. 5-8.
124. McALpine, D.M., H.K. Rodriguez, and S.P. Bell, *Coordination of replication and transcription along a Drosophila chromosome.* Genes & Development, 2004. **18**: p. 3094-3105.
125. Goldman, M.A., et al., *Replication timing of genes and middle repetitive sequences.* Science, 1984. **224**: p. 686-692.
126. Schübeler, D., et al., *Genome-wide DNA replication profile for Drosophila melanogaster: a link between transcription and replication timing* Nature Genetics, 2002. **32**: p. 438-442.
127. Iborra, F.J., et al., *Active RNA polymerases are localized within discrete transcription "factories" in human nuclei.* Journal of Cell Science, 1996. **109**: p. 1427-1436.
128. Mitchell, J.A. and P. Fraser, *Transcription factories are nuclear subcompartments that remain in the absence of transcription.* Genes & Development, 2008. **22**: p. 20-25.
129. Sadoni, N., et al., *Stable chromosomal units determines the spatial and temporal organization of DNA replication.* Journal of Cell Science, 2004. **117**(22): p. 5353-5365.
130. Cardoso, M.C., et al., *Mapping and use of sequence that targets DNA ligase I to sites of DNA replication in vivo* Journal of Cell Biology, 1997. **139**(3): p. 579-587.
131. Nakayasu, H. and R. Berezney, *Mapping replicational sites in the eucaryotic cell nucleus* The Journal of Cell Biology, 1989. **108**: p. 1-11.
132. Nakamura, H., T. Morita, and C. Sato, *Structural organization of replicon domains during DNA synthetic phase in the mammalian nucleus.* Experimental Cell Research, 1986. **165**: p. 291-297.
133. Mills, A.D., et al., *Replication occurs at discrete foci spaced thoughout nuclei replicating in vivo.* The Journal of Cell Science, 1989. **94**: p. 471-477.
134. Zink, D., et al., *Transcription-dependent spatial arrangements of CFTR and adjacent genes in human cell nuclei* The Journal of Cell Biology, 2004. **166**(6): p. 815-825.
135. Brown, K.E., et al., *Expression of α- and β-globin genes occurs within different nuclear domains in haemopoetic cells.* Nature Cell Biology, 2001. **3**: p. 602-606.

136. Chakalova, L., et al., *Replication and transcription: shaping the landscape of the genome.* Nature Reviews Genetics, 2005. **6**: p. 669-678.
137. Hassan, A.B., et al., *Replication and transcription sites are colocalized in human cells.* Journal of Cell Science, 1994. **107**: p. 425-434.
138. Wei, X., *Segregation of transcription and replicatin sites into higher order domains.* Science, 1998 **281**: p. 1502-1505.
139. Cook, P.R., *Nuclear Structure: Enhanced: Duplicating a tangled genome.* Science, 1998. **281**(5382): p. 1466-1467.
140. Berezney, R. and X. Wei, *The new paradigm: Integrating genomc function and nuclear architecture.* Journal of Cellular Biochemistry Supplements, 1998. **30**(31): p. 238-242.
141. Park, B., J., et al., *Augmentation of melanoma-specific gene expression using a tandem melanocyte-specific enhancer results in increased cytotoxicity of the purine nucleoside phosphorylase gene in melanoma.* Human Gene Therapy, 1999. **10**: p. 889-898.
142. Schaffner, G., et al., *Redundancy of information in enhancers as a principle of mammalian transcription control.* Journal of Molecular Biology 1988. **201**: p. 81-90.
143. Hamamoto, H., et al., *Tandem repeat of a transcriptional enhancer upstream of the sterol 14-α demethylase gene (CYP51) in Penicillum digitatum.* Applied and Environmental Microbiology, 2000. **66**(8): p. 3421-3426.
144. Agalioti, T., et al., *Ordered recruitment of of chromatin modifiying and general transcription factors to the IFN-β promoter.* Cell, 2000. **103**: p. 667-678.
145. Munshi, N., et al., *Coordination of a trasncriptional switch by HMGI(Y) acetylation.* Science, 2001. **293**: p. 1133-1136.
146. Panne, D., T. Maniatis, and S.C. Harrison, *An Atomic Model of the Interferon-β Enhanceosome.* Cell, 2007. **129**(6): p. 1111-1123.
147. Edelstein, L.C., et al., *NF- B-Dependent Assembly of an Enhanceosome-Like Complex on the Promoter Region of Apoptosis Inhibitor Bfl-1/A1.* Molecular and Cellular Biology, 2003. **23**(8): p. 2749-2761.
148. Barthel, R., et al., *Regulation of Tumor Necrosis Factor Alpha Gene Expression by Mycobacteria Involves the Assembly of a Unique Enhanceosome Dependent on the Coactivator Proteins CBP/p300.* Molecular and Cellular Biology, 2003. **23**(2): p. 526-533.
149. Giese, K., et al., *Assembly and function of a TCR alpha enhancer complex is dependent on LEF-1-induced DNA bending and multiple protein-protein interactions.* Genes & Development, 1995. **9**(8): p. 995-1008.
150. Hunninghake, G.W., et al., *The promoter regulatory region of the major immediate-early gene of human cytomegalovirus responds to T lymphocyte stimulation and contains functional cyclic AMP-response elements.* Journal of Virology, 1989. **63**(7): p. 3026-3033.
151. Davis, H.L., Brazolot Millan CL, and S. Watkins, *Immune-mediated destruction of transfected muscle fibers after direct gene transfer with antigen-expressing plasmid DNA.* Gene Therapy, 1997. **4**: p. 181-188.
152. Davis, H.L., et al., *DNA-mediated immunization in mice induces a potent MHC class I-restricted T lymphocyte response to the hepatitis B envelope protein.* Human Gene Therapy, 1995. **6**: p. 1447-1456.

153. Ross, D.T., et al., *Systemic variation in gene expression patterns in human cancer cell lines.* Nature, 2000. **24**: p. 227-235.

154. Ellison, G., *Further evidence to support the melanocytic origin of MDA-MB-435.* Molecular Pathology, 2002. **55**(5): p. 294-299.

155. Sellappan, S., et al., *Lineage infidelity of MDA-MB-435 cells: Expression of melanocyte protein in a brest cancer cell line.* Cancer Research, 2004. **64**: p. 3479-3485.

156. Nakshatri, H., et al., *Constitutive activation of NFκB during progression of breast cancer to hormone-independent growth.* Molecular and Cellular Biology, 1997. **17**(7): p. 3629-3639.

157. Eickhoff, J.E., *NF- B activation can mediate inhibition of human cytomegalovirus replication.* Journal of General Virology, 2005. **86**(2): p. 285-295.

158. Yurochko, A.D., et al., *The human cytomegalovirusUL55 (gB) and UL75 (gH) glycoprot ligands initiate the rapid activation of Sp1 and NF-κB during infection.* Journal of Virology, 1997. **71**(7): p. 5051-5059.

159. Yurochko, A.D., et al., *Human cytomegalovirus upregulates NF-κB activity by transactivating the NF-κB p105/50 and p65 promoters.* Journal of Virology, 1995. **69**(9): p. 5391-5400.

160. Yurochko, A.D., et al., *Induction of the trasncription factor Sp-1 during human cytomegalovirus infection mediated upregulation of the p65 and p105/50 NF-κB promoters.* Journal of Virology, 1997. **71**(6): p. 4638-4648.

161. Sambucetti, L.C., et al., *NF-κB activation of the cytomegalovirus enhancer is mediated by a viral transactivator and by T cell stimulation.* EMBO, 1989. **8**(13): p. 4251-4258.

162. Löser, P., et al., *Reactivation of the previously silenced cytomegalovirus major immediate-early promoter in the mouse liver: involvement of NF-κB.* Journal of Virology, 1998. **72**(1): p. 180-190.

163. Wolf, J.A., et al., *Long-term persistance of plasmid DNA and foreign gene expression in mouse muscle.* Human Molecular Genetics, 1992. **1**(6): p. 363-369.

164. Garg, S., et al., *The hybrid cytomegalovirus enhancer/chicken (beta)-actin promoter along with woodchuck hepatitis virus porttranscriptional regulatory elements enhances the protective efficacy of DNA vaccine* Journal of Immunology, 2004. **173**: p. 550-558.

165. Labarriere, N., et al., *Optimal T cell activation of melanoma cells depends on minimal level of antigen transcription.* The Journal of Immunology, 1997. **158**(3): p. 1238-1245.

166. Rawson, P., et al., *Immunotherapy with dendritic cells and tumor major histocompatibility complex class I-derived peptides requires a high density of antigen on tumour cells.* Cancer Research, 2000. **60**: p. 4493-4498.

167. Yew, N., et al., *High and sustained transgene expression in vivo from plasmid vectrors containing a hybrid ubiquitin promoter.* Molecular Therapy, 2001. **4**(1): p. 75-82.

168. Russ, V., et al., *Novel degradable oligoethylenimine acrylate ester-based pseudodendrimeres for in vitro and in vivo gene transfer.* Gene Therapy, 2008. **15**: p. 18-29

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# 9. Curriculum vitae

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