

## Gene Switches for Deliberate Regulation of Transgene Expression: Recent Advances in System Development and Uses

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### Abstract

It will be desirable in genetic therapies to exert temporal and/or spatial control over the expression of therapeutic genes. Temporal regulation may be achieved by placing the gene of interest under the control of one of several well-known gene switches. These gene switches comprise at least two elements, i.e., a ligand-dependent transactivator (or transinhibitor) and a transactivator responsive promoter that is functionally linked to the gene of interest. The systems respond to doxycycline, insect steroid hormones, steroid hormone antagonists, rapalogs, or various non-natural ligands. Deliberate spatial regulation may be attained by the use of promoters that are activated by physical forces which can be directed to an area in need of therapy. Best known are heat shock promoters and radiation-induced promoters. More complex gene switches are capable of both spatially and temporally regulating genes of interest. The present article reviews improvements and further refinements of the latter gene switches as well as exemplifies uses of these gene switches in research and experimental therapy. It further reports on more recently developed gene switches, including complex systems involving multiple transactivators (or transinhibitors) and/or regulating gene product functionality at the transcriptional, posttranscriptional and/or activity level.

### Introduction

Gene switches for deliberate regulation of transgenes typically comprise a transactivator or transinhibitor whose activity can be regulated and a transactivator-responsive or transinhibitor-susceptible promoter for controlling a gene of interest. The transactivators or transinhibitors typically are ligand-responsive, chimeric proteins comprising a DNA-binding domain, a ligand binding domain and a transcriptional activation domain or inhibition domain, respectively. Well known gene switches are based on tetracycline-responsive transactivators and transinhibitors, mammalian or insect steroid receptor-derived transactivators and rapamycin-induced transactivators. Other gene switches make use of endogenous transcription factors that can be deliberately activated, e.g., by physical cues, and whose transient activation is tolerated by the cell. The best known systems of this kind make use of transcription factors that can be activated by heat or ionizing radiation.

It appears that gene switches were developed originally to regulate the timing, dosing and/or spatial definition of transgene expression in gene or cell therapy. Presumably, this development occurred in response to concerns that excessive concentrations of transgene therapeutic product could have adverse effects or that expression of certain therapeutics outside of the therapeutic time window or outside of a defined region in need of therapy may have unintended negative consequences. Many preclinical animal studies were performed with this aim in mind. However, gene switches were also employed in research, e.g., for studying gene function, facilitating expression of cytotoxic proteins *in vitro* and the like.

We have last reviewed developments in the gene switch field in 2009 [1]. The present review is intended as an update that focuses on recent improvements of gene switches developed earlier as well as on discussing novel gene switches that were described subsequent to our review. It further reports on recent developments aimed at moving gene switch-regulated therapies into the clinic, discusses results of clinical trials that were undertaken and summarizes research uses of gene switches.

The main emphasis of this article is on transcription regulation. However, the same principles that were used to produce ligand-regulated transactivators or transinhibitors also were employed increasingly to subject proteins to ligand regulation of their activity. Because a regulated biological effect or therapy may be provided either by regulating the amount or the activity of an effector protein, a number of examples for ligand regulation at the effector protein level are also described.

### Small Molecule-Dependent Gene Switches

#### Gene switches comprising tetracycline repressor-derived transactivators and transinhibitors

The *E.coli* Tn10 tetracycline resistance operon consists of a tetracycline repressor protein (TetR) and a specific DNA-binding site, the tetracycline operator (TetO, also referred to as TRE). TetR binds to TetO. Tetracycline or derivatives such as doxycycline bind to TetR, causing a reversible conformational change which results in dissociation of TetR from TetO. Several different tetracycline-responsive regulatory systems were developed. In the so-called TetOff systems target gene expression is on in the absence of tetracycline and is inactivated by the addition of the ligand (Figure 1A). In the so-called TetOn systems the target gene is off in the absence of ligand and is activated upon

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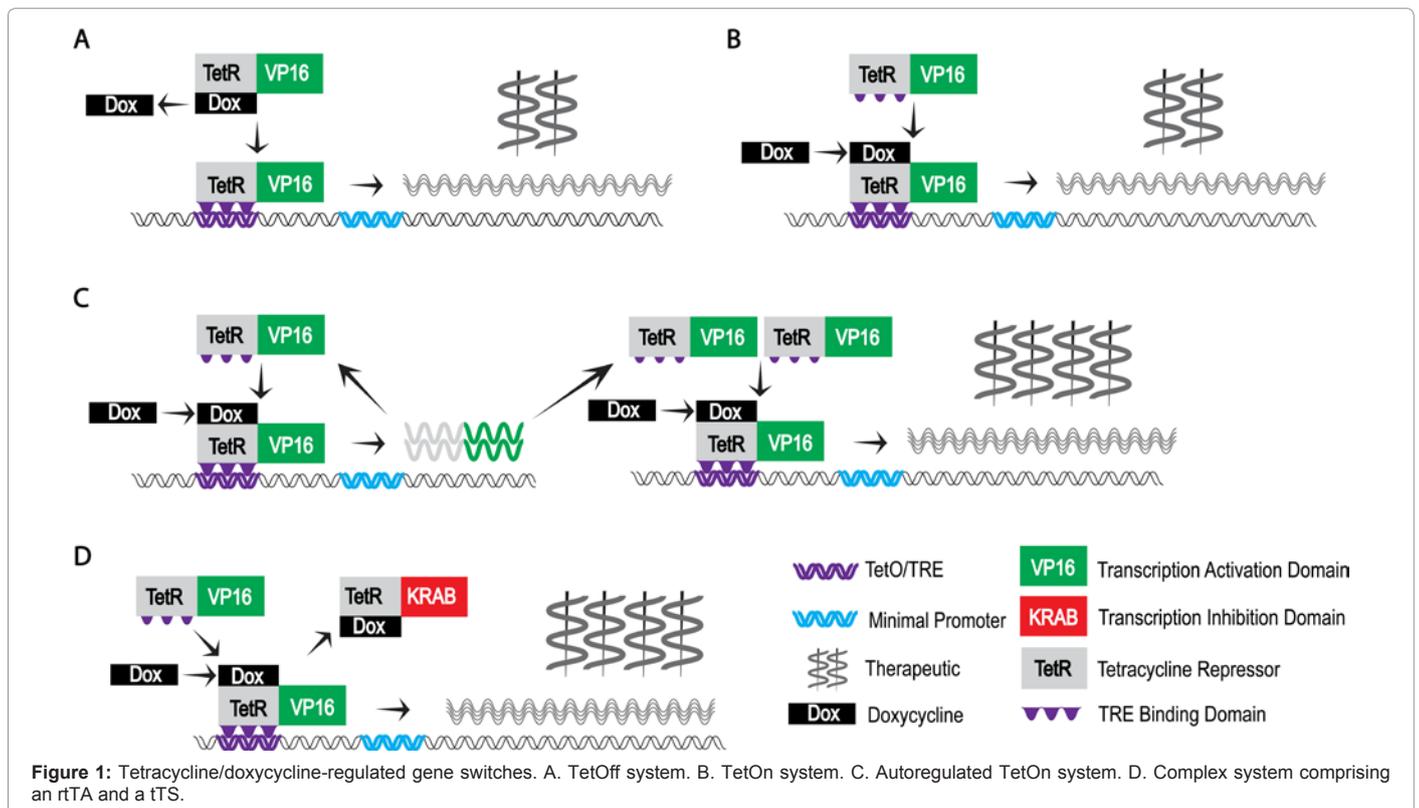
its addition (Figure 1B). The original TetOff system consisted of a chimeric transactivator composed of TetR and an activation domain from herpes simplex virus protein VP16 (tTA), and a minimal CMV promoter supplemented with seven TRE sequences for driving a gene of interest. The typical TetOn systems are based on mutants of TetR whose TRE-binding ability is induced rather than inhibited by tetracycline. The systems include a transactivator assembled from mutant TetR and VP16 (rtTA) and a responsive promoter to which a gene of interest is linked. The original TetOn system was somewhat leaky, i.e., target gene expression also occurred at some appreciable rate in the absence of tetracycline. Over the years transactivators were improved to suppress this background activity. A much used second generation transactivator has been rtTA2s-M2. In a further strategy for reducing leakiness, expression of rtTA was no longer mediated by a constitutively active promoter but a transactivator-responsive promoter (Figure 1C). Hence, in the absence of tetracycline, transactivator levels are low. Addition of tetracycline activates available transactivators, which then enhance their own expression (a feature referred to below as “auto-activation”) as well as that of the gene of interest. Alternative TetOn systems were produced that made use of the transrepressor domains (KRAB) of human zinc finger protein Kox1 or rodent zinc finger protein Kid-1. These transrepressor domains are known to inhibit all RNA polymerases II and III within a range of about 3 kb. The transinhibitors of these systems comprise TetR and a KRAB domain of Kox-1 or Kid-1 (referred to as tTS or tTS<sup>Kid</sup>, respectively), and an active promoter supplemented with TRE sequences. In attempts to further reduce background activity, the two types of TetOn systems were combined. Hence, combined systems consist of an rtTA, a tTS and a transactivator-responsive promoter for controlling transcription of a gene of interest (Figure 1D). In the absence of tetracycline, tTS binds to the promoter of the gene of interest, further suppressing background expression. In the presence of tetracycline, tTS is inactivated and rtTA

is activated. Consequently, tTS is replaced by rtTA on the promoter, resulting in induced transcription from the promoter. For additional information, the reader may also consult the 2009 review article by Stieger and colleagues [2].

### System development and improvements

In a recent development, an auto-activated TetOn system was adapted for liver-specific gene therapy [3]. A bidirectional promoter unit was constructed comprising two albumin promoters in a head-head configuration separated by a sequence element containing 7 TetO sites. To one of the albumin promoters was functionally joined an rtTA-M2 gene [4] and to the other was linked a gene of interest. The unit was delivered by means of an AAV8 viral vector. Experiments with a luciferase reporter gene as the gene of interest showed >100 fold inducibility, which was maintained over multiple cycles of deactivation and reactivation. Expression was essentially confined to liver upon intravenous administration of the virus in a mouse model. When using an IL-12 gene as the regulated gene, the viral system was highly effective against hepatic tumors in a preventative as well as a therapeutic setting. Notably, mice “cured” of tumor developed an efficient T-cell memory response to tumor cells.

Leakiness was dramatically reduced in new “all-in-one” retroviral and transposon vectors that exhibited dynamic ranges (induced to uninduced expression of target gene) of up to 25000 fold [5]. “All-in-one” vectors refer to vectors that comprise genes for both a transactivator and a regulated gene. The preferred vectors contained a constitutively expressed rtTA-M2 gene and a transactivator-responsive reporter gene. The increases in dynamic range were primarily achieved through the development of improved transactivator-responsive promoters that allowed for nearly as strong doxycycline-induced expression but far lower background activity compared to the standard tetracycline repressor-responsive promoter [6].



It may be desirable to irreversibly terminate transgene expression subsequent to a therapeutic regimen. This may be achieved by the use of a tet-O HAC-based vector that allows for regulation of transgene persistence in growing cells [7]. Tet-O HAC is a human artificial chromosome containing TetO sites inserted in its alphoid DNA array that replicates as a single-copy episome in human cells [8]. Expression of transinhibitor tTS resulted in a rapid loss of kinetochore function and loss of Tet-O HAC. Tet-O HAC was converted into a gene delivery vector by introduction of a loxP cassette for transgene insertion. The new vector was tested following introduction of an EGFP transgene cassette by Cre-loxP recombination (tet-O-EGFP HAC). Expression of the transgene could be detected in cells containing tet-O-EGFP HAC, which exhibited a high level of stability (50% retention after 2 months). An alternative approach to irreversibly terminating transgene activity was based on nonviral replicon vector pEPI that comprises an SV40 origin of replication and a chromosomal scaffold/matrix attached region (S/MAR) [9]. The vector further contains a CMV promoter driving a gene of interest, e.g., an eGFP reporter gene. Replication of this vector in mammalian cells apparently depends on transcription from the CMV promoter that extends into the S/MAR region. Insertion of an rtTA expression cassette (in a tail-to-tail arrangement with the gene of interest) as well as replacement of the CMV promoter with a transactivator-responsive promoter resulted in vector pEPI-Tet On [10]. When tested in CHO cells or in mice, eGFP expression from pEPI-Tet On was enhanced several fold in the presence of doxycycline. In the absence of doxycycline, the vector was gradually lost from growing cells.

A pair of high-capacity adenovectors for glioblastoma therapy were constructed, one containing a constitutively expressed gene for HSV thymidine kinase (HC-Ad-TK), and the other constitutively expressed rtTA and tTS genes as well as a gene encoding fms-like tyrosine kinase ligand 3 (Flt3L) controlled by a TetO-containing promoter (HC-Ad-TetOn-Flt3L) [11; 12 for methods]. The latter vector was capable of expressing Flt3L in a doxycycline-inducible fashion. Delivery of the vector pair into intracranial glioblastoma multiforme resulted in tumor shrinkage and enhanced survival in a large fraction of animals. None of the sham-treated animals survived.

An improved lentiviral vector for regulated expression of transgenes in neurons comprised several elements that had previously been optimized [13]. A first transcription unit consisted of a luciferase gene (example transgene) controlled by improved tetracycline repressor-responsive promoter TRE-tight1 [14]. The latter promoter was derived from second-generation promoter TRE<sub>tight</sub> that contains a modified tetracycline response element consisting of seven repeats of a 36 bp sequence including the 19 bp TetO sequence (Clontech Laboratories). A third generation promoter with an even lower basal activity, P<sub>TRE3G</sub>, has also been made available by Clontech. Following an observation of Osti and colleagues [15], chicken chromatin insulator cHS4 was used to separate this transcription unit from the second transcription unit, which consisted of a CMV promoter-driven modified rtTA (V16) gene [16,17]. The resulting vector showed excellent doxycycline regulation of luciferase expression *in vitro* in HeLa cells and neurons, and *in vivo* in spinal neurons. De-activation and re-activation of luciferase expression could be demonstrated. More recently, other lentiviral vectors, termed “polyswitch lentivectors”, were described that also allow for tight doxycycline regulation of a gene of interest, contain acceptor sites for easy recombination cloning of a gene of interest and express living colors and a selectable marker [18].

Gene silencing through RNAi is an approach that makes it possible

to carry out loss-of-function studies in mammalian organisms. A recent study reports the development of a new generation of lentivectors called pINDUCER for inducible RNAi *in vivo* [19]. Basic versions of these vectors comprise two expression cassettes, one expressed constitutively from a ubiquitin C promoter and containing genes for rtTA3 and a reporter joined by an IRES, and the other controlled by a TRE-containing promoter containing a fusion gene consisting of sequences coding for turboRFP and a sequence encoding a short hairpin RNA (shRNA) of choice embedded in microRNA-30 (miR30) sequences. Transactivator rtTA3 is an improved version of rtTA2s-M2 harboring further mutations F86Y and A209T. rtTA3 has improved doxycycline sensitivity and activity [20,21]. pINDUCER vectors were shown to be highly inducible in models of epithelial cancer and mammary development. A lentiviral vector of the series was also found capable of delivering BIRC4 shRNA in a doxycycline-induced fashion to BIRC4-dependent human breast tumor xenografts in nude mice, causing a substantial diminution of tumor growth. The pINDUCER system should be useful for large-scale *in vivo* genetic screening in models of human epithelial cancers. See also ref. 22 for another study that described similar technology. In a recent study retroviral vectors expressing shRNAs in a tetracycline-inducible fashion were used in a genetic screen that identified PTPN12 tyrosine phosphatase as a tumor suppressor in triple-negative breast cancer [23].

An all-in-one plasmid-based vector for expressing proteins of interest in a tetracycline-inducible fashion in pancreatic beta cells of rodents comprised a rat insulin promoter driving the expression of a gene of interest as well as a tTS gene linked to the gene of interest via IRES [24]. A TRE<sub>tight</sub> sequence element was placed upstream from the insulin promoter. In the absence of tetracycline, residual transinhibitor was expected to bind to the TRE<sub>tight</sub> element, thereby inhibiting insulin promoter activity. In the presence of tetracycline, transinhibitor should be released, resulting in enhanced expression of the gene of interest and of transinhibitor. The system was tested in Min6 and  $\beta$ TC mouse pancreatic beta cell lines and was found capable of conferring tight tetracycline regulation on the gene of interest.

Immune responses against Tet regulatory elements had been detected subsequent to intramuscular administration of vectors expressing Tet regulators. To investigate whether similar immune responses were induced in the brain, TetOff AAV vectors expressing potential therapeutic transgenes were administered to rats by intrastriatal injection [25]. Transgene expression could be demonstrated. Addition of doxycycline to the drinking water depressed expression by about 10 fold. Using a newly developed ELISA, antibodies against tTA could not be detected 5 or 9 weeks, respectively, after vector introduction in the brain. Intradermal vector injection served as a positive control in these experiments. Hence, TetOff AAV vectors did not elicit immune responses to Tet regulators in the brain and may therefore be safe vectors for the therapy of brain diseases such as Parkinson's disease. These conclusions are compatible with those of an earlier study, in which expression from an intrastrially administered TetOn high-capacity adenovector was not affected by pre-existing immunity against rtTA [26]. It is noted that a similar absence of immune reactions against Tet regulators was observed in the eye [25,27].

A TetOn transgenic rat line was developed that expressed the rtTA-M2 transactivator from the ubiquitously active ROSA26 promoter [28]. Transactivator was detected in all major organs, including brain, heart, lung, liver, kidney and muscle. Doxycycline-inducible expression of an EGFP gene delivered by a lentivirus vector was observed in cells of testis, kidney and muscle tissues.

The transcriptomic effects of TetOn and mifepristone-inducible systems in the mouse liver in the presence and absence of ligand were examined [29]. Plasmids containing rtTA-M2 or GLp65 transactivator genes under the control of albumin promoters were introduced into the liver by hydrodynamic injection. The procedure resulted in transfection efficiencies of about 17%. The transactivator genes were expressed (one month after transfection) at a rate of about 1/6 of that of a liver reference gene (Mat1a), which is expressed at a medium-to-low level in hepatocytes. RNA from livers was prepared one month after transfection. Analysis on 430A 2.0 microarrays (Affimetrix) revealed that expression of rtTA-M2 resulted in alterations in 69 gene probe sets and that of GLp65 in 1059 gene probe sets. (The Affimetrix chip contains 22690 probe sets corresponding to about 14000 genes.) Few additional changes were observed with RNA from animals that were also exposed to doxycycline or mifepristone, respectively. Most of the expression changes detected were relatively modest (equal to or less than three fold). Functional assignments indicated that alterations were mild and of little general significance. Therefore, it appeared that the two gene switches have little impact on the liver transcriptome profile and are safe for gene therapy applications. The analysis supports a slight preference for TetOn systems over mifepristone-inducible systems.

Hematopoietic stem cells (CD34+) can be efficiently transduced by high-capacity adenovectors with serotype 35 fiber knob domains (HD-Ad5/35). A vector designed for doxycycline-induced expression of transgenes in CD34+ cells, Ad5/35.Tet-1, contains a bicistronic arrangement of a GFP-coding sequence (example transgene) and a sequence encoding a tTS [30]. Expression is driven by a constitutively active (phosphoglycerokinase gene) promoter. Coding sequences and promoter are flanked by elements containing multiple TetO sites, polyA addition sequences and chromatin insulator cHS4. Another vector, Ad5/35.Tet-2, expresses an rtTA transactivator instead of a tTS. Furthermore, expression of the GFP and rtTA genes is driven by an rtTA-responsive promoter, i.e., a minimal CMV promoter supplemented by TetO sites. Both vectors were capable of expressing GFP in an inducible fashion in primary human CD34+ cells. Ad5/35.Tet-1 supported a lower induced level of transgene expression than Ad5/35.Tet-2. However, Ad5/35.Tet-2 was only active in a subset of CD34+ cells, whereas transgene expression occurred in all CD34+ cells transduced with Ad5/35.Tet-1.

A plasmid vector designed to allow phiC31 recombinase-mediated integration of a doxycycline-activated gene switch and associated regulated gene into the genome of a host cell contained a gene of interest controlled by a CMV promoter susceptible to inactivation by TetR due to the presence of two TRE sites located between the TATA box and the transcription initiation site [31]. Downstream from the gene of interest was a BGH polyA sequence. This gene cassette was followed by two copies of a cassette consisting of a CAG promoter (a combination of the CMV early enhancer element and a chicken  $\beta$ -actin promoter), a TetR gene functionally linked to the promoter and an SV40 polyA sequence. All three genes had the same transcriptional orientation. To minimize promoter interference (especially after integration in a host cell), cassettes 1 and 2 were each followed by a cHS4 chromatin insulator sequence. Finally, the plasmid also contained an attB recognition site for phiC31. Stable cell lines containing the system were isolated and examined: stable and robust transgene expression and high induction rates upon doxycycline addition were observed.

The usefulness of regulated expression systems is often hampered by the difficulty of identifying clonal cell lines in which the regulation

of the transgene of interest is optimal. HeLa cell-derived lines were generated that carry an rtTA-M2 gene under the control of a human EF1alpha promoter and contain a locus retargetable by Flp-mediated cassette exchange [32]. Retargetable loci were selected for their ability to allow for maximal inducibility of an inserted luciferase reporter gene. The availability of these lines will facilitate a number of experiments that can be carried out in HeLa cells, e.g., gene knock-down experiments to examine gene function, etc.

Another study incorporated a tTA transactivator in a gene switch that is irreversibly triggered by a transiently activated receptor [33]. The switch should be useful as an alternative approach to measuring receptor activation that is likely to be more sensitive than assays that follow signaling events downstream from an activated receptor as well as should be largely independent from endogenous co-regulators. The assay, termed Tango assay, was developed for G protein-coupled receptors, receptor tyrosine kinases and steroid hormone receptors. As an example for a G protein-coupled receptor assay, a first construct was prepared that encoded a fusion protein consisting of a human arginine vasopressin receptor 2, a short sequence containing a cleavage site for viral proteinase TEV and a tTA transactivator. A second construct encoded an arrestin-TEV fusion protein. Upon co-introduction of these constructs into a cell containing a tTA-responsive reporter gene, ligand binding was expected to activate the fusion receptor and attract binding of the arrestin-TEV fusion protein. As a consequence, the tTA transactivator would be cleaved off the fusion protein and translocate to the nucleus to activate the reporter gene. In actual experiments, ligand exposure of cells containing the system resulted in a 200-fold increase in reporter activity.

### Tetracycline repressor-based systems controlling experimental cancer therapy

Human mammary tumor lines were stably transfected to express a gene encoding fusion protein CD8/caspase-8 under the control of a TetOn system [34]. Doxycycline-induced expression of the fusion protein caused apoptotic death of the cells. The cell lines were used to establish xenograft tumors in mice. Induction of apoptotic death created void spaces and channels in the tumors, which greatly facilitated distribution of oncolytic herpes virus throughout the tumor. This study outlines a new approach for enhancing the clinical effectiveness of oncolytic viruses.

To enhance the anti-cancer activity of a replicating adenovirus vector expressing IFN-alpha (KD3-IFN), a hepatocyte-derived tumor was co-infected with KD3-IFN and replication-defective adenovirus TetOn-TRAIL expressing tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) under the control of an rtTA [35]. Co-infection with TetOn-TRAIL was significantly more effective in slowing tumor growth in the presence of doxycycline than infection with KD3-IFN or TetOn-TRAIL alone. It is noted that ligand inducibility of the system was modest (tested by replacing TRAIL with a reporter gene): doxycycline increased expression by only about 5-10 fold.

Another study tested the concept of using a brain sodium channel (ASIC2a) containing a mutation that renders it constitutively opened for cancer therapy in the brain [36]. A HSV/EBV hybrid amplicon vector was constructed that contained expressible genes for rtTA-M2 transactivator, tTS<sup>Kid</sup> transinhibitor and the mutant channel, the gene for the mutant channel being controlled by an rtTA-responsive promoter. For delivery, the amplicon vector was packaged *in vitro* into HSV-1 virions. The system was successfully tested *in vitro* and *in vivo* in a subcutaneous mouse xenograft model of human glioma.

A TetOff system was used to time the expression of IL-4 in a study that tested the effect of IL-4 on myeloid-derived suppressor cell (MDSC) development at different stages of tumor growth [37]. MDSCs inhibit T cell activity and promote tumor growth. High-level expression of IL-4 early after subcutaneous administration of mouse fibrosarcoma cells prevented the generation of MDSCs and resulted in T cell-mediated tumor rejection. IL-4 was no longer effective, if first expressed after several days of tumor growth. For other cancer-related studies that made use of Tet regulators [38,39,40].

### **Tetracycline repressor-based systems controlling experimental therapy of neurologic disorders or neurodegenerative disease**

Striatal expression of glial cell line-derived neurotrophic factor GDNF was proposed as a potential treatment for Parkinson's disease. A recombinant AAV2/5 vector was constructed that featured a gene for transactivator tTA2 (a VP16 minimal domain transactivator, Clontech) under the control of a CAG promoter and a human GDNF cDNA gene controlled by a transactivator-responsive promoter [41]. The vector was delivered to rats by nigrostriatal injection. GDNF expression in the substantia nigra and in serum was stringently dependent on doxycycline dose. Striatal GDNF induced weight alterations. These alterations were used to demonstrate the doxycycline dependence of GDNF expression over several cycles of repression and derepression.

Spinocerebellar ataxia type 3 is caused by expansion of a CAG repeat in the MJD1 gene that encodes the ataxia-3 protein. A transgenic mouse line was established that harbored a full-length human ataxin-3 cDNA gene containing 77 CAG repeats functionally linked to a Tet regulator-responsive promoter [42]. This line was crossed with a line containing a PrP promoter-driven tTA transactivator gene (TetOff) to generate a double transgenic line capable of expression of the disease-causing protein under doxycycline control. The model was used to demonstrate that discontinuation of pathogenic ataxin-3 expression in an early disease state prevents the establishment of neuronal dysfunction characterized by reduced anxiety, hyperactivity, impaired Rotarod performance and lower body weight gain. These results suggest that early pharmacological intervention will be able to halt or at least slow disease progression.

Gene therapeutic safety is arguably enhanced by spatial restriction of therapeutic gene expression. The biodistribution of transgene expression in the CNS subsequent to stereotaxic injection of recombinant AAV2/1 vectors was compared [43]. The viral vectors comprised either a CMV promoter (the CMV vector) or a TetOn system (the TetOn vector) for regulating the activity of an EGFP gene. The latter vector included a bidirectional, TetO-containing promoter (derived from Clontech construct pBI-GL) driving an EGFP and an rtTA-M2 gene. To augment transactivator expression, the rtTA-M2-coding sequence was followed by a woodchuck hepatitis posttranscriptional regulator element sequence [44,45]. After intrastriatal injection (and doxycycline administration), EGFP was mainly expressed in neurons with both vectors. However, the two vectors differed in supporting EGFP expression in substantia nigra and the olfactory bulb, where expression was observed from the CMV vector but not the TetOn vector. The study [43] concluded that this represented a safety advantage of the TetOn vector in striatal gene therapy. Midbrain injection resulted in selective EGFP expression from the TetOn vector in tyrosine hydroxylase-positive neurons. Widespread midbrain expression of the marker gene was observed following administration of the CMV vector. Hence, the TetOn vector would appear to be better suited than the CMV vector for specific transgene expression in midbrain dopaminergic neurons.

### **Use of tetracycline repressor-based gene switches in other experimental therapies**

Aminoglycoside antibiotics are used frequently to treat serious infections, e.g., septicemia, complicated intra-abdominal or urinary tract infections and nosocomial respiratory tract infections. Such treatments are associated with ototoxicity, in which cochlea and vestibule are attacked, and auditory and vestibular hair cells are destroyed. This results in irreversible hearing deficits that can manifest themselves months after antibiotic treatment. Sustained infusion of recombinant GDNF was known to protect the cochlear structure and function from noise- and drug-induced damage, although GDNF overdosing has contrary effects. An AAV1 vector was constructed that included a constitutively expressed gene for transactivator rtTA2s-S2 [4] and a GDNF gene controlled by a transactivator-responsive promoter [46]. GDNF was synthesized in a strictly doxycycline-inducible fashion in vector-infected cells. Introduction of the vector in rat cochlea and subsequent induced expression of GDNF successfully protected cochlear structure and function against damage induced by a course of treatment with kanamycin.

A lentivirus vector was prepared that contained a constitutively expressed rtTA (presumably rtTA2s-M2) gene and an expression cassette containing a gene for keratinocyte growth factor (KGF; also known as FGF7) and an IRES-linked EGFP reporter gene controlled by a transactivator-responsive promoter [47]. Mesenchymal stromal and hematopoietic stem cells were transduced with this lentivector to yield cells capable of doxycycline-induced EGFP expression. Transplantation of lentivector-transduced hematopoietic stem cells attenuated histological damage in a mouse model of bleomycin-induced lung fibrosis.

An AAV2-based vector was developed comprising a basic fibroblast growth factor (bFGF) gene under TetOff control [48]. Vector-transduced mesenchymal stem cells could be shown to induce significant bone formation and angiogenesis in a rat calvarian defect model in the absence but not the presence of doxycycline. To develop a new modality of treatment of cartilage defects, a lentivirus was constructed comprising a gene for bone morphogenetic protein 2 (BMP-2) under the control of transactivator rtTA2s-M2 [49]. In the preferred vector, the BMP-2 gene was controlled by a PRE<sub>light</sub> promoter and the rtTA gene by a spleen focus-forming virus promoter. Stringent doxycycline inducibility of BMP-2 expression could be demonstrated in vector-transduced primary rabbit chondrocytes. Induced expression was sufficient to induce proteoglycan synthesis, a marker of chondrogenesis.

In diabetes type 1, macrophages and T lymphocytes release cytokines that induce the generation of oxygen and nitrogen radicals in pancreatic islets which promote death of insulin-producing beta cells. Expression of mitochondrial peroxiredoxin III in rat insulinoma cells, under the control of a TetOn system, protected these cells against hydrogen peroxide, proinflammatory cytokines and streptozotocin stress [50].

Leber congenital amaurosis is a rare disease caused by a nonfunctional retinal pigment epithelium-specific gene (RPE65). Preliminary results from three small clinical trials suggested that expression of an RPE65 transgene can result in an improved visual function. Because it may be important to correctly titrate the amount of RPE65 provided to the retina, AAV2 vectors were developed [27] that expressed RPE65 under the control of either the rtTA-2M or the tTA2 transactivator [4]. Transactivators were controlled by CAG promoters.

Induced expression of RPE65 from subretinally delivered AAV2 vectors in RPE65(-/-) Briard dogs resulted in a detectable recovery of retinal function, but no visual improvement. The retinal recovery was reversed upon cessation of RPE65 transgene expression. The study [27] argued that, while doxycycline-regulated RPE65 expression could be achieved, maximal levels of RPE65 produced by the fully active gene switches were below the level needed for the more substantial retinal recovery that is associated with improvement in visual function.

Two approaches were described that used doxycycline-regulated transgenes for opioids for the suppression of chronic pain. In the first approach, a three-plasmid system was assembled for achieving doxycycline-controlled expression of a proopiomelanocortin gene [51]. Proopiomelanocortin is a precursor protein that is processed in a tissue-specific fashion to yield different peptide hormones including beta-endorphin. The three plasmids were pTRE2 (Clontech) with an inserted proopiomelanocortin cDNA gene, pTet-On (Clontech) containing a CMV promoter-driven rtTA gene and pTet-tTS (Clontech) containing a CMV promoter-driven tTS<sup>Kid</sup> gene. pTet-On had previously been described as pUHD17-neo 1 [52]. The three plasmid combination was introduced into rat dorsal root ganglia by electroporation. Stringent doxycycline regulation of beta-endorphin expression was observed in the transduced cells. To determine the anti-nociceptive effects of beta-endorphin therapy, the rat chronic constrictive injury (CCI) model was used. The three plasmids were introduced intrathecally, and spinal chord cells were transduced by electroporation. Robust reductions in thermal withdrawal latency and mechanical withdrawal threshold were observed in transduced animals subjected to a doxycycline regimen, but not in animals that did not receive doxycycline. Inducible expression of beta-endorphin could be measured in the cerebrospinal fluid. The second approach involved a cell therapy for reducing chronic pain [53]. A human preproenkephalin cDNA gene was subcloned into pRevTRE (Clontech). This plasmid and pRevTet-on (Clontech) were packaged using packaging cell line PT-67. The resulting retroviruses were used to transduce an immortalized rat astrocyte cell line. The transduced cells were capable of expressing enkephalin in a doxycycline-induced fashion. The cells were introduced intrathecally into CCI rats, and changes in thermal and mechanical hyperalgesia were assessed. Substantial reduction of threshold values was observed. Reversible activation of enkephalin expression could also be demonstrated *in vivo*.

### Other uses of tetracycline-regulated systems

To answer the question whether cardiovascular precursor cells are more effective than vascular precursor cells in cardiovascular cell therapy, i.e., whether transplantation of the appropriate cell type is critical for obtaining maximally effective stem cell therapy, a murine stem cell line was established that expressed Notch 4 under the control of a TetOn system [54]. Expression of Notch 4 in Flk1+ hematopoietic and vascular progenitors of this line re-specified the cells to a cardiovascular fate [55]. Transplantation of doxycycline-exposed cells in a mouse model of surgically induced myocardial infarction resulted in a substantially greater mean ejection fraction than that produced by cells that had not been doxycycline-activated [54]. These results imply that cardiovascular precursors are more effective than vascular progenitors in improving cardiovascular function after myocardial infarction.

Regeneration of mouse beta cells was thought to be largely dependent on induction of proliferation of terminally differentiated beta cells, although pancreatic progenitor cells were known to exist around the duct, which cells may contribute to regeneration. To

answer the question whether non-beta cells can contribute to beta cell regeneration, a transgenic mouse model was established harboring an rtTA transactivator gene driven by a rat insulin II promoter and an rtTA-responsive p21 transgene [56]. Doxycycline-induced p21 expression inhibited proliferation of beta cells. Animals in which beta cell proliferation had been blocked showed better recovery from streptozotocin-induced diabetes than controls. Markers of early pancreatic development and pancreatic progenitors could be detected in islets of these mice. These results suggested that in the absence of beta cell proliferation, pancreatic progenitor cells contributed to recovery from induced diabetes. A more recent study achieved near-complete beta cell ablation by means of diphtheria toxin [57]. A large fraction of regenerated beta cells were found to be derived from glucagon-producing alpha cells. The approach chosen for lineage tracing involved the use of a TetOn system to inducibly label glucagon-producing alpha cells.

Mouse and human fibroblasts can be re-programmed to a pluripotent state by co-expression of transcription factors Oct4, Sox2, Klf4 and c-Myc. Four lentiviral vectors were prepared, each of which contained a gene for one of these transcription factors functionally linked to a CMV minimal promoter supplemented with TRE sites [58,59]. Mouse embryo fibroblasts were engineered to constitutively express transactivator rtTA-M2. Cells co-transduced with the four lentivectors could be induced by doxycycline to undergo reprogramming. Selected pluripotent cells were used to produce chimeric mice. Cells derived from these mice could be reprogrammed by doxycycline addition with an efficacy that was 25-50 times better than direct reprogramming through lentiviral transduction and selection for pluripotency markers. This system should be well suited for studies of reprogramming as well as for genetic or chemical screens for factors involved in or capable of modulating reprogramming. In another study, the same technology was employed for demonstrating that embryonic and postnatal mouse fibroblasts could be rapidly and efficiently converted to neuronal cells by expression of transcription factors Ascl1, Brn2 and Myt1l [60]. These findings are expected to have an important impact on studies of neural development, disease modeling and human regenerative medicine. With regard to their potential future application in regenerative medicine, there has been concern that any one of the many viral insertions required to convert fibroblasts to inducible pluripotent stem cells may result in unpredictable genetic dysfunction. To reduce this possible risk, different methodologies for reprogramming cells to a pluripotent state were investigated. In one such study, human embryonic fibroblasts were co-transfected with two piggyback transposons, one comprising a doxycycline-inducible expression unit encoding transcription factors Oct4, Sox2, Klf4 and c-Myc, and the other a CAG promoter-driven rtTA gene [61]. Clonal lines that displayed embryonic stem cell morphology and were positive for alkaline phosphatase were obtained.

Gene regulatory networks may be analyzed by systematically disturbing them by means of overexpression of a transcription factor and then examining the genomic consequences. One such study focused on 50 selected transcription factors [62]. Transcription factor genes were introduced by recombinase-mediated cassette exchange into mouse embryo fibroblasts containing a tetracycline-repressed gene expression system at the Rosa26 locus [63]. This system that is referred to as ROSA-TET locus comprises a tTA gene driven by the Rosa26 promoter and, flanked by loxP and loxPV sites, a hygromycin resistance gene controlled by a TRE-containing minimal CMV promoter. Cdx2 was identified as the factor that caused the most dramatic transcriptome perturbation. The cell lines generated will be a resource for future

studies of biological networks in embryo fibroblasts and mice.

To facilitate studies of kidney diseases, a transgenic mouse line was generated that expressed transactivator rtTA2s-2M under a Pax8 promoter, which directed transactivator expression to renal tubular cells [64]. Inducible kidney disease models were generated by crossing Pax8-rtTA mice with TetO-MYC mice (model of polycystic kidney disease) or TetO-Tgf-beta1 mice (renal fibrosis). Triple transgenic mice, Pax8-rtTA/LC-1/Tsc1<sup>lox/lox</sup>, when treated by doxycycline *in utero*, produced newborns with massive hyperproliferation in tubules and collecting duct epithelial cells, apparently as a consequence of the induced deletion of the tuberous sclerosis complex-1 (Tsc1) gene. These mice died a few weeks after birth with giant polycystic kidneys characteristic of autosomal recessive polycystic kidney disease. LC-1 mice contain a tetracycline-inducible Cre recombinase.

Sudden infant death syndrome (SIDS) is associated with robust morphological and biochemical deficits in serotonin neurons in the brain stem of SIDS brains, suggesting that a reduced serotonin function is a risk factor for the disease. Normal levels of serotonin neuron firing are maintained by a feedback mechanism involving serotonin autoreceptor Htr1a. A model for serotonin dysfunction was generated by crossing mice containing a Tet regulator-responsive endogenous Htr1a allele with mice expressing a TetOff transactivator under the control of the endogenous promoter of the serotonin transporter gene Slc6a4 [65]. In the absence but not in the presence of doxycycline, the double transgenic animals developed sporadic brachycardia and hypothermia, which frequently resulted in death. Interestingly, and in many ways reflective of the human condition, a time window could be defined (P25-80) within which the animals were particularly sensitive to Htr1a overexpression. This study suggests that a deficit in serotonin function is a sufficient cause for SIDS and should help to channel efforts to find a treatment for this condition.

Amplification of high-capacity adenovectors (HC-Ads) requires a helper virus (HV). Frequently, yields of HC-Ad are low, and preparations are contaminated with significant quantities of HV. Self-inactivating HV AdTetCre is an E1/E3-deleted Ad whose packaging signal is flanked by LoxP sites and that contains a constitutively expressed gene for transactivator rtTA-M2 as well as an rtTA-responsive gene for chimeric Cre-recombinase MerCreMer [66]. MerCreMer is a fusion of Cre-recombinase and two mutated estrogen receptor ligand-binding domains. The latter domains control the nuclear localization of the fusion protein. HV genome inactivation by removal of the packaging signal involves prior doxycycline-induced expression of chimeric Cre-recombinase which translocates into the nucleus in the presence of ligand 4-hydroxytamoxifen. This system was used to produce at high yield HC-Ad that was only minimally contaminated by HV.

Tet systems were also used to produce packaging cell lines for deleted viruses. HEK 293 cells expressing a TetOff transactivator were engineered to express adenovirus L4 100K, 22K and 33K proteins under doxycycline control [67]. The cell lines were capable of packaging Ad5 vectors with novel late gene (L4) deficiencies. A producer line for efficient packaging of a non-primate lentivirus vector (based on equine infectious anemia virus, abbreviated EIAV) was derived from HEK 293 cells and included regulated Gag/Pol and VSV-G envelope protein genes [68]. Conditional expression of the latter transgenes was important because VSV-G is cytotoxic and Gag/Pol may be metabolically burdensome. Regulation was achieved by means of TetR expressed from a constitutively active gene and CMV promoters for driving the transgenes, into which promoters two TetO sequences

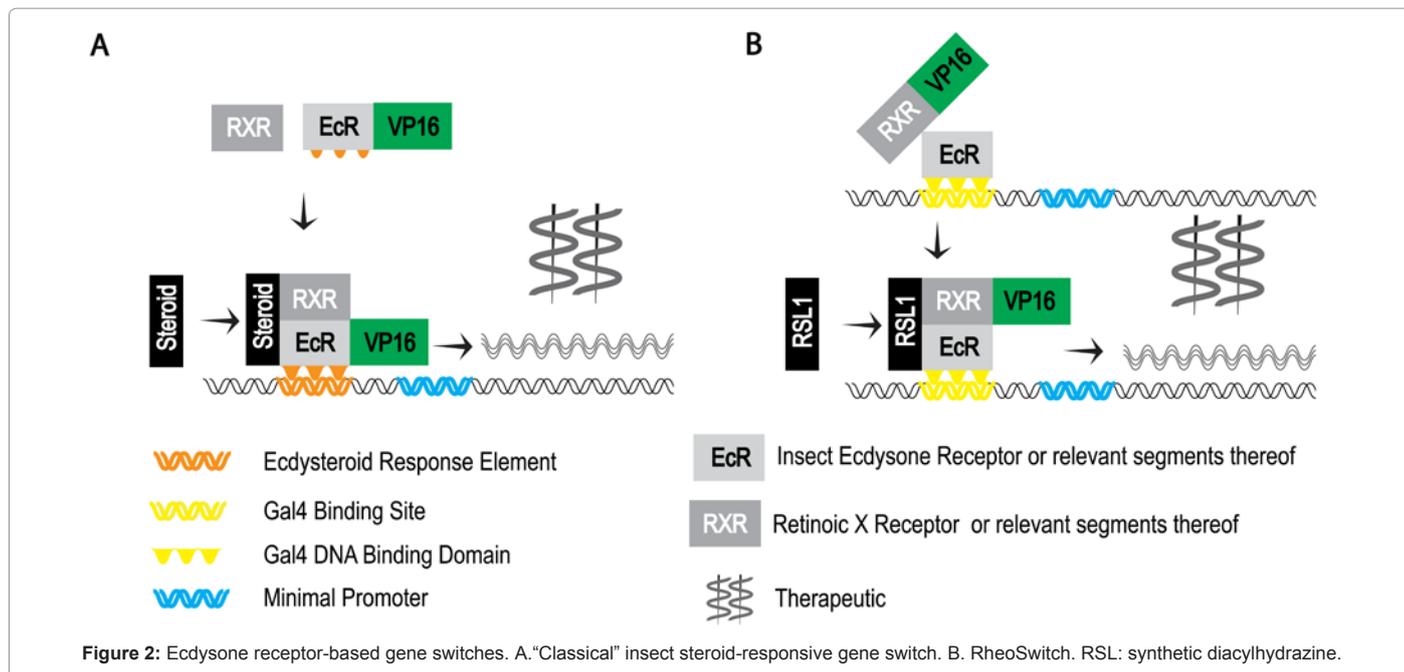
had been inserted between TATA box and transcription start site (also known as T-Rex system; Invitrogen). To validate the line, it was stably transfected with an EIAV genome cassette encoding ProSavin, a lentivirus vector intended as a gene therapeutic for Parkinson's disease. Upon induction, ProSavin was produced with a titer that was comparable to that attained with the conventional transient packaging system.

It is noted that Clontech Laboratories introduced their Tet-Express™ Inducible Expression System in February 2011. At the core of this system is a membrane-permeable version of the previously marketed Tet-Off Advanced transcription activator protein. This transactivator is capable of entering cultured cells directly and activating a TetR-responsive gene of interest.

Judging from the number of recent publications, tetracycline repressor-derived gene switches are widely employed in research. To our knowledge, they are not being incorporated in vectors intended for human therapy. The study of Mättö and colleagues reminds us that tetracycline and doxycycline are antibiotics [69]. A 10-day course of antibiotic concentrations of doxycycline (150 mg/day/human subject) resulted in a dramatic increase in bifidobacteria resistant to the antibiotic. Bifidobacteria are normal constituents of the intestinal fauna. Hence, human use of Tet systems may not be unproblematic.

### Gene switches comprising ecdysone receptor-derived transactivators

Insect ecdysone receptor (EcR) forms a dimeric transactivator with ultraspiracle protein (USP). USP is the insect homolog of vertebrate retinoid X receptor (RXR) [1]. The EcR/USP complex is stabilized by binding of ecdysteroids (insect steroids) to EcR, and the affinity of the complex for ecdysteroid response elements (EcRE) is enhanced. An early gene switch comprised chimeric receptor VpEcR and a responsive promoter. VpEcR is a fusion between *Drosophila melanogaster* EcR and an HSV VP16 transactivation domain. In a mammalian cell VpEcR forms a complex with RXR and, in the presence of an ecdysteroid, effectively binds to EcRE sequences in a promoter, resulting in activation of the promoter (Figure 2A). An improved gene switch comprised VgEcR, a mutated version of VpEcR capable of binding E/GRE sequences, and a promoter supplemented with E/GRE sequences for mediating expression of a gene of interest. E/GRE is a hybrid between a glucocorticoid response element and a response element for a type II receptor (such as EcR). This gene switch performed well in certain cells (e.g., HEK 293 cells) but needed to be supplemented with RXR from exogenous genes in other cells. A system that eliminated this requirement for extra RXR was based on chimeric *Drosophila/Bombyx* ecdysone receptor DBEcR that combined activation and DNA-binding domains of VgEcR with hinge region and ligand-binding domain of *Bombyx mori* EcR. DBEcR was found to be a potent transactivator in the presence of insect steroids such as ponasterone A or of nonsteroidal ecdysone agonists such as GS-E, a synthetic diacylhydrazine (also known as RSL1). Ecdysone receptor-based gene switches were also formulated in the two-hybrid format. G:CfE (DEF) fusion receptor comprises the D, E and F domains of *Choristoneura fumiferana* EcR (CfEcR) and a yeast GAL4 DNA-binding domain. Fusion partner V:MmR (EF) includes the E and F domains of mouse RXR and a VP16 activation domain. The dynamic range of the chimeric receptor was found to be around 9000 (based on measurements of reporter gene expression in the presence vs. absence of ligand). Nonsteroidal ligands were more effective than ecdysteroids. Subsequent improvements further increased ligand sensitivity of the system. A system consisting



of an RSL1-activated two-hybrid transactivator and a GAL4 binding site-containing target promoter for driving transgene expression is marketed by New England Biolabs under the trade name RheoSwitch (Figure 2B). [70,71,72] for detailed descriptions of selected ecdysone receptor-based gene switches.

### Research concerning system development & improvements

The field of use of ligand-dependent transactivators and transinhibitors could be greatly expanded by the incorporation of zinc finger-based DNA-binding domains. Theoretically, any endogenous gene could be targeted by such transactivators and transinhibitors. A recent study reported the construction of a retroviral vector expressing a chimeric transactivator containing the CD54-31 zinc finger DNA-binding domain, a *Drosophila melanogaster* EcR ligand-binding domain, a human RXR ligand-binding domain separated from the EcR sequences by a 30-amino acid linker and the VP64 activation domain [73]. The CD54-31 domain is known to specifically interact with a sequence within the ICAM-1 promoter. Human cells transduced with this vector substantially upregulated ICAM-1 expression in response to ponasterone A.

The most potent ligands for ecdysone receptor-based transactivators are plant-derived ecdysteroids such as ponasterone A and synthetic diacylhydrazines such as GS-E (RSL1, New England Biolabs). Ecdysteroids contain multiple OH groups and have a less than ideal ADME profile. It was hypothesized that alkylation of one or more OH groups may be tolerated from an activity point of view and may reduce hydrophilicity, resulting in an improved ADME profile [74]. A number of alkylated ecdysteroids were synthesized and tested. A key finding of this study is that ponasterone A alkylated at the 22-position may be a better gene switch actuator than its parent as well as may have an improved ADME profile.

Cell cycle dependence of induced expression of a target gene controlled by a RheoSwitch was investigated [75]. Induction of quiescence by either serum starvation or contact inhibition dramatically reduced induced gene expression in NIH3T3 cells. This phenomenon

was observed regardless of whether non-steroidal inducer RSL1 or ponasterone A were utilized. It also could not be explained by a reduction of expression of the RheoSwitch transactivator components. In contrast, no marked difference in induced expression was observed between growing and quiescent NMuMG breast epithelial cells. These results indicate that activation of a RheoSwitch-controlled target gene is cell context-dependent. The findings would appear to be highly relevant for applications in which Rheoswitch-controlled gene expression is directed to normal tissues.

A novel gene switch entirely dispenses with the requirement for expression of insect EcR sequences and is based exclusively on human RXRalpha carrying Q275C, I310M and F313I mutations in the ligand-binding domain [76]. The mutant receptor is activated by synthetic compound LG335 but not natural ligand 9-cis-retinoic acid. A fusion transactivator consisting of a GAL4 DNA-binding domain and the mutant RXR was constructed. A retrovirus vector harboring an expressible gene for the latter transactivator and a GAL4-responsive gene of interest (GFP) was prepared. In experiments with NIH3T3 cells transduced with the viral vector, ligand addition to the cultures clearly enhanced GFP expression.

### Ecdysone receptor-derived systems controlling experimental cancer therapy

Recombinant IL-12 has been long known to have antitumor activity. Unfortunately, IL-12 treatments also have considerable adverse effects. Treatment-unrelated toxicity may be avoided by intratumoral genetic IL-12 therapy. Delivery to tumors of expressible IL-12 genes or dendritic cells expressing IL-12 has produced profound antitumor effects in animal models. Both to provide a tool for discovering the effective timing of IL-12 anti-cancer therapy and to create a vector with a safety advantage, a replication-defective adenovector was constructed that comprised a version of RheoSwitch termed "Rheoswitch Therapeutic System" (RTS) and a bicistronic IL-12 target gene [77]. In RTS, coding sequences for VP16-RXR and Gal4-EcR, separated by the encephalitis virus internal ribosomal entry site, are transcribed from a human ubiquitin C gene promoter. Upstream

from this transcription unit is placed a synthetic inducible promoter that drives expression of a gene of interest, in the instant case the bicistronic IL-12 gene unit, whereby the transcriptional orientation of the gene of interest is the same as that of the transactivator gene unit. C57BL/6 dendritic cells transduced with the adenovector were found to express IL-12 in a strictly ligand-dependent fashion. Transduced dendritic cells introduced into established B16 melanomas in C57BL/6 mice caused dramatic inhibition of tumor growth, provided that ligand was administered early after dendritic cell introduction and was maintained for at least about 5 days. Additional animal studies using the conditional IL-12-expressing adenovector were reported during the 2011 annual meeting of the American Society of Gene and Cell Therapy [78]. In the 2011 annual meeting of the American Society of Clinical Oncology, results of a first clinical trial were reported, in which trial safety and clinical activity of intratumoral administration of autologous dendritic cells transduced with the conditional IL-12-expressing adenovector in combination with oral ligand were tested [79]. The treatment was generally well tolerated. Of the 7 melanoma patients treated, partial or complete regression of injected and some uninjected lesions was observed in two patients. The same two patients had intratumoral changes in IL-12-associated gene expression as well as circulating CD8<sup>+</sup> and/or CD4<sup>+</sup> cells reactive against several melanoma-associated antigens.

### **Ecdysone receptor-derived systems controlling experimental therapy of neurologic disorders or neurodegenerative disease**

Glutamate receptor subtype 5 is associated with disorders such as anxiety, depression or chronic pain. A cell line was described in which a newly cloned gene encoding mouse glutamate receptor type 5b had been placed under the control of the ponasterone A-responsive transactivator present in cell line HEK 293-EcR (Invitrogen) [80]. The cell line should be useful for *in vitro* characterization of receptor-selective agonists and antagonists.

Expansion of a polyglutamine repeat at the amino-terminal end of the huntingtin protein is the probable cause for Huntington's disease. A cell line that contained a RheoSwitch-controlled mutant huntingtin gene (comprising 72 Q polyQ length) was derived from HN10 cells [81]. (Vectors used were those available from Invitrogen Corp.) HN10 cells can be easily differentiated into post-mitotic neurons. Upon induction of mutant huntingtin expression, significant accumulation in intranuclear inclusions was observed as well as transcriptional changes resembling those occurring in animal models. Post-mitotic cells showed aconitase impairment, caspase 3 activation, neurite loss and reduced total protein characteristic of an ongoing neurodegenerative process. The cell line appears to represent a valid model of Huntington's disease and may be useful for drug screening applications.

### **Use of ecdysone receptor-based switches in other experimental therapies**

An AAV-RheoSwitch-GFP virus (obtained from Intrexon Corp., Blacksburg, VA) was used to transduce rabbit intervertebral disc cells [82]. Ligand-induced, reversible GFP expression was observed. Induced expression could be maintained for a period of more than a week. The virus (either 10<sup>8</sup> or 1.5 x 10<sup>11</sup> particles per disc) was subsequently introduced into the L2-L3, L3-L4 and L4-L5 intervertebral discs of New Zealand white rabbits. Ligand was administered for 5 days by subcutaneous injection. Animals were sacrificed either 2 h after the final ligand dose or 5 days later. Immunofluorescence analysis revealed GFP expression in the disks of all animals that had received ligand. No GFP expression was detected in adjacent untreated discs, spinal cord, dura, bone, liver or brain. The study concluded that the RheoSwitch

system should be a useful tool for controlling genetic therapies of intervertebral disc degeneration.

Another study made use of the RheoSwitch system for controlling expression of short hairpin RNAs (shRNAs) in stable cell lines [83]. Initially, a genomic microRNA (miR-122) fragment was expressed under RheoSwitch control. Correctly processed miR-122 was obtained upon induction with ligand RSL1. Downregulation of reporter genes as well as of endogenous miR-122 target genes could be demonstrated. The system was adapted to express any desired small interfering RNA (siRNA) under RheoSwitch control.

### **Mammalian steroid receptor-based gene switches**

Mifepristone-activated gene switches were developed subsequent to the identification of a mutant human progesterone receptor lacking 42 carboxy-terminal amino acids that failed to bind progesterone but instead interacted with progesterone antagonists such as mifepristone and enhanced transcription of reporter genes [84]. Mifepristone is an orally available approved drug substance also known as RU-486. An early chimeric transactivator contained an N-terminal transcriptional activation domain obtained from HSV VP16, the ligand-binding domain of the mutant receptor and a C-terminal GAL4 DNA-binding domain [85]. Improvements were made, which included the use of a transcriptional activation domain from human protein NF- $\kappa$ B in lieu of the VP16 sequence. This resulted in a partially humanized transactivator (GLp65) [86], in which the GAL4 DNA-binding domain remained the only nonhuman element. An autoinducible gene switch in which the transactivator gene was controlled by a basal promoter supplemented with GAL4 response elements was developed to further reduce undesired transactivation in the absence of mifepristone [87]. Shortening of the GAL4 DNA-binding domain resulted in transactivator GS4 which has a reduced propensity for spontaneous homodimerization and, consequently, a diminished ability to transactivate in the absence of ligand [88]. The commonly used concentrations of mifepristone for inducing gene expression are in a subclinical range (i.e., 0.1-10 nM). Still, there remain concerns that even these low doses may affect the ovarian cycle. Implanted mifepristone timed release pellets were utilized to avoid repetitive administration of the ligand [89]. A study that compared cytotoxicity of ligands of regulatable systems in a panel of normal and cancer cell lines found no toxicity of mifepristone and mifepristone A but reported some detrimental effects for doxycycline [90]. Mifepristone-regulated systems have been tested extensively. A transgenic mouse was created that harbored a gene for a mifepristone-dependent transactivator controlled by an enhancer/promoter region from the transthyretin gene to restrict expression to the liver and a human growth hormone (hGH) reporter gene linked to a GAL4-responsive promoter [91]. Serum levels of hGH were very low in the absence of mifepristone, and administration of a single dose of ligand resulted in a transient increase. Transgene expression could be reinduced by administration of a second dose of the ligand. A similar approach was taken to express inhibin A in a mifepristone-dependent fashion in mouse liver [89]. The human keratin promoter [92], the human surfactant protein-C (SP-C) promoter [93], the cardiac-specific  $\alpha$ -myosin heavy chain ( $\alpha$ MHC) promoter [94-96] and the mouse mammary tumor virus (MMTV) promoter [97] are among the promoters used in bigenic mice for tissue-restricting expression of mifepristone-dependent transactivator and, consequently, target transgenes. A HSV vector was constructed from which a beta-galactosidase reporter was expressed in a mifepristone-dependent fashion in the brain [98]. Adenoviral vectors were also used

to achieve mifepristone-regulated expression *in vivo* of hGH [86], human and mouse IL-12 [99] and vasodilatory atrial natriuretic peptide [100].

### Progesterone receptor-derived gene switches: system development

An all-in-one mifepristone-regulated system (pBRES) was developed that contains a transactivator as well as a transgene cassette [101]. Plasmids having the two cassettes in the four possible relative orientations were constructed. The transactivator, a fusion protein consisting of the yeast GAL4 DNA-binding domain, the transcriptional activation domain of the human NF- $\kappa$ B p65 subunit and a truncated ligand binding domain of the human progesterone receptor was expressed under the control of a chicken skeletal muscle alpha-actin promoter and the transgene was controlled by a minimal promoter containing six copies of 17-bp GAL4 binding sites. The *in vitro* performance of the four pBRES plasmids was tested using different inserted genes, i.e., genes for human interferon-beta, human erythropoietin, human and murine secreted alkaline phosphatase, and firefly luciferase. The system could be improved by replacement of the chicken alpha-actin promoter with a human ubiquitin B promoter which resulted in lower background levels in the absence of ligand. The pBRES plasmids were also tested *in vivo* following introduction into murine hind limb muscles by electroporation and intraperitoneal administration of mifepristone. From both *in vitro* and *in vivo* experiments it appeared that the head-to-head orientation of gene cassettes resulted in elevated background activity and, consequently, lower induction. The human interferon-beta gene in a pBRES system having the two cassettes arranged in a tail-to-tail orientation was transferred to an AAV shuttle vector. Recombinant AAV1 particles were injected into the hind limb of mice. During a one-year observation period the therapeutic gene remained capable of being switched on by administration of mifepristone. In a subsequent study, a murine interferon-beta gene (mIFN $\beta$ ) was introduced into the four different pBRES plasmids, which were electroinjected into the hind limb muscles of mice [102]. Transgene expression was followed by measuring the induction of IP-10, a chemokine that is specifically upregulated by type I interferons. All four pBRES mIFN $\beta$  vectors exhibited approximately the same level of induction, and one preferred plasmid having a tail-to-tail arrangement of gene cassettes was used for subsequent studies. Inducible expression of IP-10 could be demonstrated over a period of more than three months. Chemokine expression was proportional to plasmid and mifepristone dose. The same plasmid was also tested in a murine model of acute experimental allergic encephalomyelitis that mimics the symptoms of multiple sclerosis. Mice were immunized by subcutaneous injection of proteolipid protein (in complete Freund's adjuvant supplemented with *M. tuberculosis H37Ra*) followed by intraperitoneal injection of pertussis toxin. Plasmid administration and concomitant implantation of mifepristone pellets resulted in a significant delay in the onset of disease and a decrease in the overall severity of disease scores compared to animals that had received plasmid but not ligand.

A library of mifepristone analogs was screened to identify a ligand that had reduced anti-progesterone receptor activity [103]. Compound BLX-913 was not able to induce the pBRES system and had a 30 fold lower antiprogesterin activity than mifepristone. A homology model of the pBRES transactivator ligand-binding pocket was developed to search for steric conflicts between the inactive compound and amino acids lining the binding pocket. Site-directed mutagenesis was used to create a transactivator that can be triggered by BLX-913 and that

is similarly effective in target gene transactivation as the original mifepristone-activated factor.

To reduce the likelihood that mifepristone-regulated transactivator binds to eukaryotic promoters, an alternative transactivator was developed that contained bacterial repressor LexA sequences instead of a yeast GAL4 DNA-binding domain [104]. The operator-promoter sequence consisted of a synthetic LexA operator containing four ColE1 operator sequences that was joined to a minimal 35S promoter from Cauliflower Mosaic Virus. The system was successfully tested in transgenic zebrafish, in which EGFP reporter expression was induced upon administration of mifepristone in the water and downregulated upon ligand withdrawal. The system also performed well in transgenic zebrafish lines carrying an inducible cell-toxic K-ras<sup>V12</sup> oncogene.

To regulate the activity of the endogenous gene for vascular endothelial growth factor A (VEGF-A), a fusion gene was assembled that encoded a chimeric transactivator consisting of a synthetic zinc finger protein DNA-binding domain capable of targeting the VEGF-A gene promoter, a truncated ligand-binding domain from the human progesterone receptor and an activation domain from the p65 subunit of human NF $\kappa$ B [105]. Transient and stable transfection experiments employing different cell lines demonstrated induced, dose-dependent secretion levels of VEGF-A in the presence of mifepristone and low background expression in its absence. Induction of VEGF-A expression was reversible; VEGF-A level returned to basal within 48 hours after removal of inducer.

### Progesterone receptor-derived gene switches: experimental cancer therapy

An adenovirus capable of expressing TRAIL under the control of a mifepristone-inducible system was constructed [106]. Human lung carcinoma cells transduced with the adenovirus did not express detectable levels of TRAIL in the absence of mifepristone, but the transgene could be induced by mifepristone in a dose-dependent manner. The antitumor efficacy of the virus was tested in a mouse xenograft model of human lung tumor. Virus was injected in the tail vein, and mifepristone was administered intraperitoneally. This treatment resulted in significant suppression of tumor development when compared to animals that only received virus but not ligand.

Soluble VEGF receptor 1 (sflt1) was expected to neutralize vascular endothelial growth factor (VEGF) function and inhibit tumor growth. Two high capacity adenovectors were prepared, of which one contained a TTRB promoter (liver-specific)-controlled gene for a mifepristone-regulated transactivator and a transactivator-responsive sflt1 gene and the other a constitutively active sflt1 gene driven by an EF1alpha promoter [107]. Functional assays demonstrated that soluble receptor expressed from both vectors inhibited VEGF function. The two vectors were injected in tumor-bearing C57BL/6J mice via the tail vein; transient suppression of aggressive tumor growth was observed as a result of either constitutive or inducible expression of soluble receptor. Unexpectedly, mice receiving the virus that constitutively expressed sflt1 developed kidney damage, ascites and died thereafter. Similar outcomes were observed in mice that were injected with the virus carrying the inducible system and received mifepristone pellets. However, when inducer was administered intraperitoneally on a daily basis, which resulted in transiently elevated levels of soluble receptor, animals were free of ascites and survived. These results indicate that intermittent inhibition of VEGF function can be an effective anti-tumor strategy.

The immunomodulatory cytokine interleukin-12 (IL-12) is a potent inducer of interferon (IFN)-gamma and exerts antitumor effects against liver cancer. To be able to modulate the intensity and duration of cytokine expression, the two murine IL-12 chains were placed under the control of either a TetOn gene switch (using transactivator rtTA2s-M2) or a mifepristone-inducible system (using transactivator GLp65) [108]. To restrict production of IL-12 to the liver, transactivator expression was directed by liver-specific promoters. Plasmid containing the doxycycline-inducible IL-12 expression system was administered to immunocompetent mice by hydrodynamic injection. The mifepristone-inducible system was delivered by intravenous or intrahepatic injection of a high-capacity adenovector. Upon administration of doxycycline or mifepristone, respectively, IL-12 levels in the serum of the animals initially rose but then experienced a time-dependent substantial reduction. Inducibility could be restored after a resting period. Inhibition of murine IL-12 production was associated with diminished transactivator expression, an effect that was caused by interferon-gamma-mediated downmodulation of liver-specific promoters. To neutralize this problem, the mifepristone administration regimen was optimized [109]: mifepristone was administered intraperitoneally with stepwise increases that maintained sustained levels of IL-12 for more than 10 days in mice that had been administered high capacity adenovector intrahepatically. The induction protocol could be repeated after a resting period. The improved mifepristone dosing regimen was tested in experiments that assessed the efficacy of IL-12 gene therapy either as a monotherapy or in combination with oxaliplatin. Liver tumors were produced by direct administration of MC38Luc1 cells to the livers of syngeneic mice. Vector was introduced in the proximity of tumor nodules. Compared to animals treated only with oxaliplatin or only with IL-12 gene therapy, the combination of both strategies achieved the best therapeutic effects and long term survival. Combination of IL-12 gene therapy and oxaliplatin chemotherapy also was most effective in protecting animals against tumor rechallenge. The same adenovector expressing murine IL-12 was also tested in a model of HBV infection in woodchucks chronically infected with woodchuck hepatitis virus (WHV) [110]. Repression of liver-specific promoters appeared to be less dramatic in woodchuck than in mice. Direct intrahepatic injection of the vector in animals with chronic WHV infection and stable viremia followed by intraperitoneal administration of mifepristone resulted in high-level expression of IL-12 and IFN-gamma around the injection site. Woodchucks with low basal viremia levels (below  $10^{10}$  viral genomes/ml) exhibited an intense and sustained decrease of serum WHV DNA upon IL-12 induction. This antiviral effect was associated with induction of T-cell immunity against viral antigens and reduction of hepatic expression of transcription factor Foxp3, a key marker for regulatory T cells.

### **Progesterone receptor-derived gene switches in other disease models**

An intestinal cell line capable of insulin processing and glucose sensing was derived from mouse GTC-1 cells. The line contained a human preproinsulin gene that was controlled by a mifepristone-dependent transactivator, which was expressed from a glucose-dependent insulinotropic polypeptide (gip) promoter [111]. A second cell line was generated, in which the transactivator gene was controlled by a minimal thymidine kinase promoter supplemented with GAL4 binding sites to constitute an autoregulatory loop. While in the two cell lines human insulin content increased with mifepristone dose and was negligible in the absence of the ligand, total insulin production was higher when feedforward control of transactivator

expression was utilized. The better system was then tested in a mouse model of streptozotocin-induced diabetes. Engineered cells were encapsulated and transplanted into the peritoneal cavity of mice. Upon administration of mifepristone pellets blood glucose in streptozotocin-treated animals decreased to levels indistinguishable from those in streptozotocin-naive controls. Glucose levels in mifepristone-treated, transplanted diabetic mice remained lower than those in untreated animals during oral glucose tolerance tests.

Another study that related to weight management utilized a mifepristone-regulated transactivator expressed under the control of the gip promoter for dosing leptin [112]. A GTC-1 cell line was generated in which leptin production was stringently dependent on mifepristone dose. Transplantation of the conditionally leptin-producing gut cells under the kidney capsule of ob/ob leptin-deficient mice followed by implantation of mifepristone pellets led to reduced food intake and steady loss of body weight when compared to animals that were transplanted with engineered cells lacking the transgene component. Reduction in food intake and loss of body weight correlated with the leptin level in the circulation as revealed by experiments that compared two different mifepristone doses. Plasma insulin levels also decreased in a mifepristone dose-dependent fashion and remained significantly reduced in mice that had received leptin-producing cells. Interestingly, blood glucose levels remained lower in the group of mice transplanted with leptin-producing cells than in the control group, with the leptin-treated group remaining normoglycemic and the controls hyperglycemic. This effect lasted for 38 days after transplantation, i.e., much longer than the leptin-induced reduction of food intake and loss of body weight. However, the mifepristone-induced leptin cell therapy did not appear to induce weight loss in C57Bl/6J mice maintained on a high-fat diet.

### **Other uses of progesterone receptor-derived gene switches**

A mifepristone-inducible system was also used for preventing expression of cytotoxic and anti-HIV genes during production of lentivirus particles [113]. A transactivator-responsive promoter was subcloned into a self-inactivating lentiviral vector plasmid in either the forward or the reverse orientation with respect to the direction of viral genomic RNA. The activities of several transgenes were examined in lentivirus-infected cells that expressed a mifepristone-dependent transactivator. Infectious lentiviral particles could be produced with a human CD14-coding sequence inserted in lentiviral vector plasmid in either direction. However, the forward orientation resulted in high levels of background expression in the absence of the inducer, which was likely due to transcriptional interference by a heterologous promoter required for Tat-independent transcription of the viral RNA genome. When a cytotoxic gene was assayed, i.e., the vesicular stomatitis virus M gene, the reverse orientation but not the forward orientation resulted in high titers of virus. The reverse vector was able to overcome self inhibition caused by an anti-HIV transgene, a dominant negative mutant of human VPS4B that blocks HIV-1 release and infectivity. The study demonstrated that a lentiviral vector that transduces a harmful gene can be produced, provided that a mifepristone-inducible expression unit comprising the harmful gene is introduced in the virus in the reverse orientation.

### **Estrogen receptor-derived systems**

Estradiol-regulated expression systems are based on a fusion protein that includes a human estrogen receptor (ER) ligand-binding domain and is transcriptionally active in the cell nucleus. The ER domain sequesters the fusion protein in the cytoplasm until ligand

binds to the ER domain and translocation to the cell nucleus can occur. A completely humanized transactivator named HEA was created that incorporated a DNA-binding domain from hepatocyte nuclear factor-1, the G521R mutant of the ligand-binding domain of human ER- $\alpha$  that binds anti-estrogen 4-hydroxytamoxifen but not estradiol, and the activation domain from the p65 subunit of human NF $\kappa$ B [114]. The system exhibited robust and sustained inducibility of an erythropoietin transgene in muscle of mice that received tamoxifen, which is converted to 4-hydroxytamoxifen *in vivo*. Predictably, administration of this inducer in the context of long-term human gene therapy will raise safety concerns, because the doses needed to activate HEA (in mice) are comparable to those used in clinical practice. Prolonged treatment with tamoxifen is associated with an increased risk for endometrial cancer due to its partial agonist activity on endogenous ER- $\alpha$ . These concerns were met by the identification of an ER ligand-binding domain double mutant (L384M, M421G) with a decreased affinity for estradiol and enhanced binding to compounds that do not interact with estrogen receptors [115]. A triple mutant including the G521R substitution was insensitive to physiological concentrations of estradiol and had nanomolar affinity for novel ligand CMP8. The latter mutated ER ligand-binding domain was grafted onto chimeric transactivator HEA. The improved transactivator induced a human secreted alkaline phosphatase reporter gene by about 2000 fold in the presence of CMP8, but remained inactive in the presence of estradiol.

### Regulation of protein activity based on a steroid receptor-ligand interaction

Regulation by 4-hydroxytamoxifen has been exploited in inducible gain-of-function and loss-of-function studies using transgenic animals [116]. In these systems, one transgenic mouse strain carries a gene for a 4-hydroxytamoxifen-regulated Cre or FLP recombinase, i.e., an estrogen receptor ligand-binding domain-recombinase fusion protein. The second strain carries a conditional allele of a gene of interest. When a mouse inherits both transgenes and is exposed to 4-hydroxytamoxifen, the recombinase is translocated to the nucleus where it inactivates the gene of interest by removing an essential exon flanked by LoxP or FRT sites, respectively, from the gene or activates gene expression by removing a stop cassette. Using the same technology, temporal regulation of reporter genes was obtained upon electroporation of plasmids carrying a reporter gene into the embryonic mouse brain or postnatal retina [117]. Spatio-temporal control of transgene expression could be achieved by means of ligand-regulated recombinase genes that were driven by cell- or tissue-specific promoters; transgene expression could be so confined, e.g., to rod photoreceptors, bipolar cells, amacrine cells and glia [43], melanocytes [118], epithelial cells [119], osteoblasts [120] and podocytes [121]. A mifepristone-inducible Cre recombinase was obtained by fusion with the ligand-binding domain of a mutant human progesterone receptor [122]. More recently, high-efficiency fusion variants of FLP and PhiC31 recombinases were engineered. These fusion recombinases mediate site-directed gene insertion in the presence of mifepristone but exhibit low levels of activity in the absence of ligand [123].

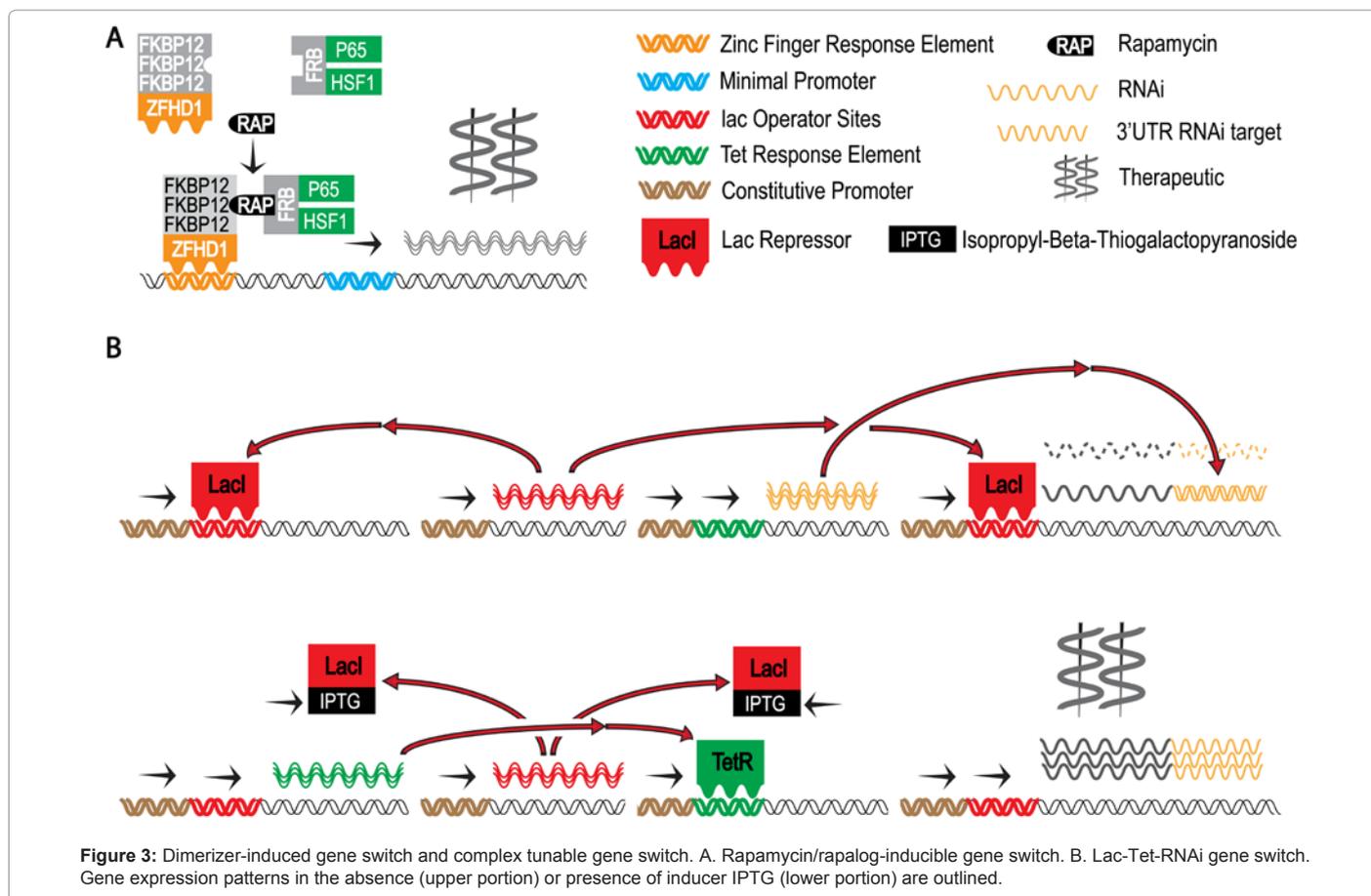
A recent study attempted to direct inducible Cre recombinase activity to keratin-positive cells of the airways [124]. Bigenic reporter mice were created by crossing a strain that expressed a truncated, mifepristone-inducible progesterone receptor-Cre recombinase fusion protein under the control of a keratin 5 or 14 promoter with a Rosa26 reporter strain in which a loxP-neomycin-loxP sequence separated constitutively active Rosa26 promoter and beta-galactosidase reporter gene (R26-LacZ). Other bigenic reporter mice generated expressed a

Cre fusion protein that contained a shortened progesterone receptor sequence to reduce background recombinase activity. A solvent system of 20% acetone/80% sesame oil with isoflurane anesthesia was established for delivering mifepristone to the trachea. Ligand administration induced significant tracheal Cre activity as shown by beta-galactosidase staining. The fusion protein with the shortened progesterone receptor segment exhibited the most tightly regulated and robust recombinase activity. In another study a gene for a fusion protein was assembled that consisted of a green fluorescent protein, Cre recombinase and a progesterone receptor ligand-binding domain [125]. Because of the low background activity and natural sensitivity to mifepristone of the lactoferrin promoter, the fusion protein gene was placed under the control of a truncated lactoferrin promoter. A transgenic mouse line was created, and crossed to Cre reporter strain R26-LacZ. Only the salivary glands of bigenic mice that had received implants of mifepristone pellets displayed X-gal staining, suggesting that the fusion protein was expressed and activated specifically in salivary glands.

To minimize basal transgene expression, another study combined ligand regulation at the transcriptional and protein activity levels [126]. Bigenic mouse lines were generated by breeding a transgenic mouse line that expressed chimeric factor Glp65 under the control of an epidermal-specific keratin 14 promoter (which was used to direct gene expression to the keratinocytes of the basal epidermis and hair follicles) with a transgenic mouse line that carried a GLP65-responsive gene for inducible caspase-3 or caspase-9 precursors. The Glp65 fusion protein was exclusively expressed in skin keratinocytes, which in the presence of mifepristone initiated caspase precursor expression. Administration of lipid-permeable rapalog AP20187 (see below) caused dimerization of inactive caspase precursor molecules, which dimerization resulted in their self activation. The double induction system was tested in neonates by delivering mifepristone *in utero*, along with progesterone to counter the abortion side effect, followed by topical administration of AP20187. The skin of the so treated neonates showed peeling, appeared dehydrated and exhibited strong induced expression of caspase 3 and 9 when compared to the skin of control littermates.

### Dimerizer-activated gene switches

Dimerizer-activated gene switches make use of “heterodimeric” transcription factors in which DNA-binding and activation domains are on separate polypeptides that have no intrinsic affinity for one another but can be held together by a small dimerizer molecule. The original gene switch made use of small immunosuppressive molecule rapamycin which interacts with immunophilin FKBP12 and as rapamycin-FKBP12 complex with mTOR/FRAP. FRAP is a phosphoinositide-3-kinase homolog that is involved in the control of cell growth and division. A fully “humanized” dimerizer-activated transactivator was engineered (Figure 3A): a first fusion protein comprised human zinc finger and homeodomain-binding domain ZFHD1 and three copies of FKBP12, and a second fusion protein included a 100-residue domain of FRAP (FRB) and a transcription activation domain from the p65 subunit of human NF $\kappa$ B. Subsequently, an activation domain from human heat shock factor HSF1 was added. A minimal IL-2 promoter supplemented with 12 ZFHD1 binding sites served to control expression of a chosen target gene. Stringent regulation by rapamycin of target gene expression was demonstrated in various *in vitro* and *in vivo* models. The system was made more attractive by the development of rapalogs that are non immunosuppressive analogs of rapamycin which retained the ability to function as dimerizers. The technology is presently commercialized under the trade name ARGENT by Clontech



Laboratories. A second dimerizer-activated gene switch uses small molecule FK506 as dimerizer [1,127,128,129].

To further improve control of expression of secreted therapeutic proteins, a system was devised in which not only transcription but also exocytosis was rapalog-regulated [130]. The system consisted of two lentiviral vectors. Both vectors contained the central Flap sequence to improve transduction of non dividing cells [131]. The first vector, the transactivator expression vector, comprised a CMV promoter for directing expression of a bicistronic cassette containing coding sequences for the two subunits of dimerizer-activated transactivator separated by an internal ribosome entry sequence derived from the encephalomyocarditis virus. The second vector, the trophic factor secretion vector, included a transactivator-responsive promoter that mediated expression of a SS-4xFKBP-36M-FCS-target polypeptide fusion protein. The fusion protein-coding sequence was followed by a woodchuck hepatitis virus responsive element to enhance production of the fusion protein. SS refers to a signaling sequence, 4xFKBP-36M to four tandem repeats of FKBP12 carrying the F36M mutation (transforming the FKBP into a conditional aggregation domain), and FCS to a furin cleavage site [132]. As target polypeptide either GFP or neurotrophic factor GDNF was employed. *In vitro*, cotransduction of the two vectors resulted in robust expression of target proteins in the presence of rapalog AP21967, which proteins were effectively secreted. The proteins were essentially undetectable in the absence of rapalog. Induction of target protein expression was shown to be reversible. Analogous results were observed when the vectors were co-introduced into the striata of mice.

It is noted that in recent studies rapalogs appear to have been used primarily to regulate protein activity rather than gene expression. In one such study, an adenovector was prepared that expressed iCaspase under the control of an endothelial cell-specific vascular endothelial growth factor receptor-2 (VEGFR2) promoter [133]. iCaspase is a fusion protein containing two copies of FKBP12 variant V36 and procaspase-9 [134]. The adenovector caused apoptosis in the presence of rapalog AP20187 in human dermal microvascular endothelial cells but not in several human tumor cell lines. *In vivo* experiments showed that intra-tumoral delivery of the vector followed by i.p. administration of rapalog resulted in ablation of tumor microvessels and inhibition of tumor growth. Another study tackled the question whether activation of protein kinase Akt2 is sufficient to cause translocation of glucose transporter 4 to the cell surface, thereby enhancing insulin-regulated glucose uptake into muscle and fat cells [135]. 3T3-L1 adipocytes were engineered to express a fusion protein containing full-length Akt2 and rapamycin-binding domain FRB. To recruit the Akt2 fusion protein to the cell membrane, the cells also expressed a fusion protein containing two copies of FKBP12 to which was added N-terminally a Src myristoylation signal. Rapalog addition resulted in activation of the fusion Akt2 as evidenced by phosphorylation of the factor at characteristic sites as well as phosphorylation of downstream substrates. Transient activation of the fusion Akt2 also increased glucose transport and glucose transporter 4 translocation to the plasma membrane, validating the hypothesis. An alternative insulin therapy involving insulin-secreting keratinocytes was evaluated in yet another study [136]. Keratinocytes were transduced with a lentivirus vector that expressed a fusion protein containing several copies of a self-dimerizing

variant of FKBP12 and proinsulin preceded by a furin cleavage signal. The transduced keratinocytes could be induced by rapamycin to secrete insulin. Insulin secretion was observed within 30 min of rapamycin addition and ceased 2-3 hours after rapamycin removal. Transplanted lentivector-transduced keratinocytes secreted active insulin for weeks and were capable of repeated rapamycin stimulation of insulin secretion. Induced insulin secretion reversed hyperglycemia in athymic mice made “diabetic” by exposure to streptozotocin. These results showed that insulin produced in a regulated fashion by skin cells can control hyperglycemia. Finally, dimerization technology was employed to study the function of dynein light chains LC8 and TcTex1 [137]. The latter LCs bind as dimers to dimers of dynein intermediate chains (IC). The authors prepared LC traps which are fusion proteins between a self-dimerizing variant of FKBP12 and an LC-interacting sequence from IC. Evidence was obtained that these LC trap proteins dimerize and bind to LCs in the presence of rapalog. The system was employed to demonstrate that LCs are involved in lysosomal, endosomal and Golgi organization, but apparently are not critical to mitotic progression despite the central role of dynein in cell division.

### Regulation or coregulation by siRNAs and miRNAs

A problem common to most mammalian gene regulation systems is residual target gene expression when the systems are turned off. This is due to basal promoter activity as well as to random interactions of transactivators with their binding sites. Recently developed strategies make use of RNAi to more stringently control transgene expression. A tunable genetic switch termed LTRi (Lac-Tet-RNAi) couples repressor proteins and RNAi target modules to deactivate a transgene [138] (Figure 3B). In this system, bacterial repressor LacI is expressed constitutively from a cytomegalovirus promoter. In the absence of ligand isopropyl-beta-thiogalactopyranoside (IPTG), LacI binds to lac operator sites in the transgene, repressing transgene transcription. LacI also binds to lac operator sites located in a tetR gene, repressing its expression. In the absence of TetR, a short hairpin RNA (shRNA) is transcribed from a tetO site-containing active promoter. This shRNA binds to a complementary synthetic sequence element (originating from the *E. coli* beta-galactosidase gene) present in the 3'UTR of transgene mRNA, targeting for degradation any mRNA molecule made by leaky transcription. In the presence of IPTG, LacI is inactivated, and target and TetR genes are transcribed at their respective maximal rates. TetR so made binds to the TetO sites upstream of the RNAi gene, repressing transcription of the shRNA. The robustness of the LTRi system was tested in stably transfected cells, using an EGFP reporter gene as the transgene. The circuit showed greater than 99% repression in the off state and could be repeatedly switched between induced and uninduced states. The level of transgene expression was dependent on IPTG dose. To demonstrate how tightly transgenes can be regulated by the LTRi system, genes such as the highly toxic alpha chain of diphtheria toxin, the Cre recombinase and the proapoptotic gene bax were placed under its control.

The induction profile of several gene control switches was improved by a strategy that relied on the fact that the effectiveness of silencing increases with siRNA-to-target ratio [139]. siRNA was expressed from an intronic sequence within a gene for a ligand-activated transactivator, and siRNA sequence specific tags were placed in the transgene to enable silencing by bound siRNA. This approach succeeded in reducing the leakiness of an erythromycin and a tetracycline-responsive gene switch. Transfection experiments demonstrated the ability of the siRNA-based silencing system to control expression of two highly toxic gene products, the apoptotic RIP death domain and *M. esculenta*

derived linamarase, which converts the innocuous linamarin into gaseous cyanide. Autoregulated silencing vectors were constructed by introducing an RNAi-coding sequence into a transactivator gene and by placing this gene under the control of a promoter that is responsive to the transactivator. Intronic encoded siRNA was also used as an element of a three component mammalian oscillator circuit that is capable of mimicking circadian regulation [140]. The first component consisted of a tTA gene controlled by a tetracycline-responsive promoter. An untranslated *Photinus pyralis* firefly luciferase-derived sequence tag was added at the 5' end of the tTA-coding sequence. The presence of this sequence tag enabled interference-based silencing of the tTA transcript by a luciferase-specific siRNA. The second component comprised a gene for a macrolide-responsive transactivator expressed from a tTA-responsive promoter. The latter transactivator gene included an intronic sequence that encoded luciferase-specific siRNA. The third component of the system consisted of a gene for a destabilized variant of yellow fluorescent protein d2EYFP controlled by a macrolide-responsive promoter. The activity of the system can be modulated by tetracycline and erythromycin. Leaky transcription from the tetracycline-responsive promoter leads to positive feedback, resulting in high levels of tTA as well as in increased expression of siRNA and macrolide-responsive transactivator. The macrolide-responsive transactivator will activate the d2EYFP gene. The siRNA mediates the breakdown of tTA, turning off the network. As a consequence, levels of d2EYFP and siRNA decrease, allowing the circuit to restart. Co-transfection assays indicated variations in cell fluorescence intensity that displayed an oscillatory behavior with a period of 26 hours.

New systems were recently described that exploit microRNAs (miRNAs) for transgene regulation [141]. miRNAs are small single-stranded, non-coding RNAs transcribed from DNA that is not translated, which RNAs function as regulators based on complementarity to mRNA transcripts of coding genes. Binding of a miRNA to a target mRNA results in translational repression and “gene silencing”. By inserting complementary miRNA target sites into the 3' untranslated region of transgene cDNAs, transgene expression can be subjected to endogenous miRNA regulation. Intrinsic differences in miRNA expression patterns can be exploited to finely control transgene expression [142]. However, a threshold level of miRNA must be reached for target mRNA suppression to occur.

Certain miRNAs are expressed broadly in the human body, whereas others display differential expression in developmental, tissue, or cell type-specific manners. A baculoviral vector for glioma suicide therapy was constructed harboring a herpes simplex virus thymidine kinase (HSVtk) gene controlled by a glial fibrillary acidic promoter for restricting transgene expression to the glial cell lineage [143]. The vector contained target sequences for three miRNAs that are enriched in astrocytes but downregulated in glioma cells. This strategy resulted in a significant improvement of *in vivo* selectivity over a control vector lacking the target sequences as observed in experiments involving injection of vector into the striatum of glioblastoma-bearing nude mice followed by daily intraperitoneal injection of ganciclovir for 5 days. In another study, the specificity of hepatic miRNA miR-122 was used to suppress transgene expression in the liver after intravascular administration of an AAV vector [144]. An analogous approach was used to restrict transgene expression to the central nervous system, detargeting liver, heart, and skeletal muscle, i.e., the tissues that are most efficiently transduced by systemically administered AAV vectors [145].

Overexpression of hypothalamic brain-derived neurotrophic factor

(BDNF) results in weight loss. In a study aimed at developing a system for sustainable weight management, an AAV vector was constructed that contained a gene for BDNF controlled by a constitutive promoter [146]. The construct also expressed a specific miRNA directed against BDNF transcripts under the direction of the BDNF-responsive *AgRP* promoter. BDNF expression led to weight loss, increased *AgRP* activity and BDNF miRNA expression. The therapeutic efficacy of the dual cassette vector was tested in *db/db* mice, a genetic model of diabetes and obesity. Administration of the vector resulted in an initial weight reduction which was subsequently maintained, indicating efficient autoregulation of the BDNF transgene. A vector encoding BDNF and a scrambled miRNA caused progressive weight loss. The above-discussed studies demonstrate that incorporation of a miRNA-based component can significantly contribute to transcriptional targeting or control of transgene expression.

## Transgene Activation by Focused Heat or Ionizing Radiation

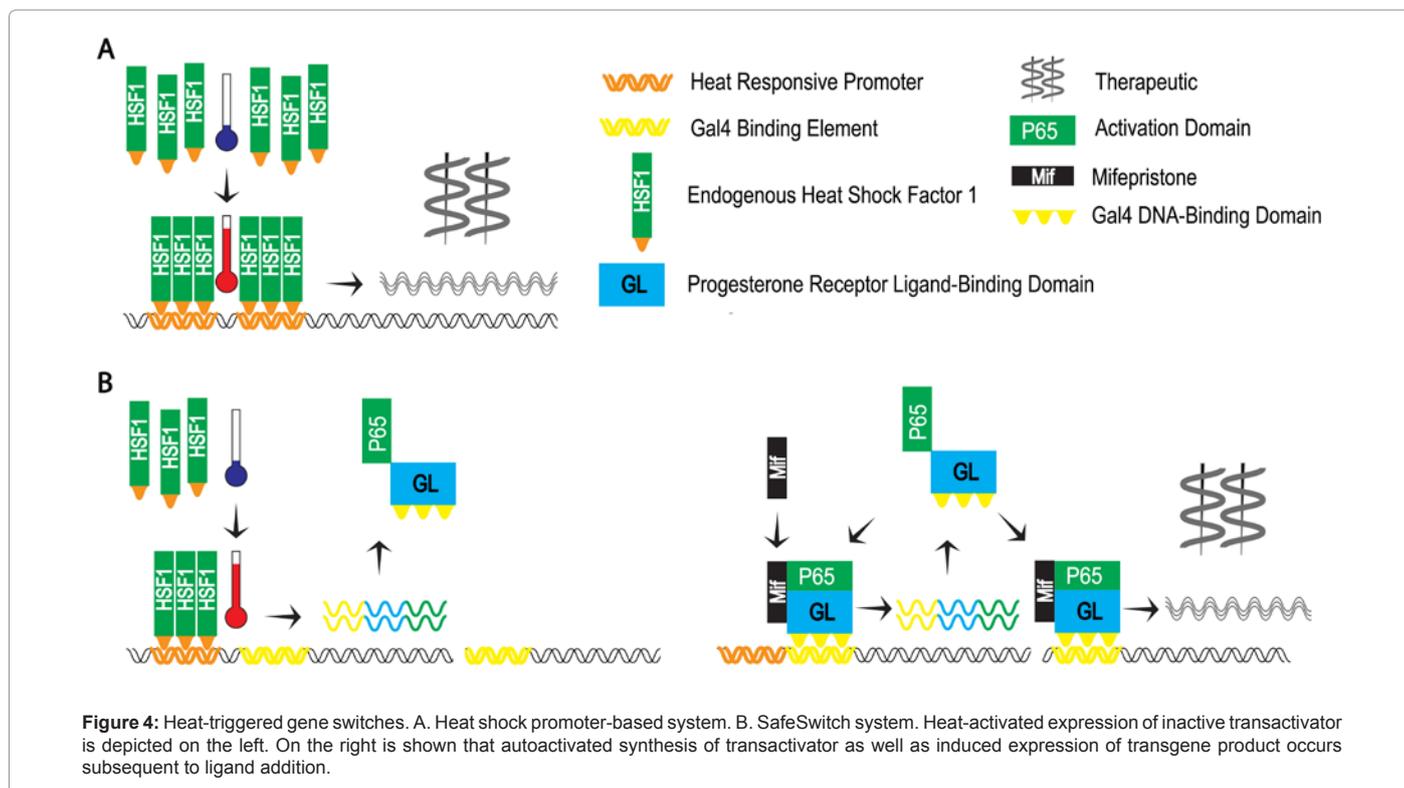
### Heat shock promoters and heat shock promoter-based systems

A common response of all living organisms to abnormally high temperatures or other environmental or metabolic stresses is the induction of a group of highly conserved proteins, termed heat shock proteins (HSPs). Stimuli that induce HSP expression include radiation, heavy metals, ischemia, nitric oxide radicals and the like, whereby elevated temperature arguably is the most robust inducer of hsp gene expression. All the stimuli share the ability to activate heat shock transcription factors (HSFs). Different hsp promoters differ in their inducibility. Based on reporter assays, the promoter of the human *hsp70B* gene (HSP A7) is one of the most highly heat-inducible promoters [1,147,148]. It is characterized by a very low basal activity

that increases several thousand fold in a cell subjected to a sublethal heat treatment (heat shock) [149]. It is noted that the promoter of the highly related *hsp70B'* gene (HSP A6) has closely similar properties to the human *hsp70B* promoter. The architecture of hsp promoters shows considerable variation, but all heat-inducible promoters contain one or more sequence elements termed heat shock elements (HSEs) that are binding sites for heat shock transcription factors (Figure 4A). HSEs are the elements that confer heat regulation on the promoters and are comprised of three or more modules of the pentamer sequence NGAAN arranged in alternating orientation. Mammalian cells express several types of HSF, but heat induction of hsp genes cannot be observed in the absence of HSF1 [150]. In unstressed cells, HSF1 is largely present in an inactive form. Upon stress exposure, HSF1 is induced to homotrimerize and acquires the ability to bind to HSEs. Concentration of HSF1 in the nucleus and acquisition of transcriptional competence are further events that occur nearly simultaneously with homotrimerization. Activation of HSF1 is transient. HSF1 and, consequently, hsp promoters are inactivated within a few hours after an activating heat treatment.

Simple methods for administering heat to a cell or an experimental animal involve contacting with a heated surface or liquid. More sophisticated methods rely on microwave or infrared radiation, or ultrasound. As heat can be administered in a focused fashion, hsp promoters can be potentially used for spatially controlling transgene expression. In fact, several studies demonstrated that spatial accuracy of tissue heating can be achieved by focused ultrasound radiation guided by online MR thermometry [151]. Focused ultrasound induced hyperthermia was found to be capable of precisely inducing expression of a target gene under the control of an *hsp70B* promoter in the liver, a deep seated organ [152].

HSF1 is expressed ubiquitously, and the mechanism for heat



induction of hsp promoters exists in virtually every cell type. Thus, it is possible to use these promoters to uniformly control therapeutic gene activity throughout an organ comprised of different tissues or a tumor. In addition, peak induced activity of a hsp promoter is a function of both temperature and length of temperature exposure, allowing modulation of a therapeutic outcome by changing parameters of the activating treatment. As a generality, hsp promoters appear to be more efficient than tissue-restricted promoters.

Several groups developed *in vivo* models of localized activation of human hsp70B promoter-controlled transgenes [153,154,155,156]. Most of these studies related to cancer therapies in which the hsp70B promoter was employed for directing expression of cytokine, suicide or membrane fusion-inducing genes.

In a recent study, the human hsp70B' promoter, an artificial promoter comprising five HSEs arranged in tandem and a combination promoter, in which the five HSEs were situated upstream from the hsp70B' promoter, were compared [157]. A green fluorescent reporter gene was linked to each of the promoters, and activities were tested in transfected human pancreatic tumor cell lines. The combination promoter produced the highest reporter activity and was subsequently employed for controlling the expression of a mildly toxic variant of diphtheria toxin A in stably transfected cells. Heat shock completely arrested cell growth. The construct was also tested in human PSN1 tumor cells that were inoculated subcutaneously in nude mice. Tumor heating was by directly contacting the skin with a heated surface; animals underwent two 15-min heat treatments at 42°C administered 72 hours apart. The heat treatments induced significant reductions in tumor volumes compared to unheated tumors, indicating that the engineered promoter is useful for controlling expression of toxic/therapeutic products *in vivo*.

Heat inducibility of an hsp70B' promoter-driven luciferase reporter gene present either in a replication-deficient adenovirus or in a melanoma-targeted oncolytic adenovirus (Ad2xTyr) was analyzed in another study [158]. Heat treatment induced reporter expression from the replication-deficient adenoviral vector by about 8000-fold. Heat inducibility of luciferase expression was also strong in Ad2xTyr-infected permissive cells, but regulation of hsp70B' promoter activity was all but lost in the course of viral replication. Insertion of upstream insulator elements did not protect the promoter against this dysregulation. These results caution against an unquestioned use of inducible promoters in replicating systems, even if these promoters had worked well in replication-deficient vectors.

During recent years, efforts were made to develop systems capable of codelivering thermal energy and heat-inducible vectors. One approach involved the use of Mn-Zn ferrite magnetic nanoparticles that generate thermal energy when exposed to an alternating magnetic field (AMF) [159]. The nanoparticles were surface-modified with poly(ethylenimine) and loaded with plasmid DNA containing a hsp70B promoter-controlled bacterial beta-galactosidase gene. Cultured cells were subject to magnetofection with the resulting complexes and were subsequently exposed to AMF (230 kHz) for 60 min. Medium temperature increased as a function of nanoparticle dose. Induced beta-galactosidase activity could be observed in AMF-treated cells. The complexes were also injected into human tumors in nude mice; AMF treatment induced reporter expression, although this induction was less dramatic than that previously observed in cultured cells. While the feasibility of the approach was established by the study, concerns remain regarding the potential systemic toxicity of inorganic nanoparticles.

A self-inactivating retroviral vector was constructed that comprised IRES-linked human sodium iodide symporter (hNIS) and GFP cDNA genes controlled by a hsp70A promoter [160]. Heat treatment (42°C for 60 min) of stably vector-transduced human RG2 glioma cells produced a 39 fold increase in GFP-positive cells. Uptake experiments with Na<sup>131</sup>I and <sup>99m</sup>TcO<sub>4</sub> indicated accumulation of radiotracers after heat induction. Subcutaneous xenografts of stably vector-transduced RG2 cells in nude mice were subjected to heat treatment in a circulating water bath at 42°C for 60 min, and fluorescence, single-label scintigraphic imaging and <sup>124</sup>I-iodide micro-positron emission tomography (microPET) were performed 24 hours later. Low levels of GFP fluorescence and <sup>99m</sup>TcO<sub>4</sub> accumulation were recorded prior to heat. A marked increase in both fluorescence and <sup>99m</sup>TcO<sub>4</sub> uptake was observed after heat exposure. microPET imaging undertaken subsequent to intravenous injection of <sup>124</sup>I-iodide revealed induction of hNIS expression.

Thymidine kinase activity produced by a replication-deficient type 5 adenovector containing an hsp70B promoter-linked gene for a HSV1-TK-EFGP fusion protein was evaluated by a tritiated penciclovir uptake assay in human head and neck squamous cell carcinoma SCC-9 cells [161]. Increased cell-associated radioactivity was observed one day after heating at 41°C for 1 hour. Analogous results were obtained when EGFP expression was analyzed. Thymidine kinase activity was also examined *in vivo* in mice bearing subcutaneous SCC-9 xenografts that were directly injected with the adenovector. A temperature probe was placed in the tumor, and hyperthermia was delivered by means of an ultrasound transducer. A biodistribution study showed specific tumor uptake of tritiated penciclovir after heating at 41°C for 1 hour. *In vivo* expression of thymidine kinase was monitored noninvasively by microPET imaging after injection of 9-(4-[<sup>18</sup>F]-fluoro-3-hydroxymethylbutyl) guanine substrate. Significant differences were observed between heated and non-heated tumors. These experiments demonstrated that PET imaging can be used to monitor heat induction of thymidine kinase gene therapy.

As also alluded to before, heat has become an attractive means for controlling gene expression due to the availability of technology for externally inducing local hyperthermia by high-intensity focused ultrasound radiation (HIFU) and measuring temperature distribution in the targeted tissues by magnetic resonance temperature imaging (MRI). In a recent study that was aimed at comparing local temperature distribution and heat-induced transgene expression, thigh muscles of transgenic NFL-1 mice that harbor a firefly luciferase gene driven by a mouse hsp70 promoter (HSPa1b) were heat-treated by MRI-guided HIFU [162]. Control of reporter gene expression was achieved by adjustment of the HIFU power based on MR thermometry. Luciferase activity was followed by bioluminescence imaging. These experiments revealed a close correspondence between spatial distribution of temperature and reporter gene expression. The noninvasiveness of the heating approach was evaluated by histological analysis of the heated tissue. No damage was observed for a 43°C/2 min hyperthermia protocol, whereas increasing the duration of the HIFU treatment to 8 min resulted in significant tissue alterations.

Owing to autoregulation, hsp promoters do not remain active for periods exceeding a few hours, and gene products typically reach peak concentration about one day after the activating heat treatment. Sustained expression of a hsp promoter-controlled therapeutic gene in a patient would require daily or twice daily heat treatment, resulting in a regimen that is impractical. Inadvertent activation of hsp promoter-controlled therapeutic transgenes in non-target tissues could be an

important problem, e.g., in the context of cytotoxic or angiogenic therapies. Such activation could arise from an elevation in body temperature caused by disease, strenuous exercise, intoxication by heavy metals and other toxicants, certain pharmacological interventions, or ischemic events [163-171]. As exclusive delivery of a transgene to a desired tissue or body region it is not possible with currently available technology [172], this could result in undesired transgene expression and adverse effects in major organs. To activate a therapeutic gene only at the time and only for the duration required for effective therapy and to restrict expression of the gene to the tissue/organ in need of therapy, we designed regulatory circuits that combined an hsp70B promoter and a small molecule-activated gene switch [173]. These gene switches consist of (a) a ligand-activated transactivator expressed under the dual control of a promoter or promoter cassette that is responsive to heat and the transactivator and (b) a promoter responsive to the transactivator for controlling a gene of interest. In a cell containing such a gene switch and a target gene neither transactivator nor target gene are expected to be expressed in the absence of ligand and/or without an activating heat treatment. When the cell is exposed to a transient elevation of ambient temperature, endogenous HSF1 will be activated and will transactivate the ligand-activated transactivator gene. Subsequent to the heat exposure, HSF1 activity will decline and return to basal levels. In the absence of ligand, the transactivator will remain inactive, and the target gene will remain silent. In the presence of ligand, transactivator synthesized in response to the transient heat treatment will be activated and will begin transactivating any gene that is controlled by a transactivator-responsive promoter, i.e. the transgene will be expressed, and the transactivator will autoactivate its own gene. This feedforward mechanism is expected to sustain transactivator levels after HSF1 has been inactivated. Rate of synthesis and half life of the transactivator as well as the doubling time of the cell will determine the concentration of transactivator present in the cell, which concentration will eventually stabilize. In the presence of ligand, expression of the transgene will be maintained for an extended period. The system can be turned off by withdrawal of ligand. The main advantage of these gene switches is that they cannot be activated in the absence of the specific ligand, providing a solution to the problem that hsp promoters are subject to inadvertent activation in nontarget locations under certain physiological or pathological conditions.

Gene switches of this kind employing different ligand-activated transactivators were built and tested [173]. A particular two component system, termed SafeSwitch, employed mifepristone-activated chimeric transactivator GLp65 (Figure 4B). The first component consisted of a GLp65 gene under the control of a promoter cassette that combined a human hsp70B promoter and a GAL4-responsive promoter. This promoter cassette conferred stringent heat and autoactivated expression on the linked transactivator gene. The second component was a GAL4-responsive promoter that was linked to a luciferase reporter gene. Transient and stable transfection experiments established that SafeSwitch remains stably in the inactive state prior to heat activation or in the absence of mifepristone, is highly active for an extended period subsequent to a single transient heat treatment in the presence of mifepristone, allows modulation of target gene expression as a function of the intensity of the activating heat treatment and is inactivated by withdrawal of mifepristone. Plasmids encoding the two components of SafeSwitch were introduced into gastrocnemius muscles of adult C57BL/6 mice by electroinjection. Heat activation combined with administration of mifepristone resulted in about a 1000-fold induction of reporter activity one day after heat treatment. In animals that received a daily dose of mifepristone, reporter activity induced by a single heat treatment could be maintained at a high level

for at least 6 days. Discontinuation of mifepristone administration deactivated the gene switch. More recently, we built another heat-triggered, small molecule-activated gene switch that employs a dimerizer-controlled chimeric transactivator comprising modules derived from human proteins FKBP12 and FRAP for inducibly joining DNA-binding and activation domains [174]. The system was tested in transfection experiments using luciferase as the target gene. The gene switch showed the desired properties of only being activated by heat in the presence of rapamycin or rapalog AP21967, of maintaining high level reporter expression for several days after heat activation, and of being silenced by removal of ligand. Cytotoxicity from HSV thymidine kinase (in the presence of ganciclovir) or fusogenic GALV expression could be adequately controlled by the heat and rapamycin-dependent gene switch. Heat-activated, rapamycin-dependent gene switch and SafeSwitch supported comparable levels of target gene activity. The two heat activated gene switches may be used in combination for precisely controlling transgenes that need to be expressed in a sequential fashion.

### Radiation-inducible promoters

A group of genes, the so-called “immediate early genes“, react rapidly to ionizing radiation (IR). These genes encode transcription factors such as c-FOS, c-JUN, AP-1, NFkB and EGR-1. IR results in upregulation of the egr-1 promoter within 15 min; the promoter remains active thereafter for about three hours. The egr-1 promoter contains sequence elements referred to as CarG sequences that are responsible for the enhanced promoter activity subsequent to IR [175]. As induction by IR can be abrogated by antioxidants, it is believed that the promoter senses reactive oxygen species. Consistent with this hypothesis, the egr-1 promoter is also induced by neutrons, radioisotopes, photons and various chemotherapy agents including cisplatin, 5-fluorouracil, gemcitabine, paclitaxel, doxorubicin, cyclophosphamide and temozolomide. Subsequent to the proposal that a radiation-inducible promoter such as the egr-1 promoter could be utilized for targeted cytotoxic tumor therapy, a number of preclinical studies were carried out, many of which employed the TNF-alpha gene as the cytotoxic gene. These studies suggested that intratumoral delivery of a radiation-inducible TNF-alpha gene (by means of a replication-defective adenovector) substantially enhances anti-tumor activity of IR. This therapeutic approach was subsequently taken to the clinic. The vector used in the clinical experiments, which was named TNFerade, was a type 5 adenovector with E1, E3 and E4 deletions containing an egr-1 promoter-controlled TNF-alpha gene. Several phase I trials of combination IR and TNFerade therapy were carried out on subjects with various types of tumors. Generally, the results showed improved tumor responses compared to historical controls. Side effects were fever, chills and pain at the injection site. A successful phase II trial of IR, TNFerade and 5-fluorouracil was subsequently carried out in patients with advanced non-metastatic pancreatic cancers. A maximal tolerated dose of  $4 \times 10^{11}$  pfu TNFerade was defined. Based on the encouraging results of this trial, a phase III randomized trial was initiated. Unfortunately, the trial had to be stopped in March 2010 by GenVec, the company that had underwritten clinical development, subsequent to an interim analysis indicating that the required statistical significance for registration could not be attained. As TNFerade had previously also produced positive results in early trials of esophageal cancer, head and neck cancer, rectal cancer and soft tissue sarcomas, pivotal studies in these cancer types may be warranted.

### Other Inducible Systems

Phloretin, a non-toxic flavonoid found in apples, binds to the

TtgR-operator complex releasing cognate repressor TtgR from the specific operator  $O_{TtgR}$ . This results in induction of TtgABC production and effective pump-mediated efflux of the flavonoid from *Pseudomonas putida*. Based on the phloretin-sensitive TtgR- $O_{TtgR}$  interaction in *P. putida*, a synthetic mammalian phloretin-adjustable control system named PEACE was assembled [176]. A transactivator was created by fusing TtgR to the VP16 transactivation domain. In the absence of phloretin, the synthetic transactivator was able to bind to and activate a promoter containing  $O_{TtgR}$  sequences linked to a minimal cytomegalovirus promoter. Addition of phloretin releases the transactivator from the operator sequences. The system was tested extensively in different mammalian cell lines and human primary cells and was found to be capable of stringently controlling a secreted alkaline phosphatase (SEAP) reporter gene. Phloretin was tested as a potential transdermal transgene inducer. A CHO cell line harbouring a SEAP reporter gene under the control of the PEACE system was microencapsulated in alginate-poly(L-lysine)-alginate beads and implanted subcutaneously in mice. Phloretin-containing cream was applied to the shaved skin area near the implantation site. After 72 hours, reporter gene activity was high in animals that had not received phloretin and was reduced, to a degree depending on phloretin dose, in animals that had been treated with the flavonoid.

The ability of *Chlamydia pneumoniae* to sense physiological signals and synchronize its metabolism with the host cell was exploited for the design of an L-arginine-regulated gene switch [177]. ArgR, an arginine-binding apo-repressor that functions as a biosensor, specifically interacts with arginine-responsive transactivator (ARG box) operators and represses the *glnPQ* operon that encodes a putative arginine transport system. The N-terminal domain of ArgR contains the DNA-binding domain, whereas the C-terminal half specifically binds L-arginine. A two-component L-arginine-regulated gene switch (ART) was assembled. The first component, encoded by a constitutively active gene, was a chimeric transactivator comprising ArgR and a transactivation domain from human NFkB subunit p65 or viral protein VP16. The second component was constructed by cloning an ArgR-specific operator module comprising two ARG boxes 5' of a minimal version of the human cytomegalovirus promoter. Cotransfection experiments showed that at a low L-arginine concentration, the transactivator was in a low-affinity DNA-binding state and did not interact with its specific operator sequence. At an elevated L-arginine concentration, the transactivator assumed a high-affinity conformation and activated transcription. The gene switch was tested in mice that had been transplanted i.p. with microencapsulated engineered cells harboring ART as well as a SEAP reporter gene. The system performed well as SEAP levels in the serum correlated with administered arginine dose.

Other bacterial transcriptional repressors were also employed for engineering synthetic circuits. A uric acid-responsive expression network (UREX) was built around bacterial repressor HucR from *Deinococcus radiodurans* that in the absence but not in the presence of uric acid binds to operator sequence *hucO* [178]. A transinhibitor construct encoded a fusion protein consisting of HucR and a KRAB transsilencing domain. A reporter construct included a SEAP reporter gene that was controlled by a simian virus 40 promoter containing multiple *hucO* modules. Cultures co-transfected with transinhibitor and reporter constructs exhibited very low basal expression of reporter in the absence of uric acid; uric acid treatment derepressed reporter expression in a dose-dependent manner. The sensitivity of the UREX circuit to uric acid could be increased by addition of an expression construct for human urate-anion transporter URAT1. A mammalian

cell line stably transfected with the latter three constructs was used to demonstrate that the UREX gene switch was capable of responding to pathological changes in urate levels in urate oxidase-deficient mice which develop hyperuricemia with human-like symptoms. (Urate oxidase converts urate into the more soluble and renally secretable allantoin.) Urate oxidase-deficient mice that had received transgenic cells intraperitoneally exhibited high levels of SEAP. SEAP activities were dramatically lower in animals that had also been treated with urate-reducing agent allopurinol. To autoregulate uric acid levels, a codon-optimized uricase/urate oxidase (Uox) gene from *Aspergillus flavus* was placed under the control of the three component UREX system. *In vitro* experiments indicated that feedback control of uric acid levels could be achieved and suggested that the approach could be utilized for reducing pathologic urate levels to physiological levels in an autocontrolled fashion.

Melanopsin, a member of the opsin family, triggers an intracellular calcium increase in response to blue light. This increased calcium level leads to calcium-dependent activation of calcineurin and calcineurin-mediated mobilization of transcription factor nuclear factor of activated T cells (NFAT). A synthetic mammalian light-controlled transcription system was recently constructed that consisted of a constitutive melanopsin expression vector and a NFAT-responsive SEAP reporter construct [179]. Cotransfection experiments showed that blue-light induced reporter expression. Reporter levels were dependent on the intensity and duration of light pulses administered. Reporter expression could also be light-controlled in a standard bioreactor setting. In mice containing intraperitoneal implants of engineered cells capable of light-triggered SEAP expression, blue light was delivered to the implanted cells by means of optical fibers. Measurements of SEAP levels in sera from irradiated and non-irradiated mice indicated that light-triggered expression was achieved *in vivo*. The system was also tested in a therapeutic setting in which expression of a glucagon-like peptide-1 variant, shGLP-1, was controlled by a NFAT-responsive promoter. Light-triggered expression of shGLP-1 was able to induce secretion of insulin *in vitro*. Transgenic cells harboring a shGLP-1 gene controlled by the light-inducible system were microencapsulated and implanted subcutaneously into diabetic db/db mice. After the animals were illuminated, insulin levels increased significantly. Furthermore, blood glucose levels remained well controlled after intraperitoneal administration of glucose.

## Concluding Remarks

Today, systems responding to chemical or physical stimuli are available for effectively timing and spatially restricting expression of a transgene. During recent years, some of the "classic" gene switches, including those based on tetracycline repressor-, ecdysone receptor- or mammalian steroid receptor-derived transactivators, have been refined to reduce leakiness in the absence of ligand. Some of these gene switches were introduced in high capacity vectors to avoid the need for using multiple separate components, were incorporated in self-inactivating vectors capable of irreversibly terminating transgene expression subsequent to a therapeutic intervention or were engineered to target specific tissues or cells. Use of zinc finger-based DNA-binding domains in regulatable transactivators or transinhibitors made it possible to target any endogenous gene. Recently developed strategies incorporated RNAi or miRNA regulation into transcriptional targeting vectors for improving stringency of transgene regulation. Ligands were modified to elevate sensitivity of regulation or achieve better ADME profiles, and transactivators that do not interact with natural ligands were constructed and successfully tested. Furthermore, novel gene

switches were constructed that are controlled by inducers such as phloretin, arginine, uric acid or blue light.

Inducible systems were incorporated in experimental cancer and neurodegenerative disease therapies to confine transgene expression to the therapeutic time window or avoid expression in locations in which transgene expression is not desired. The results of a first clinical trial using a replication-defective adenovector harboring a RheoSwitch-controlled cytokine gene were reported recently, in which trial regression of melanoma lesions was seen. Hence, regulatable systems have reached the clinic and may be on their way of finding their place in human genetic medicine. Other disorders that have been experimentally controlled by inducible systems include diabetes, multiple sclerosis, intervertebral disc degeneration, lung fibrosis and hyperuricemia.

In summary, a tremendous research effort has been dedicated to the development of regulatable switches. As a result, a multitude of promoters, transactivators, transinhibitors and other elements have been validated and are available not only for the design of gene therapy strategies but also for exploration of the functional roles of genes involved in the onset as well as progression of many diseases/disorders.

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