Chapter 4

Regulated Gene Therapy

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Abstract

Gene therapy represents a promising approach for the treatment of monogenic and multifactorial neurological disorders. It can be used to replace a missing gene and mutated gene or downregulate a causal gene. Despite the versatility of gene therapy, one of the main limitations lies in the irreversibility of the process: once delivered to target cells, the gene of interest is constitutively expressed and cannot be removed. Therefore, efficient, safe and long-term gene modification requires a system allowing fine control of transgene expression.

Different systems have been developed over the past decades to regulate transgene expression after in vivo delivery, either at transcriptional or post-translational levels. The purpose of this chapter is to give an overview on current regulatory system used in the context of gene therapy for neurological disorders. Systems using external regulation of transgenes using antibiotics are commonly used to control either gene expression using tetracycline-controlled transcription or protein levels using destabilizing domain technology. Alternatively, specific promoters of genes that are regulated by disease mechanisms, increasing expression as the disease progresses or decreasing expression as disease regresses, are also examined. Overall, this chapter discusses advantages and drawbacks of current molecular methods for regulated gene therapy in the central nervous system.

Key words Tet-responsive, Doxycycline, Promoter, Zinc finger-based transcription factor, Destabilizing domain, Trimethoprim

1 Introduction

The possibility to regulate transgene expression has been discussed in the gene therapy field for a long time (*see*, e.g., [1, 2]). In clinical settings, regulated transgene expression would allow for increased or decreased transgene levels in response to clinical need. Regulating transgene expression would ideally provide a means to avoid adverse effects due to continuous overexpression of therapeutic genes. Furthermore, the ability to turn transgene expression off and on offers experimental advantages when studying causal effects of gene transfer in disease models.

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Many different regulated gene expression systems have been developed and most operate at transcriptional levels. In this chapter we discuss three different approaches to achieve regulation of genes by gene therapy. Two are active at the transcriptional level and target either transgenic or endogenous genes. The third example regulates protein stability rather than transcriptional activity and represents a novel approach to transgene regulation that may be utilized for gene therapy to the brain.

1.1 Inducible Promoters: Tetracycline-Controlled Transgene Expression

Various drug-dependent induced technologies have been developed to control gene expression in mammalian cells. However, the most common and widely used remains the tetracycline-controlled promoter activity developed by Gossen and colleagues more than 20 years ago [3, 4]. The tetracycline systems take advantage of the tetracycline-resistance operon derived from the Tn10-resistant *E. coli* strain. In these bacteria, tetracycline-resistant mediated promoters are repressed by the binding of the tetracycline-dependent repressor (TetR) on the tetracycline operator (TetO). In the presence of the antibiotic tetracycline, the TetR is prevented from binding its operator, thus allowing transcription of the genes. Two main variants of controlled expression were developed based on this mechanism: the Tet-Off and Tet-On system. The first one uses a fusion of DNA-binding domain of the TetR, obtained from the Tn10 *E. coli*, and the C-terminal transcription activation domain of virion protein 16 of herpes simplex virus (VP16). The resulting DNA is placed under the control of a tissue/cell-specific promoter, therefore allowing expression of the tetracycline-controlled transactivator (tTA) in desired cell types. Controllable expression of a gene of interest is obtained by placing the target gene under control of a minimal promoter sequence of the cytomegalovirus promoter (CMV) fused with TetO. In the absence of the antibiotics tetracycline, the tTA, expressed in a cell-specific manner, will bind to the TetO, thus initiating the transcription of the target gene (Fig. 1). Administration of tetracycline switches off the system. Indeed, by binding the tTA, tetracycline induces conformational changes, preventing tTA from binding and activating the TetO, therefore blocking the transcription of the downstream target gene. In opposition, the Tet-On system required the presence of tetracycline to allow transcription of the target gene. Indeed, the reverse tetracycline-controlled transcriptional activator system (Tet-On), although based on the same principle, has the complete opposite effect. In more details, a mutant Tet repressor was fused to VP16, altogether coding for the rtTA, which can bind to TetO only after conformational changes occurring while binding tetracycline.

Although both systems are commonly used in neuroscience research, it is considered preferable to use a Tet-On approach for the development of gene therapy for the treatment of neurologi-

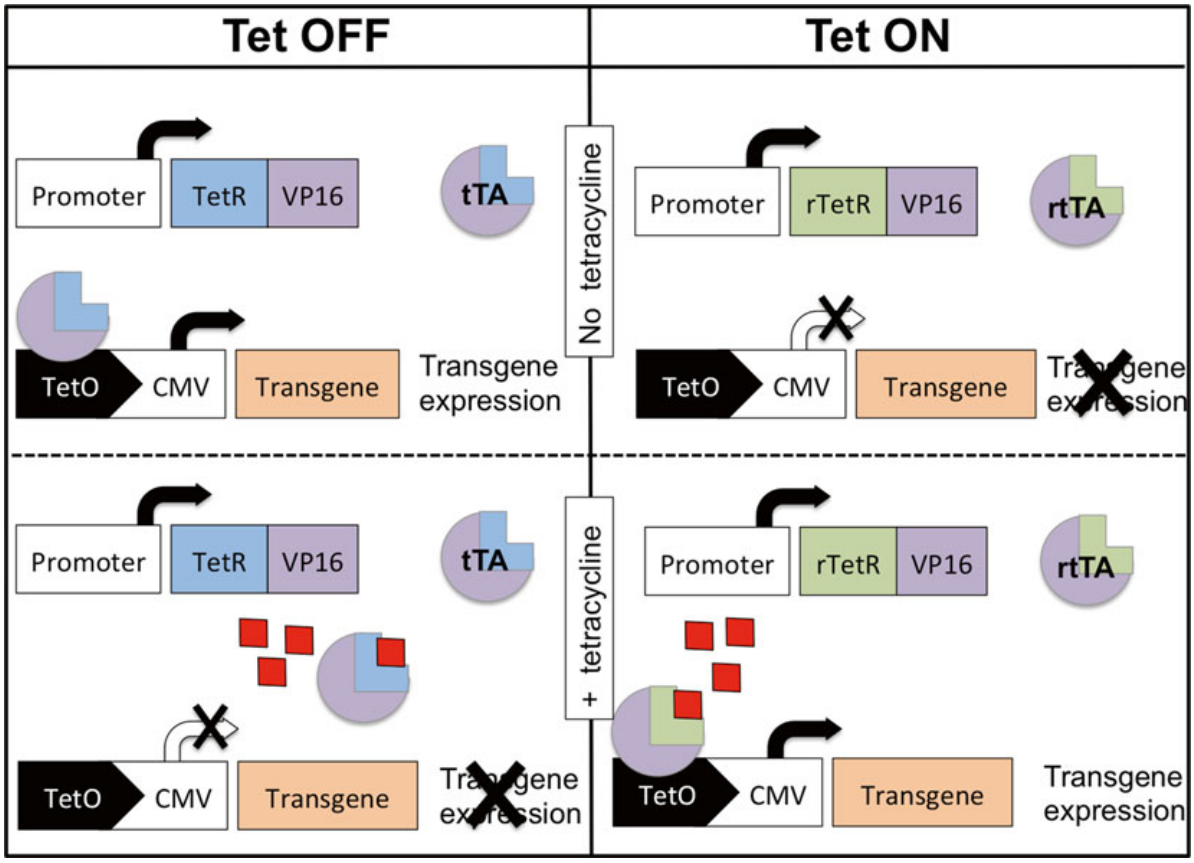

Fig. 1 Schematic representation of the Tet-Off (*left*) and Tet-On (*right*) system, in the presence (*higher panel*) or absence (*lower panel*) of tetracycline (*red squares*). Tet-Off: the transactivator (tTA) is expressed under the control of a specific promoter. In the absence of tetracycline, it binds the operator (TetO) and activates the CMV promoter, leading to transgene expression, while in the presence of tetracycline, the tTA undergoes conformation changes and is no longer able to bind the TetO which ends the transcription of the transgene. Tet-On: in the absence of tetracycline, the reverse transactivator (rtTA) is unable to bind the TetO and no transcription can occur, when in the presence of tetracycline it changes conformation and is enabled to bind the TetO, allowing transcription of the transgene

cal disorders. Indeed, an approach where transgene expression is normally repressed and will only occur when patients are submitted to treatment with the inducer is considered safer. The original inducing drug used to activate the Tet-On system was a tetracycline, but other derivatives have been used. Among theme, doxycycline, another antibiotic, is currently the most widely used as it has a low cost and a long half-life and crosses the blood-brain barrier easily [5]. However, it has been shown that the half-life of doxycycline can be reduced by 50 % when co-administered with other neurological treatments [6]. Patients suffering from neurological disorders are usually treated with various cocktails of drugs. It is therefore important to bear in mind that inducers remain active drugs, which could interact with other treatments that the patients might be on. Doxycycline has very limited side effect and

has been safely used in the clinic. In rodents, it can be administered by gavage (20–50 mg/day) or through drinking water (200 μg to 2 mg/ml) [7–9]. Importantly, if the doxycycline is administered via drinking water, sucrose (2–5 %) should be added to cover the bitter taste of the drug and water bottles should be changed every other day as the drug loses stability over time. In the clinic, doxycycline is administered orally, with doses between 100 and 200 mg/day for adults. It is of course important that the dose of inducer required to reach therapeutic level of transgene expression remains below that threshold. Prolonged use of antibiotics as inducer rises important issues, not only in terms of side effect for the patients, but it also increases the risk of promoting the development of antibiotic resistance in bacteria strains [10]. For that reason, scientists have been looking at alternatives. The use of the doxycycline metabolite (4-epidoxycyclin) or the tetracycline agonist (GR333076X), which has no antibiotic properties, is a promising option.

1.2 The Five Golden Rules for a Clinically Relevant Inducible Transgene Expression System

1. Absence of basal expression: In order to be safe, the expression should be as close to zero as possible in absence of the inducing drug ("off" state). It is indeed crucial to ensure that the residual level of expression of transgene remains below therapeutic action so the system can be shut down in case of adverse effects.
2. Rapid, dose-dependent induction: The expression of the transgene should occur rapidly after administration of the inducing drug. The level of expression of the transgene should be dependent of the dose dependency inducer administered. The inducer should be have a long half-life and be able to cross the blood-brain barrier. Finally, protein levels should be within the therapeutical range.
3. Quick shut down: In order to better manage potential side effects, the expression of the transgene should stop rapidly after discontinuation of the drug treatment. However, the stability of the therapeutic molecule will also influence the duration of ongoing adverse events.
4. Specificity: The transgene should be expressed in a discrete area and/or cells types in the brain. This will ensure maximal and localized effect while reducing the risk of adverse effect.
5. Limited immune response: The delivery of viral vectors into the brain requires surgical intervention, thus compromising the blood-brain barrier. The rupture of the wall, normally isolating the brain from circulating white blood cells, might trigger an immune response against the exogenous protein. To minimize immunogenicity, it is important to use human genes and to avoid contamination with animal products (e.g., serum in culture medium).

1.3 In a Nutshell

Main advantages: The Tet system allows fine control of level of expression of the transgene and can be shut down if necessary. Doxycycline is commonly used in the clinic to treat infections; it is a potent, low-cost, cheap, and safe inducer. Finally, the Tet system has been extensively characterized and tested in animal models of neurological disorder (for review, *see* ref. 11).

Main drawbacks: One of the main challenges associated with inducible expression approaches for gene therapy is the leakiness of the system. The existence of basal level of expression of the transgene in "off state" raises serious concern about the controllability of the system. Research is currently ongoing to improve tightness of the system, including reduction of nonspecific transactivator-TetO binding. The second issue concerns the triggering of an immune response and inflammation by the Tet transactivator. As the majority of the population have been in contact with the herpes simplex virus from which the VP16 part of the rTA has been derived, this system can be particularly immunogenic [12].

Important things to consider: The Tet systems comprise two elements, the rtTA and the transgene cassettes (Fig. 1) that can be delivered either separately or by the same vector. However, it is possible that altogether, these constructs exceed the cloning capacity of certain viral vectors (e.g. adeno associated virus ≈4.5 kb). Although the use of a dual-vector approach is possible, it results in reduced expression of the transgene, as each cell has to be transduced by the two vectors to allow gene expression. The single vector approach is therefore highly recommended. Different configurations of the Tet system have been developed and adapted for single vector approaches: either using right-facing cistrons, where the transgene and the rtTA are placed one after the other, or using a bidirectional promoter to drive the expression of the transgene, on one side, and the rtTA on the other side. Finally, the areas of the brain and the cell types to be targeted should be carefully chosen as ectopic expression can influence efficacy and safety of the treatment.

1.4 Autoregulated Promoters

An autoregulated system could be an alternative to using a drug-regulated system, such as the tetracycline and rapamycin systems. These have the advantage that no proteins of nonmammalian origin have to be over expressed and no exogenous administration of a regulating drug is required. Instead, autoregulated systems are based on a promoter or regulatory elements from an endogenous gene. Examples of promoters that have been used in autoregulated vectors for CNS gene therapy are the glial fibrillary acidic protein (GFAP) promoter and the enkephalin (ENK) promoter [13]. GFAP is expressed in astrocytes and is upregulated in the gliotic reaction following a lesion. ENK has been shown to be upregulated in striatal neurons of the indirect pathway following dopamine depletion in Parkinson's disease (PD). The authors showed that vectors contain-

ing these promoters have a similar expression pattern in rat striatum as the endogenous proteins in animals subjected to lesions or dopamine depletion, respectively. The transgene expression was also responsive to decreases in inflammation and restoration of dopamine levels. The hypoxia-responsive element (HRE) from the erythropoietin gene has also been used to achieve autoregulated transgene expression in the CNS [14]. In this system, nine copies of the HRE sequence were coupled to a SV40 minimal promoter. No transgene expression was detected in healthy mouse brain, but transgene expression could be detected following transient middle cerebral artery occlusion. These results show that promoter elements or promoters of genes regulated by disease or changes in the cells environment could be used to create autoregulated vectors.

Zinc finger-based transcription factors (ZFTFs) can be used to regulate the transcription of endogenous genes. ZFTF consists of several connected zinc fingers, which determine binding specificity, a nuclear localisation signal and an activating or repressing domain. A ZFTF consisting of six zinc fingers recognizes an 18-base pair sequence and is regarded as specific for one site in the human genome. Most studies using ZFTF in the CNS have used ZFTF designed to target and upregulate the endogenous vascular endothelial factor (VEGF) gene. VEGF plays a role in angiogenesis, but has also been shown to have neuroprotective and neurotrophic effects [15]. Beneficial effect on cell survival and motor behavior has so far been reported in rat models of stroke, spinal cord injury, and traumatic brain injury following injection of viral vectors carrying the VEGF ZFTF gene [16–18]. ZFTF has also been used in studies on the neurodegenerative disorders Huntington's disease (HD) and PD. In the HD study, the authors used a ZFTF designed to target extended CAG repeats [19]. By this approach, they were able to specifically knockdown the mutant huntingtin allele and improve motor behavior in an HD mouse model. In the PD study, the authors used a ZFTF designed to upregulate the endogenous glial cell line-derived neurotrophic factor (GDNF) gene [20]. This potent neurotrophic factor has been shown to promote the survival of dopaminergic neurons. Expression of the GDNF ZFTF in a rat model of PD reduced the loss of dopaminergic neurons and improved motor behavior.

Transcription activator-like effector based transcription factors (TALE-TFs) can also be used to regulate the transcription of an endogenous gene. TALE-TFs consist of several connected DNA-binding repeats derived from natural TALEs found in *Xanthomonas*, a nuclear localization signal and an activating or repressing domain. The DNA-binding domain of TALE-TFs is more modular than the domain found in ZFTF. While each finger in a ZFTF recognize three to four base pairs and neighboring fingers affect each other, each DNA-binding repeat in a TALE-TF recognise only one base pair without any influence from neighboring repeats. The use of TALE-TFs to regulate endogenous genes is still a fairly new tech-

nology and studies using TALEs in the CNS has therefore been few. However, a recent study combined light-inducible transcriptional effector technology with a customised TALE DNA-binding domain to create an optically controlled TALE-TF [21]. The authors used a two-component system where the first component contained the TALE DNA-binding domain coupled to CIB1 and the second component contained a light-sensitive cryptochrome 2 protein coupled to an activator. Upon exposure to blue light, CIB1 and cryotochrome 2 combine to create a functional TALE-TF. The study showed that this technology could be used to upregulate the endogenous metabotropic glutamate receptor 2 gene (Grm2) in the mouse prefrontal cortex. Upregulation of Grm2 was also possible in a more traditional TALE-TF setting using a TALE DNA-binding domain coupled directly to the activator.

Both ZFTF and TALE-TFs have the advantage that all splice variants of the gene is produced since both technologies function at the level of transcription. This is essential when overexpressing certain genes. For instance, overexpression of VEGF using cDNA for only one splice variant leads to the formation of leaky vessels. By contrast, overexpression of all the splice variants using a ZFTF designed to target the endogenous gene leads to new fully functional vessels [22].

1.5 Destabilizing Domains

It is also possible to regulate gene expression at the protein level using destabilizing domains (DD). These are protein domains that have been mutated to be readily ubiquitinated and consequently targeted for destruction to the ubiquitin-proteasome system. Depending on the protein used to create the DD, it is possible to have small-molecule ligands that shield the DD from degradation, thereby stabilizing it. Creating a fusion protein containing the DD and a protein of interest will target the whole fusion protein to degradation that can be rescued in the present of the shielding small molecule ligands. Therefore, the DD system can be used to regulate gene expression by regulating protein stability of proteins fused to DD.

To date, three different proteins have been used to engineer DD: FK506- and rapamycin-binding protein (FKBP) using the synthetic ligand shield-1 as the stabilizing ligand [23], *E. coli* dihydrofolate reductase (DHFR) using trimethoprim (TMP) as the stabilizing ligand [24] and estrogen receptor ligand binding domain (ERLBD) using hydroxytamoxifen (4OHT) as the ligand [25]. From the three DD, DHFR and ERLBD can be used to regulate gene expression in the brain as TMP [26] and 4OHT [27] can cross the blood-brain barrier.

Initial characterization of YFP fused to DHFR-based DD (YFP-DD) [24] showed that YFP-DD was efficiently regulated in the brain of rats. In a following study using YFP-DD [28], it was shown that YFP-DD expression could be reversibly regulated with peak expression 3 weeks after TMP treatment was initiated and returned to background levels 3 weeks after TMP treatment ceased.

Furthermore, placing the DD in the C or N terminus of the fusion protein influenced the stability and induction of YFP-DD. C-terminal placement of DD led to more consistent expression after induction and lower background expression when the system was turned off. Moreover, the C-terminal YFP-DD could be regulated in a dose-response manner between 0.01 and 0.2 mg/ml TMP.

The DHFR-DD system has also been used to regulate GDNF. The first-generation GDNF DD fusion proteins (GDNF-DD) resulted in limited induction of C and N terminal GDNF-DD [28]. Subsequent analysis [29] indicated that the C-terminal GDNF-DD was not efficiently processed and the N-terminal GDNF-DD had impaired secretion. To address these issues, second generation of GDNF-DD were created where the N-terminal placement of DD was optimized and an additional furin-cleavage site was added to the DD placed on the C-terminal DD.

In vitro validation assays indicated that while second-generation N-terminal GDNF-DD was efficiently secreted, it had a high background expression when the system was not induced. On the other hand, the second-generation C-terminal GDNF-DD was efficiently secreted and had a negligible background when the system was off.

Second-generation C-terminal GDNF-DD was validated in vivo in the striatum of rats. Three weeks of TMP induction was sufficient to elicit a robust GDNF-DD expression. When compared to wild-type GDNF, GDNF-DD secreted 4–6 times less protein [29]. However, the amount of secreted GDNF-DD was functional as it was sufficient to activate signaling pathways in target cells. The group of GDNF-DD animals that did not receive TMP had only minimal expression of GDNF-DD that was not functional.

GDNF-DD was also tested in a 6-hydroxydopamine induced model of PD and when induced showed neuroprotective effects comparable to wild-type GDNF. Animals where GDNF-DD was not induced showed low levels of GDNF-DD that was not functional. Moreover, the GDNF-DD animals not given TMP were comparable to YFP-DD control animals. This suggested that second-generation GDNF-DD could be regulated to therapeutic levels in vivo and exhibited a very tight regulation in vivo, event in neurodegenerative disease models.

The DD system has several advantages as the system needs only the fusion protein and has negligible expression when the system is not induced. There are also considerations for the use of the DD system. The system has a lower dynamic range of induction when compared to tetracycline-based inducible systems; therefore it is suitable for secreted proteins or proteins that do not require very high levels of expression. Moreover, the design of the fusion protein is empirical and the ideal placement of the DD needs to be validated. Due to the posttranslational nature of the regulation, the regulated proteins may be detected at low levels and need to be validated using functional assays to ensure that any residual expres-

sion inert. Although, the use of DD system in the brain is still in its infancy, preliminary studies show great promise and the system seems especially suited for gene regulation in the brain.

1.6 Guidelines for Creation and Validation of DD-Regulated Proteins

Designing DD fusion proteins: N and C-terminal DD placements need to be designed and validated for every protein of interest to assess optimal DD placement. Also glycine linkers need to be added [24] ensure minimal steric hindrance from the DD. For secreted proteins an extra furin cleavage site can be added [29].

In vitro validation: After cloning, production of viral vectors and transduction of cells, the DD can be induced using a concentration of 10 μM TMP for 24 h [23, 24, 28, 29]. A functional assay should be designed to ensure that the fusion between the protein of interest and DD is functional and that there is no leakage of the DD when it is not induced. This is of special importance for proteins that go through the secretory pathway as a reservoir of DD will be present at the endoplasmic reticulum [29, 30]. Although, this DD reservoir is not functional, it needs to be considered when validating candidates.

In vivo TMP treatments: For in vivo induction, 0.01–0.5 mg/ml TMP should be given in the drinking water of animals, continuously for at least 3 weeks to ensure a robust induction [28, 29]. YFP-DD studies indicate the animals need at least 3 weeks without TMP that to ensure that expression of DD reverts to basal levels. Similarly, to the in vitro situation, there will be a low nonfunctional expression of proteins fused to DD, particularly in the case of secreted proteins [29]. Although this background expression is inert, it needs to be accounted and assessed using functional assays in vivo.

2 Summary

The possibility to regulate gene expression by gene therapy is indeed a promising future avenue for gene therapy to the brain. It will, however, need further development and characterization to become a viable clinical option. The work includes analysis of immunological responses, regulation of repeated cycles and over long periods of time, and of course many efficacy parameters in relevant in vivo models.

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