

# Gene transfer: the challenge of regulated gene expression

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**Gene therapy is expected to have a major impact on human healthcare in the future. However, precise regulation of therapeutic gene expression *in vivo* is still a challenge. Natural and synthetic enhancer-promoters (EPs) can be utilized to drive gene transcription in a temporal, spatial or environmental signal-inducible manner in response to heat shock, hypoxia, radiation, chemotherapy, epigenetic agents or viral infection. To allow tightly regulated expression, a regulatable gene-expression system can also be implemented. Most of these systems are based on small molecule (drug)-responsive artificial transactivators. In this review, we aim to provide a brief overview of the classes of EPs and regulatable systems, along with lessons learned from these studies. We highlight the potential applications in gene transfer, gene therapy for cancer and genetic disease and the future challenges for clinical applications.**

## Introduction

Gene transfer has become an essential technique for studies of gene function *in vivo*, expression of protein or RNA molecules, and for the development of gene therapy approaches for cancer and genetic disease. Gene therapy is expected to become a promising therapeutic modality for treating and managing a diverse array of diseases. In gene therapy, it is desirable to express a specific protein or RNA molecule at the right level, right place and right time [1–3]. Enhancer-promoters (EPs), as major *cis*-acting genetic elements in the regulation of gene expression, play key roles in conferring temporal, spatial, developmental or environmental regulation of gene expression in the organism (Box 1). A natural, composite or synthetic EP, derived from the genomes of mammals, viruses or other organisms, might be sufficient in many applications. However, when stringent gene regulation is needed, the use of a regulatable expression system might be necessary (Table 1).

Several regulatory gene-expression systems are based on small molecule-responsive transactivators derived from either bacterial tetracycline (Tet) repressor, insect or mammalian steroid receptors, or mammalian FKBP12/FRAP (see Glossary). Some systems have been incorporated into various vectors to facilitate regulated gene expression *in vitro* and *in vivo* and to further improve the kinetics of gene regulation. Among them, the Tet-regulated system, which

uses non-viral and viral vectors, is the most widely exploited vehicle for controlled gene expression (Box 2). The development of this system and its applications for gene therapy and transgenic animal modeling have been reviewed extensively [2,4,5].

We aim to provide a brief overview of various natural and synthetic EPs and their specific applications, emphasizing what lessons have been learned in recent studies. We then discuss regulatable systems for controlled gene expression and strategies and potential problems for optimizing transgene expression. These principles might be helpful for those who are interested in studies of gene function, stem cell gene transfer, gene therapy for cancer and genetic diseases, and expression of therapeutic proteins.

## Lessons learned from gene transfer studies

Several lessons have been learned in the course of gene transfer studies. First, some so-called ‘ubiquitous’ EPs might not actually be ubiquitous in their activities *in vivo*. For example, an early transgenic study with RSV EP, which was presumed to be ubiquitous, demonstrated

## Glossary

**Cre/loxP system:** a genetic tool for controlling site-specific recombination events in genomic DNA. The system begins with the Cre protein, a site-specific DNA recombinase. Cre can catalyze the recombination of DNA between specific sites in a DNA molecule. These sites, known as *loxP* sequences, contain specific binding sites for Cre that surround a directional core sequence where recombination can occur.

**Enhancer-promoter (EP):** a promoter is a site on DNA to which RNA polymerase can bind and initiate transcription. An enhancer is a *cis*-regulatory sequence that can elevate levels of transcription from an adjacent promoter. More details are presented in Box 1. Some commonly used EPs are derived from human cytomegalovirus immediate early transcription unit (CMV); Rous sarcoma virus long terminal repeat (RSV); human elongation factor 1 $\alpha$  gene (EF1 $\alpha$ ); human phosphoglycerate kinase-1 gene (PGK); human telomerase reverse transcriptase (hTERT) gene; and a hybrid EP unit with the CMV enhancer linked to the chicken  $\beta$ -actin gene promoter (CAG).

**Gene transfer:** the insertion of unrelated genetic information in the form of DNA into cells.

**Mammalian target of rapamycin (mTOR):** an atypical serine/threonine kinase that has a central role in the regulation of cell proliferation, growth, differentiation, migration and survival. mTOR is also named FRAP (FK506-binding protein 12 [FKBP-12] and rapamycin-associated protein).

**RNA interference (RNAi):** the phenomenon of small double-stranded RNA (referred as small interference RNA or siRNA) inducing efficient sequence-specific silencing of gene expression.

**Small molecules for gene regulation:** some small molecules (drugs) can bind to certain transcription factors and regulate their activities in cells, and thus they can be utilized to modulate gene expression. Examples of these drugs include doxycycline (Dox), mifepristone (MFP) and tetracycline (Tet).

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### Box 1. Transcriptional regulatory elements

Transcriptional regulatory elements are composed of both *cis*-acting and *trans*-acting components. The *cis*-acting elements include promoters, enhancers and silencers. Other *cis*-acting elements, such as the locus control region and 3' specific elements, might also dictate expression of a particular gene. The *trans*-acting factors are proteins of the basal transcription machinery, transcription factors binding to promoters, enhancers and silencers, and proteins involved in the regulation of the chromatin structure.

In eukaryotic cells, each of the three RNA polymerases has its own type of promoter. The RNA polymerase II (*pol* II) promoters are mostly used to express protein-coding genes, whereas *pol* III promoters are frequently used to express small RNA molecules for RNA interference (RNAi).

The *pol* II promoter is a region of DNA to which RNA polymerase II binds before initiating the transcription of DNA into mRNA. A synthetic promoter is a DNA segment composed of synthetic elements for expressing the desired gene in a specific manner. These elements include a 'TATA' region basal promoter and upstream elements incorporating several binding motifs and novel flanking sequences for binding specific transcription factors. Enhancers can significantly elevate levels of transcription from an adjacent promoter. They can be located upstream or downstream of the promoter, in some cases several thousand base-pairs away from the target promoter.

Transcription factors are composed of several functional domains: (i) a DNA-binding domain that determines the binding specificity to DNA; (ii) a transactivation domain that interacts with the basal transcription machinery to affect the initiation of transcription; and (iii) in some cases a third domain called a regulatory domain, which binds a hormone or other small molecule and regulates the protein activity.

tissue-specific expression that occurred preferentially in tendon, bone and muscle [6]. Similarly, a recent study on delivering genes driven by either RSV or CMV EP in adenovirus (Ad) vectors in primary cell cultures showed cell-type-specific expression [7]. Yet another study found that the EF1 $\alpha$  EP directed robust transgene expression at every stage of mouse embryonic stem cell (ESC) differentiation, whereas the CMV, CAG and PGK EPs drove transgene expression at significant levels only during late stages [8]. The second lesson is that promoters influence the kinetics of transgene expression and might influence the expression of endogenous genes if integrated into host chromosomes [3,8,9]. Third, an exogenous promoter inserted into a viral vector might affect the expression of viral genes in the viral genome, thus enhancing the immu-

### Box 2. Viral vectors for gene transfer

The most commonly used viral vectors for gene transfer include adenoviruses (Ads), adeno-associated viruses (AAVs), retroviruses and lentiviruses.

#### Adenoviruses (Ads)

Ads are non-enveloped linear double-stranded DNA viruses. They can transduce both dividing and quiescent cells. Helper-dependent or 'guttled' Ad vectors are completely devoid of all viral coding sequences and were demonstrated to be significantly safer compared to early-generation adenoviral vectors, which still contain part of the adenoviral coding sequences. These vectors have been used in gene transfer for basic studies of gene functions and for gene therapy of genetic disease and cancer. Ads with E1A driven by tumor-specific promoters have often been explored as oncolytic viruses.

#### Adeno-associated viruses (AAVs)

AAVs are replication-defective, single-stranded DNA viruses. They depend on the help provided by coinfection with Ads for their replication. They are not known to cause disease in humans. In the absence of a helper, AAVs establish latency by preferential integration into a specific site on human chromosome 19. AAV vectors are frequently used for gene therapy for genetic disease and cancer.

#### Retroviruses

Retroviruses are single-stranded RNA viruses that can integrate into the genome of cells, which results in stable replication and transmission to all the progeny of these cells. Retroviruses can only transduce dividing cells.

#### Lentiviruses

Lentiviruses are a genus of retroviruses that cause persistent infection that can typically result in chronic progressive disease. HIV, SIV and FIV are all examples of lentiviruses. Lentiviral vectors can transduce both proliferating and resting cells. Lentiviral vectors can integrate into the host genome to achieve sustained gene expression and can be easily grown to titers sufficient for clinical use; therefore, they might have broad applications in gene therapy.

nogenicity of the vector [10]. In one case, the EP of CAG, but not that of EF1 $\alpha$ , activated the protein IX (pIX) promoter and enhanced production of pIX, an Ad transcriptional activator. This might be one of the main causes of Ad-induced immune responses [10]. Fourth, the use of multiple heterologous promoters in a single vector might result in mutual interference among promoters [11]. Finally, the biologically active products, such as virus-

**Table 1. General properties of different types of enhancer-promoters (EPs)**

Type	Advantages	Disadvantages
Natural EPs	<ul style="list-style-type: none"> <li>• Constitutive low or high levels</li> <li>• Tissue-, developmental-stage- and cell-cycle-phase-specific promoters for specific genes</li> <li>• Useful for long-term gene therapy</li> </ul>	<ul style="list-style-type: none"> <li>• Ubiquitously active EPs might lead to unwanted gene expression in non-targeted tissues</li> <li>• Untoward immune responses to transgene products, viral vectors and other adverse effects</li> <li>• Levels of transgene expression might not be optimal</li> <li>• Hard to fine-tune the activity of the promoter</li> </ul>
Synthetic EPs	<ul style="list-style-type: none"> <li>• Responsive to particular environmental signals, including patho-physiological signals</li> <li>• Might be useful for cancer therapy</li> </ul>	<ul style="list-style-type: none"> <li>• Endogenous genes might be affected by the same environmental signal(s) and thus side effects are produced if the signal is artificially generated</li> </ul>
Inducible gene-expression systems	<ul style="list-style-type: none"> <li>• Spatial, temporal and quantitative control of expression by a small molecule (drug)</li> <li>• Especially useful for expression of cytokines, chemokines and toxic gene products</li> <li>• Minimum safety risk due to stringent control</li> <li>• Doxycycline, mifepristone and rapamycin are clinically approved drugs</li> </ul>	<ul style="list-style-type: none"> <li>• 'Leakiness' might still be an issue for some systems</li> <li>• Artificial transactivators might be immunogenic</li> <li>• Effectiveness in humans needs to be validated</li> </ul>

encoded proteins, transcription factors and cytokines, expressed from gene-transfer vectors might change the cellular physiology and result in feedback to the tissue-specific cellular and viral promoters, thus obscuring the tissue specificity and activity of the promoter [12–15]. Taken together, these ‘lessons’ dictate that careful considerations must be exercised when selecting an EP to drive a transgene in gene transfer.

### Natural, composite and synthetic EPs

#### *Ubiquitously active EPs*

The ubiquitously active EPs, such as those of CMV, RSV, PGK and EF1 $\alpha$ , have been frequently used in gene transfer. EF1 $\alpha$  provides long-term, sustained, high levels of gene expression *in vivo* [16–18]. CMV often provides transient high levels, whereas RSV provides sustained high levels of gene expression *in vivo* but for shorter periods than EF1 $\alpha$  [3,16,19–21]. The transient expression activity of CMV might vary in different viral vectors and in different tissues. In liver, adenoviral infection activates nuclear factor- $\kappa$ B (NF- $\kappa$ B), and thus transiently activates the CMV that contains multiple binding sites for NF- $\kappa$ B [22]. In muscle, DNA methylation of the promoter region caused loss of the promoter activity over time in Ad vectors [23]. For lentiviral vectors, the CMV and CAG promoter-dependent silencing in human ESCs is dependent on viral DNA integration, even though the exact mechanisms have not been determined [24]. However, many studies have shown that a gradual loss of retroviral gene expression is caused by one or more epigenetic modifications of the viral DNA after its integration [25].

To combine the strength of each enhancer and promoter unit, some EP units from different sources have been swapped, resulting in chimeric EPs. The most widely used is the so-called CAG promoter, which combines a CMV immediate early enhancer, a chicken  $\beta$ -actin gene promoter and a rabbit  $\beta$ -globin splice acceptor. This hybrid EP drives strong gene expression in several tissues, via viral or non-viral vectors [9,17,21,26]. Another application of the CMV enhancer has been to combine it with a tissue-specific promoter for tissue-specific enhancement of gene expression [27,28].

#### *More-specific EPs*

These EPs include those whose transcriptional activities are tissue-specific, developmental-stage-specific or cell-cycle-regulated. They are potentially useful for transgene expression in a tissue-specific, developmental-stage-specific or cell-cycle-phase-specific manner, respectively. As an example, albumin and other hepatocyte-specific promoters could be used to drive transgene expression for gene therapy of liver diseases [29]. Among the tissue-specific EPs, tumor-specific EPs are a special group [30]. Several genes are expressed only in tumor cells, and their transcriptional regulatory sequences have been defined as tumor-specific. Nevertheless, most of these promoters are tissue- or cell-type-specific rather than tumor-specific, although it is possible for them to be more active in tumor cells than in their normal counterparts. Examples of this group are  $\alpha$ -fetoprotein for liver cancer, prostate-specific antigen (PSA) for prostate cancer and carcinoembryonic

antigen (CEA) for gastric cancers. By contrast, promoters of human survivin and hTERT genes are active in many types of cancer.

#### *Environmental-signal-regulated EPs*

Investigators have come up with natural and synthetic EPs that are responsive to specific patho-physiological or environmental signals for transgene expression. These environmental signals might include heat shock, hypoxia, radiation, chemotherapy and viral infection, among others.

**Hypoxia-inducible EPs.** The state of hypoxia triggers a signaling pathway that is mediated by transcription factors such as hypoxia inducible factor-1 binding to a specific enhancer, the hypoxia response element (HRE) [31]. These observations have led to the use of HREs for driving gene expression in several target tissues, from tumors to cardiac muscle [32]. Most recently, an adeno-associated virus (AAV) vector with a hypoxia-responsive synthetic promoter for inducing the expression of vascular endothelial growth factor was constructed for ischemic heart disease therapy [33]. In another study, hypoxia-dependent oncolytic Ads armed with an interleukin-4 (IL-4) gene driven by a bidirectional synthetic promoter were constructed and tested in hypoxic tumors [34].

**Radiation- and/or chemotherapy-inducible EPs.** Cancer patients frequently undergo chemotherapy and radiation therapy. It would be ideal if multiple therapies could work synergistically. The early growth response (Egr-1) gene promoter contains specific *cis*-acting sequences termed CARG elements. Ionizing radiation and DNA-damaging chemotherapeutic agents can readily induce the Egr-1 promoter via the CARG elements [35–37]. The first application of this system utilized an Ad vector, Ad.Egr.TNF, in which the pro-apoptotic cytokine tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) was under the control of CARG elements. Upon radiation or chemotherapy, TNF- $\alpha$  was induced [36–38]. Indeed, several chemotherapeutic agents, including resveratrol, are effective inducers of CARG-driven TNF- $\alpha$  [39]. Ad.Egr.TNF has been in phase I and II trials and demonstrates synergistic antitumor effects with anticancer agents [39–41]. It is currently in a phase II and III clinical trial for local advanced pancreatic cancer.

Another CARG-element-containing synthetic promoter (pE9) was made to drive production of inducible nitric oxide synthase (iNOS), which radiosensitized hypoxic tumor cells to radiation [42]. Similarly, a p21(WAF1)-promoter-driven iNOS gene therapy significantly sensitized both p53 wild-type RIF-1 tumors and p53 mutant HT29 tumors to fractionated radiotherapy [43]. Creco and colleagues have constructed hypoxia- and radiation-activated Cre/loxP ‘molecular switch’ vectors using synthetic promoters. These vectors represent a promising strategy for targeted gene therapy of solid tumors [44].

**Hyperthermia-inducible EPs.** Heat-shock protein (hsp) promoters have been used for gene therapy strategies. An Ad vector expressing feline IL-12 under a heat-inducible hsp70B promoter directed localized feline IL-12 expression in soft tissue sarcomas and limited

systemic toxicity via hyperthermia induction [45]. Focal gene induction in the liver of rats was tested by a heat-inducible promoter using focused ultrasound hyperthermia under the control of magnetic resonance imaging (MRI) [46]. The promoter of the human multidrug resistance gene harbors defined heat-responsive elements and has been exploited for heat-inducible expression of TNF- $\alpha$  for cancer gene therapy [47].

**Epigenetically regulatable EPs.** Several promoters, such as the CMV EP, a synthetic Tet-controlled minimal promoter that is derived from the CMV, are subject to epigenetic silencing when stably introduced into cells via gene-transfer vector for long-term transgene expression [23,48]. These promoters could potentially be reactivated by epigenetic agents, such as inhibitors of DNA methyltransferases and/or histone deacetylases [23,48]. An artificial epigenetic transgene switch is very useful for stringent gene regulation in mammalian cells [49]. In cancer therapy, it is possible to use epigenetic modifiers to induce expression of endogenous genes and transgenes in conjunction with immunotherapy [50]. However, it is worth noting that the epigenetic modulators might not be specific for a particular gene, thus caution must be exercised in their application, especially in non-cancerous diseases.

**Virus-specific promoters.** In contrast to the commonly used CMV and RSV EPs, other viral promoters are highly active only in the presence of certain viral proteins and are thus quite specific for the particular virus. This property could be leveraged for virus-specific gene expression. By using the upstream regulatory promoter region (URR) from human papillomavirus type 16 (HPV-16) to drive Ad *E1a* gene, an oncolytic Ad was shown to be highly selective for HPV-associated neoplasms due to the fact that expression of E1A driven by the HPV-derived URR is dependent on the expression of HPV proteins [51].

### A Gal4-VP16-based binary system for transcription amplification

The pioneering work by Ptashne and associates in the late 1980s demonstrated that the yeast transcription factor Gal4 and its derivatives, such as GAL4-VP16, can function to activate gene transcription in mammalian cells [52]. Later, Gal4 and its derivatives were shown to activate certain viral origins of DNA replication as well [53]. This early work laid solid foundations for further future development. Since then, GAL4-derivatives have been used as tools for investigating the mechanisms of gene regulation and for regulating the expression of therapeutic genes in model systems *in vivo*.

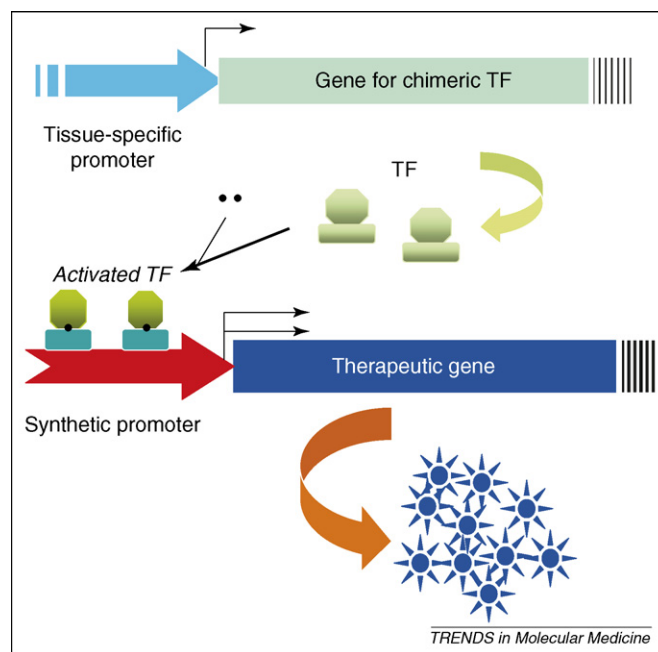
The Gal4-based systems have translated into two types of applications. The first is the amplification of the activity of a weak tissue- or tumor-specific promoter to express a transgene at high levels in a tissue-specific manner. We have shown that in such a binary system, a weak tumor-specific promoter drives the potent transcriptional activator Gal4-VP16, which in turn binds to a second promoter to drive a marker gene or therapeutic gene [54]. This binary expression system was further applied in CEA-expressing tumor models [55,56]. Other investigators have applied

such transcriptional signal amplification systems to improve the specificity and quality of *in vivo* noninvasive imaging of cancer, using prostate- or pancreatic-cancer-specific promoters to drive GAL4-VP16 in those particular types of cancer, as well as for therapeutic purposes [57,58]. The second application is its integration into small-molecule-inducible gene-expression systems, as described below.

### Small-molecule-inducible gene-expression systems

The ability to regulate gene expression in gene-transfer vectors by small molecules would be desirable. One way to achieve this goal is to introduce artificial transcription factors that target a synthetic promoter that drives the gene of interest (Figure 1). Regulating the activity of these transcription factors by exogenously administered small-molecule inducers would allow gene function to be turned on or off as desired, as well as providing precise dose-response and the ability to reverse the regulation event. In therapeutic settings for several diseases, regulated expression of therapeutic genes might be required to ensure both efficacy and safety.

Four regulatable expression systems have been extensively explored: (i) The Tet-inducible systems, known as Tet-Off (tTA) and Tet-On (rtTA), have been well studied both *in vitro* and *in vivo* and have been reviewed exten-



**Figure 1.** A schematic illustration of a typical small-molecule-inducible gene-expression system. The first promoter drives the expression of a chimeric or synthetic transcription factor (TF) gene. This TF contains a DNA-binding domain, a transactivation domain and, importantly, a regulatory domain that, upon binding of a small molecule (shown as small black circles), would change the conformation of the whole protein, making it functionally active (or inactive in the Tet-Off system). Once it is activated by a small molecule or drug, it binds to the binding sites on the second synthetic promoter to drive the expression of a therapeutic gene. Typically, the first promoter is weak but tissue-specific or tumor-specific, and thus it produces a limited quantity of chimeric TF. However, due to the high potency of the chimeric TF (e.g. one that is fused with the highly potent VP16 transactivation domain), the second synthetic promoter containing the binding sites for the TF is highly active and thus generates high levels of therapeutic proteins (shown as blue stars). Thus, a typical binary inducible gene-expression system possesses two important properties: small-molecule inducibility and a two-step transcriptional amplification with retained tissue specificity.

sively [2,4,5,59]. The original Tet-regulatable element (TRE)-based vectors suffered from disadvantages such as high basal expression 'leakiness' and low efficiency of regulation [60]. An improved TRE, marketed by Clontech as 'TRE-tight', has been developed to achieve high inducibility combined with undetectable levels of leak expression [61]. (ii) The progesterone-inducible system developed by O'Malley and associates uses a chimeric regulator whose activity depends on binding to mifepristone (MFP). This was achieved by fusing the ligand-binding domain of the human progesterone receptor hPRB891 to the yeast Gal4 DNA-binding domain and the herpes simplex virus (HSV) protein VP16 activation domain [62]. (iii) The ecdysone-inducible system first developed by Evans and associates [63]. (iv) A rapamycin-based protein dimerization system [64]. Although these inducible systems have been improved over time and have been applied to numerous preclinical studies, thus far they have not been tested in clinical trials.

### Applications in stem cell gene transfer and molecular medicine

#### *Gene transfer in stem cells*

Efficient genetic modification of animal and human ESCs is required to realize the full scientific and potential therapeutic use of these cells. Viral vectors such as lentivirus and AAV have been used for gene transfer in ESCs [8,17,18,24]. As for non-viral vectors, the Sleeping Beauty (SB) transposon system mediates stable gene transfer in human ESCs [65]. Human artificial chromosomes (HACs) can carry genomic loci with regulatory elements, thus allowing for the expression of transgenes in a genetic environment similar to the chromosome [66]. As a proof of concept, a HAC with a genomic human p53 gene was introduced into mouse p53<sup>-/-</sup> multipotent germline stem cells, resulting in functional restoration of p53 via micro-cell-mediated chromosome transfer [67].

Several EPs display dynamic activities in undifferentiated and differentiated ESCs [8,17,18]. Interestingly, transgenes delivered by lentiviral vector are suppressed in human ESCs in a promoter-dependent manner [24]. The EF1 $\alpha$  EP has been demonstrated to be one of the best for gene expression in ESCs in multiple studies [8,18,24]. Interestingly, EF1 $\alpha$  directed robust transgene expression throughout every stage of mouse ESC differentiation, whereas CMV, CAG and PGK drove transgene expression at a significant level only during late stages [8].

ESCs from mouse, monkey and human have been engineered with Tet regulatory systems for inducible expression of various proteins or RNA interference (RNAi) [68–71]. These types of genetically modified stem cells are valuable tools for basic stem cell science and might possess the potential for therapeutic applications [72,73]. Human mesenchymal stem cells can be readily isolated from bone marrow and differentiate into multiple tissues, making them a promising target for future cell and gene therapy applications. Extending the lifespan of human mesenchymal stem cells by ectopic expression of hTERT driven by the CMV EP in a lentiviral vector might be an attractive and safe way to generate appropriate numbers of cells for therapeutic applications [74]. Based on these studies, EPs

affect the level and kinetics of transgene expression in stem cells. In summary, EPs behave very differently in terms of kinetics and the strength and persistence of transgene expression in stem cells, and the cells' differentiation state might up- or downregulate the activity of the promoter in use. Thus, it is crucial to choose the most suitable EPs for the genetic engineering of stem cells. The factors to consider when selecting an appropriate EP include the particular transgene to be expressed, the biological effect of its product and the overall goals of the study.

#### *Gene therapy of cancer, including oncolytic virotherapy*

To date, only two gene-therapy products are available for clinical use. They are a p53-overexpressing Ad (Gendicine<sup>TM</sup>) and an E1B-55K-gene-deleted oncolytic Ad (Oncorine<sup>TM</sup>), both of which have been approved in China [75,76]. Gendicine is a recombinant human serotype 5 Ad with the E1 region replaced by an expression cassette, in which the wild-type human p53 gene is driven by an RSV EP [75]. In addition, as mentioned earlier, Ad.Egr.TNF (marketed as TNFerade<sup>TM</sup> by GenVec [39–41]), has been in a multi-center phase II and III randomized controlled trial in combination with chemoradiation in patients with locally advanced pancreatic cancer [77].

The development of preclinical studies has been best exemplified by the use of the hTERT promoter in hundreds of studies since 2000, shortly after its initial characterization [78,79]. The initial studies used the tumor-specificity of the promoter to drive suicide genes such as Bax and caspase-8 in cancer cells [80–82]. Later on, the hTERT promoter was incorporated into oncolytic Ads, where it was used to control tumor-specific replication via the E1A gene, which is an essential gene for viral replication [83–85]. This approach, therefore, appears to be very promising [86]. Surprisingly, however, a recent meticulous study revealed that this tumor-specific promoter does not drive E1A expression in as specific a manner as expected when used in oncolytic Ads. E1A expression and viral DNA replication were detected in both cancer and normal cells [13]. Evidence showed that there is a feedback regulation by E1A that obscures the specificity of tumor-specific promoters in the context of oncolytic Ad [13]. This is reminiscent of other studies in which tumor- or tumor-associated-virus-specific promoters failed during the development of tumor-selective oncolytic viruses [12,14,87]. All four studies highlight one of the lessons summarized earlier – viral proteins might modulate cellular and viral promoters, thus obscuring their specificity.

Synthetic or hybrid EPs have been designed to improve both the strength and tumor-specificity for gene expression. Bartlett and associates showed that by ligating the tyrosinase gene enhancer elements in tandem (Tyrex2), a Tyrex2-linked promoter could direct strong melanoma-specific gene expression in an Ad [12]. Nettelbeck *et al.* constructed a melanoma-specific oncolytic Ad in which both E1 and E4 regions were controlled by such a synthetic EP [88]. Fang and associates constructed another hybrid EP by linking the CMV enhancer to the hTERT basal promoter. This hybrid EP drives E1A expression and thus Ad replication in a tumor-specific manner. The resulting

virus suppressed tumor growth and provided a survival benefit in a subcutaneous tumor model [27]. The Wnt/ $\beta$ -catenin/T-cell factor (Tcf) pathway is aberrantly upregulated in the majority of colorectal cancers. Kuroda and colleagues have generated a transcriptionally targeted oncolytic HSV (bM24-TE) for this type of cancer. The synthetic TE EP contains 12 tandem repeats of Tcf-responsive elements followed by the minimal CMV promoter, which is linked upstream with an intronic enhancer element. Thus, this TE EP is very active in tumor cells with strong  $\beta$ -catenin/Tcf signaling, driving ICP4 expression and thus viral DNA replication [89].

#### Applications of inducible transgene expression *in vivo*

The following *in vivo* preclinical studies illustrate the potential of utilizing inducible gene-expression systems for gene therapy with enhanced efficacy and minimized toxicity.

Human monoclonal antibodies (mAbs) have been used successfully for treating diseases including cancer and autoimmunity, and there are ~20 US FDA-approved therapeutic mAbs on the market. However, serious bottlenecks in the development process remain, primarily due to the time and cost in developmental and production stages. In a recent study, Fang and colleagues employed a modification of the 2A self-processing sequence derived from the foot-and-mouth disease virus to express a full-length mAb. This mAb was expressed from a single coding sequence driven by the CAG promoter in an AAV vector. The authors have provided convincing evidence that *in vivo* therapeutic mAb gene transfer is possible at the preclinical level [90]. In a follow-up study, they deployed a gene-transfer system that allowed high-level inducible expression of unmodified mAbs *in vivo* [91]. They have used a modified dimerizer-regulated gene-regulation system in which all protein components are of human origin and expression is con-

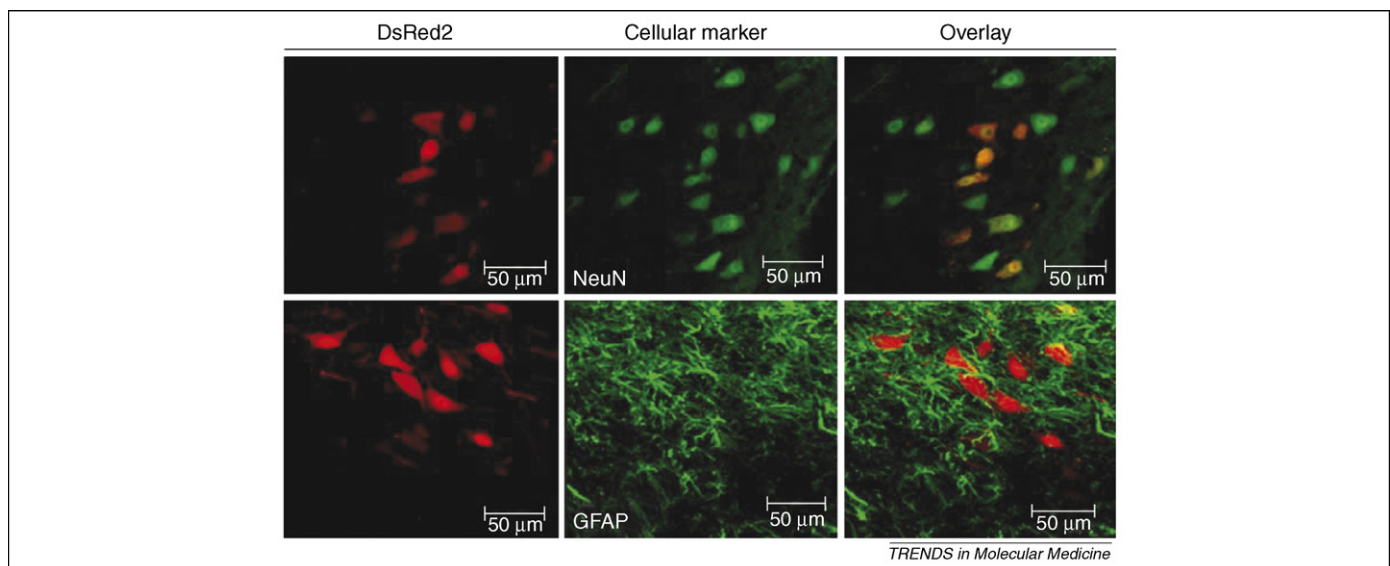
trolled by rapamycin analogs. High antibody expression levels (>1 mg/ml) were achieved after multiple induction cycles *in vivo*. As expected, the antibody production was efficiently turned off after discontinuation of the inducer drug.

Recent studies have investigated inducible expression of various transgenes in the brains of animals. In one study, Kasparov, Wang and coworkers made a GAL4-NF- $\kappa$ B-fusion-protein-based two-step amplification system and showed that this binary system can drive sustained high-level transgene expression in neurons [92]. By combining them with a Tet-Off system, the authors used lentiviral vectors to deliver genes with cell-type specificity and Tet regulation in rat brain (Figure 2) [93]. In another study, the authors developed two lentiviral vectors with two different regulatory systems for transgene induction by nonimmunosuppressive rapamycin analogs. This allowed simultaneous control of expression and of exocytosis of secreted therapeutic polypeptides [94].

#### Concluding remarks

Successful delivery of a gene-transfer vector to a targeted tissue and/or organ and subsequent expression of the transgene(s) would determine the successful outcome of any gene-transfer study and thus any subsequent therapeutic applications. As a key component of any gene-transfer vector, the utilization of appropriate EPs directly dictates the success of the gene transfer. Each type of EP has various advantages and disadvantages (Table 1). Thus, caution should be taken when choosing an appropriate EP or inducible system for transgene expression. The choice might vary depending on the vectors used, the routes of administration, the experimental systems applied and the aims of the gene transfer.

For conventional applications, a natural or composite EP might be sufficient to drive the transcription of a gene of



**Figure 2.** The activity and specificity of a TA-enhanced Tet-Off (tTA) regulatory system in the rat brain via lentiviral transfer. Two lentiviruses were used. The first (LV-Tretight-DsRed2) contains Tretight promoter (Tet-regulatable) to drive the marker gene DsRed. The second virus (LV-2xSYN-tTA) uses human synapsin 1 (SYN) promoter to drive expression of a chimeric transactivator consisting of the transactivation domain of the NF- $\kappa$ B p65 protein fused to the DNA-binding domain of the yeast GAL4 protein, and the GAL4-binding sites containing SYN promoter to drive tTA. These viruses were injected into the rat brain. Seven days later, brain tissues were collected, processed and immunostained against NeuN and GFAP for the visualization of neurons and astroglial cells, respectively. Essentially, all DsRed2-positive cells in the LV-Tretight-DsRed2/LV-2xSYN-tTA-injected rats were NeuN-positive, whereas none stained positively for GFAP, indicating that the transgene was expressed exclusively in neurons. This figure is adapted from Ref. [93]. Copyright John Wiley and Sons Limited. Reproduced with permission.

interest. As we have discussed above, these natural and composite EPs might drive gene expression ubiquitously or selectively in somatic tissues or in cancer. Synthetic EPs have been designed to respond to a specific environmental signal(s), such as stress, hypoxia, radiation, chemicals, epigenetic signals or hormones. The specific disease to be addressed might dictate the choice of EP for the expression of a therapeutic gene in a gene-transfer vector.

Stringent regulation of therapeutic genes within specific cells or in a localized anatomical region – by means of a cell-type- or tissue-specific promoter coupled with inducible expression systems in targeted vectors – will significantly decrease the potential safety risks. This is especially important for the safe replication of oncolytic viruses armed with suicide genes. It is even more critical for gene therapy of chronic diseases such as neurodegenerative disorders. Striking progress has been made in many preclinical studies (e.g. Ref. [93]). Some of the remaining challenges include: leaky gene expression in the off state; lack of penetration of the inducer to the target tissue and/or organ; and undesirable immune responses to the synthetic transcriptional proteins encoded by the inducible gene-expression systems and viral proteins from viral vectors [2,59,95–97] (Box 3). Although these problems can be further addressed in preclinical studies, inducible expression systems must be tested in clinical trials in order for this field to move forward. A key step towards the successful clinical development of inducible systems will be to investigate the use in humans of these systems driven by well-tolerated drugs with excellent safety profiles.

In addition, with the aid of systems biology and bioinformatics, it is foreseeable that we might achieve the specific and targeted gene regulation through intelligent gene-expression networks [98,99]. Such advances could facilitate the development of gene-transfer vectors armed with natural, chimeric and/or completely synthetic EPs in combination with synthetic and humanized transcription factors that can be regulated by small-molecule drugs for applications where stringent control of gene expression is needed. These synthetic systems might allow the maximal control of gene expression, offering the required spatial and temporal expression of therapeutic genes in experimental systems and, ultimately, in human patients.

### Box 3. Some outstanding questions

- How can the problem of immunity against the viral vector and transgene products be overcome to achieve long-term persistence of the viral vector?
- Will further improvements in gene regulation help to improve the safety of long-term administration of gene-transfer vectors?
- Can the problem of the frequent leakiness of regulatable gene-expression systems be addressed?
- Can small molecules or drugs penetrate efficiently to the target tissue and/or organ?
- For oncolytic virotherapy, will an oncolytic virus efficiently spread within solid tumors after systemic delivery?
- When will it be possible to test a drug-inducible expression system in clinical trials?

### Disclosure statement

D.L.B. has financial interests in Jennerex Biotherapeutics, Inc., a company developing oncolytic viruses for cancer treatment.

### Acknowledgements

We thank the reviewers for their excellent suggestions, which have been incorporated into the revised manuscript. We also thank Mark E. O'Malley for critical reading of the manuscript. This work was supported in part by the David C. Koch Regional Therapy Cancer Center. Some original studies conducted in the authors' laboratories were funded by grants from the National Institutes of Health.

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