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# Gene therapy for pituitary tumors: from preclinical models to clinical implementation

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## Abstract

Gene therapy, which entails the use of nucleic acids as drugs, is a new approach to treat disease. Gene therapy has been successfully implemented in several preclinical animal models, including several paradigms of experimental pituitary tumors. In spite of these successes, several critical issues need to be addressed before gene therapy can become a clinical reality for the treatment of pituitary tumors. These include the development of safer and more effective gene delivery vectors, the uncovering of novel therapeutic targets, the development of molecular switches which will allow turning therapeutic transgene expression “on” and “off” as and when it is needed, and the ability to scale up the vector preparations devoid of any putative contaminants. There are still many basic science developments that must take place in order to allow this new therapeutic technology to make its way successfully into the clinical arena to treat pituitary disease. We envisage these developments taking place within the next five years, gene therapy for pituitary tumors will then form part of the armamentarium available to better treat and manage pituitary tumors.

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## 1. Introduction

Pituitary tumors are very common, occurring in about 11% of the population, as determined from autopsy studies. Pituitary tumors are mostly slow growing, thereby gaining the classification of ‘benign,’ yet this histologic designation belies the true clinical impact of pituitary tumors; the regularity with which they encroach on critical neural structures, coupled with distressing endocrinopathies they frequently induce, legitimizes their status as sources of significant morbidity and, occasionally, mortality [82]. Small pituitary tumors may cause clinical syndromes due to oversecretion of hormones, i.e., growth hormone, adrenocorticotrophic hormone, prolactin, etc. Larger pituitary tumors may also elicit hypersecretion of specific hormones, but can in addition give rise to a variety of problems due to mass effects. Tumors exhibiting suprasellar extensions

may compress the optic chiasm or hypothalamic structures and the hypothalamo-pituitary stalk with resulting hypopituitarism and other complications.

Pituitary tumors are generally classified clinically by the cell of origin (Table 1), i.e., the hormones or subunits they produce; their size, i.e., whether they are microadenomas (< 10 mm in diameter), macroadenomas (> 10 mm in diameter), or macroadenomas with extrasellar extension; and by their invasiveness of surrounding structures. For most of the different pituitary tumor types, the degree of hormone hypersecretion dictates the severity of the clinical syndromes, for example in acromegaly and Cushing’s disease. For other tumor types, such as gonadotrophin-secreting adenomas, symptoms relate primarily to the size of the tumor mass and not to the hormone overproduction.

### 1.1. Current treatment for pituitary tumors

Any treatment of pituitary tumors must address both endocrine malfunction and oncological concerns. The

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Table 1  
Classification of pituitary adenomas

Adenoma cell origin	Hormone secreted	Clinical syndrome
Lactotroph	PRL	Hypogandism, galactorrhea <sup>a</sup>
Gonadotroph	FSH, LH, subunits	Silent or hypogonadism
Somatotroph	GH	Acromegaly, gigantism
Corticotroph	ACTH	Cushing's disease
Mixed growth hormone and prolactin cell	GH, PRL	Acromegaly, hypogonadism
Other plurihormonal cell	Any	
Acidophil stem cell	PRL, GH	Hypogonadism, acromegaly
Mammotroph	PRL, GH	Hypogonadism, acromegaly
Thyrotroph	TSH	Hyperthyroidism
Null cell	None	Pituitary failure
Oncocytoma	None	Pituitary failure

Hormone-secreting tumors are listed in decreasing frequency of appearance. Some tumors can cause local pressure effects, including visual disturbances, cranial nerve palsy, and headache.

<sup>a</sup> Females present with amenorrhea and infertility and males present with impotence or infertility.

three treatments currently employed, i.e., surgical therapy, medical therapy, and radiation therapy each offset symptoms of the disease but in the majority of cases, no single treatment is sufficiently effective in providing a complete cure. As such, treatment of pituitary tumors has become a multi-disciplinary endeavor, usually employing surgical therapy to eliminate the tumor mass, medical therapy to treat mainly endocrine symptoms and also shrink the tumor mass, and radiation therapy to slowly offset both oncological and endocrine concerns. Treatments are implemented on a case-by-case basis according to which treatment will appropriately manage a particular tumor type. Surgical therapy is commonly the first modality implemented, except when treating prolactinomas, in which case medical therapy will be the primary treatment option [82]. By combining different treatment modalities it is possible to reduce symptoms associated with pituitary tumors that do not respond to a single treatment strategy. However, current therapies are limited in that they manage symptoms without being truly curative, are not effective in treating all pituitary tumor types, and carry with them the possibility of inducing numerous adverse side effects. These shortcomings leave room for improvements in the treatment and management of pituitary tumors [8].

### 1.2. Gene therapy as a possible new approach

There has been much effort dedicated to developing new, more effective treatments against pituitary tumors. Although, treatment of these tumors has rapidly evolved benefiting from refinements in endocrine assays, imaging technology, transsphenoidal microsurgery, receptor-mediated pharmacotherapy, and radiotherapy we believe that gene therapy will prove a very useful addition to the treatment options for these tumors. The increasing interest in gene therapy as new treatment against pituitary tumors “has stemmed from the fact that cur-

rent treatment modalities have mostly addressed the consequences of the underlying defects, or tried to relieve the symptoms, as opposed to trying to eliminate or correct the cause of the disease” [9]. Gene therapy constitutes a novel approach to pituitary tumor treatment, which aims to use nucleic acids as “drugs” to treat disease. Below we will review the main gene therapy systems currently available and discuss their use, advantages, and disadvantages.

## 2. Viral vectors for gene delivery

The aim of gene therapy is to introduce therapeutic genes into tissues, leading to efficient and stable expression of the therapeutic gene products and minimizing any adverse effects. The two main gene delivery systems are viral and non-viral. Viruses can easily enter cells and express genetic material, therefore are much more efficient vectors when compared to non-viral systems. The most commonly used viral vectors for gene therapy applications are adenovirus (Ad), adeno-associated virus (AAV), herpes simplex virus type 1 derived vectors (HSV-1), and lentivirus vectors (Table 2). Important parameters to be considered when comparing gene therapy vectors include: size limit for transgene expression, ease of production of virus at high-titers, transduction efficiency, ability to infect dividing and/or quiescent cells, stability of transgene expression, potential to integrate into the host chromosomes, cell-type specificity, vector associated toxicity and immunogenicity (Table 3).

Any putative gene transfer vector will have advantages and limitations (Table 3). Due to the fact that Ad vectors are commonly chosen vectors for gene transfer into the anterior pituitary gland, both in vitro and in vivo, we will focus the rest of our review on the use of these vectors and also the results obtained with them in preclinical animal models of pituitary tumors.

Table 2  
Gene transfer vehicles used in gene therapy applications

	Adenovirus	'Gutless' adenovirus	HSV-l/r	HSV-l/a	Adeno-associated virus	Retrovirus (murine and human derived)	Vaccinia virus	Microinjection	Transfection
Size (kb)	36	36	152	10–30	4.68	3.5–9.2	186	Unlimited	Unlimited
Cloning capacity (kb)	7.5	~30	30	10–30	2–4.5	~8	30	Unlimited	Unlimited
Transduction									
In vivo?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No
In vitro?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Vaccination	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Vector titres (pfu/ml)	10 <sup>12</sup>	10 <sup>11</sup>	10 <sup>8</sup>	10 <sup>8</sup>	10 <sup>9</sup>	10 <sup>7</sup>	10 <sup>6</sup> –10 <sup>8</sup>	—	—

HSV-l/r, herpes simplex type 1-recombinant vector; HSV-l/a, herpes simplex type 1-amplicon; pfu/ml, plaque forming units per ml; gutless helper-dependent adenovirus vector.

Table 3  
Advantages and disadvantages of viral vectors for gene therapy

Virus	Advantages	Disadvantages
Adenovirus	Broad cell tropism, infection of dividing and non-dividing cells, easy to produce at high titer	Inflammatory and immune responses, transient expression in the pituitary
HD-Ad	Broad cell tropism, infection of dividing and non-dividing cells, less inflammatory and cellular immune response, long-term transgene expression	Difficult to produce in large-scale, inflammatory response
AAV	Broad cell tropism, infection of dividing and non-dividing cells, integration into host genome	Difficult to produce pure preparations at high titers, discrete immune response
Lentivirus	Infection of dividing and non-dividing cells, integration into host genome	Risk of insertion mutagenesis, risk of seroconversion
HSV-1	Broad cell tropism, latency in neurons, very stable	Highly toxic

### 2.1. Adenovirus vectors

Adenoviruses are a family of DNA viruses characterized by an icosahedral, non-enveloped capsid containing a linear double-stranded genome. The genome of human Ads, serotype 2 and serotype 5, both of subclass C, is approximately 36 kb long, and encodes genes that are classified into early (E1–E4) and late (L1–L5), depending on whether they are expressed before or after DNA replication [75]. At one end of its genome, the nominal left end, there is an inverted terminal repeat (ITR) necessary for initiation of viral DNA replication, and an adjacent DNA packaging signal, while a second ITR is found at the right end of the genome.

Viral infection is initiated when the Ad fiber protein binds to the coxsackievirus and adenovirus receptor (CAR) on the cell surface [2] followed by a secondary interaction between Ad penton and  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$  integrins [92]. Ad is internalized by endocytosis, triggered by the penton-intergrin interaction, and escapes from the early endosome prior to formation of the lysosome. The virion translocates to the nucleus along the microtubule network, during which time there is a sequential disassembly of the Ad virion and Ad hexon remains at the nuclear membrane while the DNA is released into the nucleus and remains episomal [46].

The most common first generation adenovirus vectors developed for gene therapy are based on the Ad2 and

Ad5 serotypes, made replication defective through replacing E1 region by transcription cassettes containing either marker or therapeutic genes. Thus, 90% of the wild-type Ad genome is retained in the vectors [17]. The recombinant E1-deleted Ad vector genomes are then transfected into human 293 cells that express the E1 proteins in trans, allowing for E1-deleted Ad vector replication and packaging. E1-deleted Ad vectors have a number of attractive attributes, one of the most important ones is their relative ease for scale up at very high titers, approximately 10<sup>12</sup> infectious units (IU)/ml. Other attractive features include the ability to infect many different cell types, both dividing and post-mitotic, terminally differentiated cells. They also have an extremely low probability of random integration into the host chromosomes [32], and a large cloning capacity of around 8 kb with full E1 and E3 deletions.

In spite of the E1 deletion, first generation Ad vectors have residual expression of viral genes that lead to a strong host immune response, resulting in generation of high titer, neutralizing anti-capsid antibodies that inhibit re-infection with the same serotype of Ad vector [61]. In addition, at high viral doses, this residual expression leads to cellular cytotoxicity which can result in an immune-mediated loss of the Ad vector transduced cells [13,62,78]. Injection the first generation recombinant Ad vectors into the brain parenchyma causes acute cellular- and cytokine-mediated inflammatory

responses, although these do not affect stability of transgene expression [84]. Transgene expression from first generation Ad is abolished in the presence of a peripheral immunization against these vectors [7,84]. Adenovirus induced cytotoxicity is only seen when high vector doses,  $\geq 10^8$  are used to infect the target tissue [85].

To overcome this limitation, a series of Ad vectors with multiple deletions have been developed. In order to propagate multiply deleted Ad vectors, packaging cell lines must be developed that trans-complement the growth of these vectors. Cell lines co-expressing Ad E1 and E4 genes; E1 and E2a (single-strand DNA binding protein, ssDBP) genes; the EI and pre-terminal protein (pTP) genes; E1 and protease genes, have been generated and used to package the E1, E4 deleted Ad vectors [30]; the E1, E2a deleted Ad vectors [5]; the E1, E2b deleted Ad vectors [36]; and the E1 and protease deleted Ad vectors [35], respectively. These Ad vectors further reduced the acute toxicity and inflammatory responses and increased the transgene cloning capacity, but still have disadvantages, i.e., viral replication was also accompanied by expression of Ad proteins, and reduced length of transgene expression was observed in the presence of an immune response against Ad.

Recently, a new generation of helper-dependent adenoviral vectors (also known as high-capacity, ‘gutless’ or ‘guttled’ vectors; HD-Ad) have been developed. These HD-Ad are devoid of all viral coding sequences [40,68] and have a minimum requirement for the extreme termini of the linear adenovirus genome, containing only those *cis*-acting elements for viral DNA replication and packaging, mainly the inverted terminal repeat sequences (ITR) and packaging signal. Since these elements are contained  $\sim 500$  bp from the ends of the genome [29], helper-dependent vectors have the potential to range in size from a few hundred base pairs to carry up to  $\sim 32$  kb of the foreign DNA, which is close to the size of the native genome. HD-Ad is co-propagated with an E1-deleted helper virus, which provides in *trans* all of the proteins required of the packaging of the vector (Fig. 1).

Up to now, several systems have been developed to prevent packaging of the helper viral genomes during the HD-Ad vector rescue/amplification process in order to limit the helper virus contamination. The Cre/loxP-based system for the generation of HD-Ad involves the use of a first generation helper virus, where the packaging signal is flanked by loxP recognition sites [31].

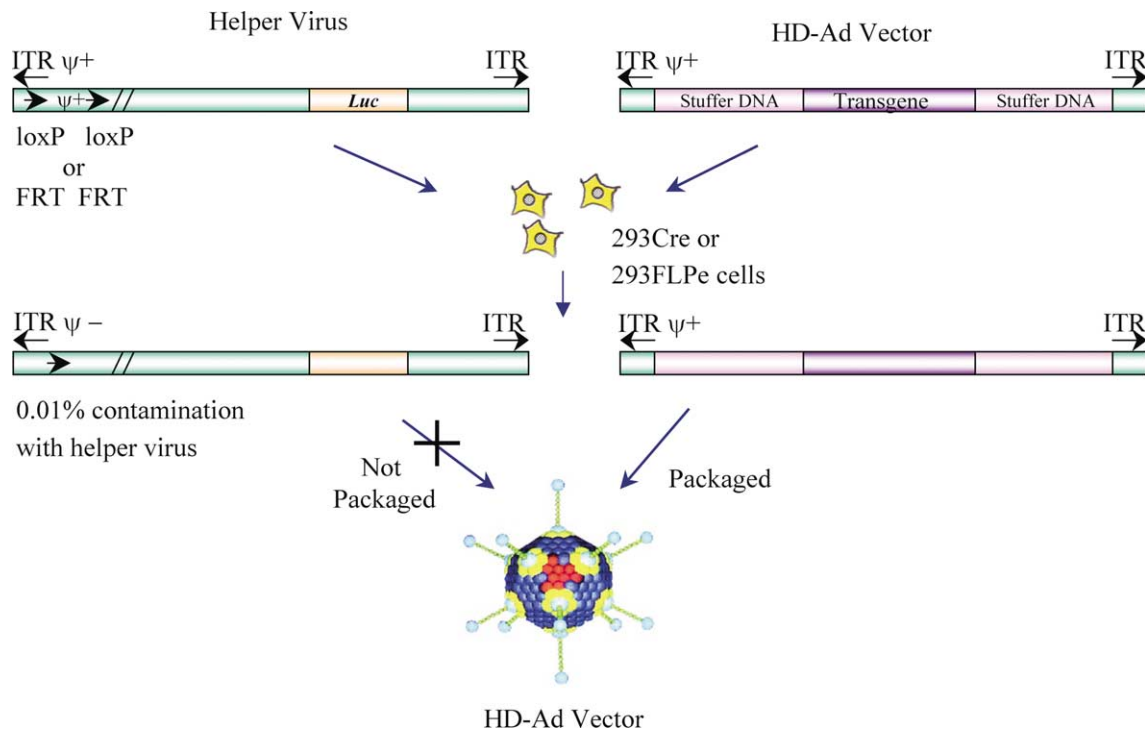


Fig. 1. Schematic representation for the production of helper-dependent adenovirus vectors. The helper virus is an E1-deleted adenovirus that contains a packaging signal ( $\psi$ ) flanked by loxP or frt recombination sites. The helper-dependent adenovirus vector genome (HD-Ad vector genome) is constructed as a plasmid containing the transgene, stuffer DNA and the adenovirus *cis*-elements, mainly the inverted terminal repeats (ITR) and packing signal. Upon co-transfection the HD-Ad vector and infection of the helper virus into a 293-derived cell line that stably expresses the Cre or Flpe recombinase, the packaging signal of the helper virus is excised, rendering the helper virus DNA unpackageable. The helper virus provides the adenoviral functions that are required for replicating the vector DNA, for producing viral structural proteins, and for the packaging of the vector DNA into virions. The titer of the helper-dependent adenovirus vectors is increased by serial passages through 293-derived cell line expressing Cre or Flpe, infected with the helper virus. A purification step using CsCl centrifugation can further reduce the contamination of the gutless Ad vector with helper virus to 0.01%.

Infection of Cre-expressing 293 cells with the helper virus results in excision of the viral packaging signal, rendering the helper virus DNA un-packagable, but still able to replicate and provide helper functions for HD-Ad vector propagation (Fig. 1) [11]. Purification by caesium chloride centrifugation is necessary to reduce the titer of the helper virus to negligible levels, typically ranging from 0.1 to 0.01% of the HD-Ad vector titer [62]. Recently, another Flp/frt system has been developed. The Flp recombinase was used in place of Cre, and shown to excise the frt-flanked packaging signal in the helper virus very efficiently [50,66,88]. The most recent improvement to this system is the development a new Cre-expressing cell line based on E2T, an E1 and E2a-complementary cell line. Thus an E1 and E2a double-deleted helper virus can be used with the new cell line to produce HD-Ad vector with low helper contamination, further improving the HD vector safety [98].

Compared with first-generation adenovirus vectors, the HD-Ad vector can efficiently transduce a wide variety of cell types from numerous species in a cell cycle-independent manner as first generation Ad vectors. HD-Ad vectors have the added advantage of increased cloning capacity, reduced toxicity and immune responses, and prolonged stable transgene expression in vivo [74,84,86]. The main limitations of HD-Ad vectors are difficulties in large-scale production and helper virus contamination [50]. If these can be overcome by new HD-Ad purification techniques, the HD-Ad vectors will become a critical viral vector for gene therapy applications (Table 3).

### 2.2. Adeno-associated virus (AAV) vector

AAV is a simple, linear, single-stranded DNA parvovirus, which is non-pathogenic to humans and is being developed as a gene therapy vector for the treatment of numerous diseases, such as diabetes, obesity, lactose intolerance, hemophilia B, and blindness.

AAV has two genes: *rep*, which encodes for replication and integration functions of the virus; and *cap*, which encodes for the structural components of the virus. On either side of *rep* and *cap* are two inverted terminal repeats (ITRs), which define the beginning and the end of the virus and contain DNA sequences needed to pack the viral genome into the capsids [81]. AAVs have the capacity of establish latency in the host cells and to integrate their DNA into a 4 kb region of human chromosome 19, designated *AAVSI* [39]. Recombinant AAV (rAAV) vectors provide long-term transgene expression without major immune or toxic responses, although neutralizing antibodies are generated. They can infect and integrate in a wide range of cells including dividing and post-mitotic cells.

The disadvantage of AAVs is their relatively small packaging capacity, approximately 4.7 kb. Because of

this size limitation, the *rep* and *cap* genes were removed from first-generation AAV based vectors to make room for the therapeutic or marker genes. It was later discovered that the *rep* gene, or at least one of its products, the Rep68 or Rep78 protein, is required for preferential integration of AAV [1]. Recent developments in AAV gene therapy vector construction allow the inclusion of the *rep* gene into a second generation AAV vector. The packaging capacity of these vectors has been extended by harnessing the observation that AAV genomes concatamerize after transduction. When two vectors, one encoding for the first half and the other encoding the second half of a protein, were transduced into cells, head-to-tail stitching of the viral genomes resulted in the reconstitution of a functional gene, effectively increasing the size of the gene that can be delivered [36,64,96]. However, this approach requires the use of two separate vectors and it will only work if the multiplicity of infection (m.o.i.) is high enough to ensure co-infection with both vectors. Another problem with AAV vectors is that it has been difficult to scale them up, and no cell lines have been reported that stably produce high-titer AAV vectors carrying a therapeutic gene (Table 3). The size limitations imposed with these vectors are likely to constitute a barrier towards developing regulatable and/or cell-type specific gene therapy approaches.

### 2.3. Lentivirus vector

Lentiviruses, such as human immunodeficiency virus (HIV), are part of the retrovirus family, but have acquired the unusual property of transducing non-dividing cells, therefore can be used in the CNS [65] and possibly the pituitary gland, although this has not yet been tested.

The first generation lentiviral vectors relied largely on substitution of viral Env protein with vesicular stomatitis virus G protein (VSVG), which relieved them of their dependence on CD4, the T-cell receptor protein required for lentivirus infection. Instead, the vectors showed a wider tropism by infecting cells known not to express CD4 protein, including neurons, hepatocytes, muscle fibers, and retinal cells. Although the first-generation vectors fulfilled many of the criteria of an ideal vector, they were viewed with great caution because of the possibility of recombination and generation of infectious HIV. To minimize some of these concerns, the lentivirus vectors have been deleted off as many viral accessory genes as possible, (i.e., genes associated with pathogenicity, *nef*, *vif*, *vpr*, and *vpu*) while still maintaining the key feature of infection of non-dividing cells [99]. An extra feature that has improved lentiviral vectors include the central polypurine tract, which allows internal initiation of second-strand DNA synthesis and probably aids in the transport of the pre-integration complex to the nucleus [97]. The development of self-inactivating lentiviral vectors in which the U3 region of

the 3' long terminal repeat has been deleted resulting in inactivation of the viral promoter substantially improved the biosafety of HIV-1-derived vectors [100,59]. These modifications reduced the risk of appearance of replication-competent viruses through recombination. Also, the viral genome has been cloned into four separate plasmids to further limit the formation of replication competent viruses [20].

Some non-human lentiviruses, such as simian immunodeficiency virus, feline immunodeficiency virus and equine infectious anaemia virus, have been used to generate efficient vectors capable of transducing non-dividing cells. There are no clinical trials with lentiviral vectors at present. Like other integrating vectors, the lentiviral vectors will have the disadvantage of non-specific integration in the host chromosomes (Table 3). The duration of expression of transgenes mediated by lentiviral vectors also needs further testing. Other modifications of lentivirus vectors which will be useful in gene therapy applications include pseudotyping with cell-type-specific envelope proteins [3,54,90] and development of tetracycline-regulatable gene expression systems [89].

#### 2.4. *Herpes simplex virus-1 (HSV-1) derived vector*

Herpes simplex virus-1 is an enveloped double-stranded DNA virus, with a genome of 152 kb in size and containing unique sequence flanked by repeat regions. HSV-1 has been exploited for gene transfer in different *in vitro* and *in vivo* models. Despite its wide transduction spectrum, it has been especially used in the central nervous system because of its ability to persist in a latent state in neurons. It has also been used to transduce the anterior pituitary gland *in vitro* [28] and *in vivo* [4]. Its genome structure allows large inserts (up to 30 kb). HSV-1 vectors that express transgenes stably for up to 18 months in dorsal root ganglia and brain stem neurons have been produced. Whether similar vectors could also be used for long-term transgene expression the pituitary gland still remains to be tested. The major drawback, which limits their application, is their toxicity and immune responses [41]. The immune response and toxicity can be reduced by using “amplicons.” HSV amplicons are prepared with a plasmid vector containing only HSV origin of replication and appropriate packaging elements. The HSV sequence from the plasmid replicates and is packaged into virions by providing the viral genes *in trans*. [80]. The first generation of amplicons was obtained using a temperature-sensitive mutant of HSV-1 as helper virus [24]. The resulting stocks were contaminated with helper virus, which expressed some viral genes and also reverted to wild-type at relatively high frequency. This caused the death of 10% of the treated animals which presents signs of encephalitis [21] and necrosis within the striatum [67].

‘Novel helper-virus free’ amplicons have been produced by using multiple restriction fragments of the helper virus genome lacking the packaging signals [15,23]. Amplicons have a large cloning capacity allowing insertion of multiple genes and transcriptional units (Table 3).

Among the viral vectors described previously, none is, at present, ideal for human gene therapy, the advantages and disadvantages of each vector are summarized in Table 3. Chimeric vectors have been developed with the aim of exploiting the most advantageous properties of each vector system such as AAV/Ad [73] or AAV/HSV-1 [15].

Significant progress in vector development is also occurring in the area of tissue- or cell-specific expression. This targeting can be achieved by two strategies. The first one involves engineering the viral capsids for binding to the cellular receptors that subsequently mediate viral entry. This type of engineering has been reported for adenoviral [91] and for AAV vectors [27]. The second strategy relies on choosing an appropriate promoter to restrict the expression of the transgene to predetermined cell types. Our laboratory has harnessed this approach to restrict transgene expression to neurons [76] glial cells [76] and lactotrophic, gonadotrophic, and corticotrophic cells within the anterior pituitary gland [42–44,77,78].

Although tissue- or cell-specific expression will continue to elicit interest, we envisage that regulated expression of the transgene will become an important focus for practitioners of gene therapy. The most widely used regulatable system is based on bacterial tetracycline resistance regulation (the Tet system) where transgene expression can be switched “on” and “off” in the absence or presence of tetracycline or its analogue, doxycycline [38,76,77,93].

### 3. Gene therapy strategies for the treatment of pituitary tumors

The majority of pituitary tumors are clinically well-managed diseases and normally, long term survival and often cure of the affected patients can be achieved. In spite of this, for some very large and locally invasive, hormone secreting pituitary tumors, treatments are far from ideal and often, a “cure” cannot be achieved. Below we will briefly discuss several treatment strategies, which could be implemented as gene therapy approaches for pituitary tumors which are resistant to currently available strategies. Many of these strategies are also being pursued for the treatment of other forms of cancer, either in preclinical animal models or in clinical trials (reviewed in [10]).

One of the commonly employed cancer gene therapy strategies uses the thymidine kinase from herpes simplex

type I (HSV1-TK). HSV1-TK converts nucleoside analogues, i.e., ganciclovir and acyclovir, into their phosphorylated metabolites, which are then incorporated into replicating DNA, causing cell death of proliferating cells. This phenomenon can be observed both in vitro and in vivo [60]. This strategy can be further enhanced by what is known as ‘bystander’ effect, which causes the death of non-transduced cells due to the transfer of phosphorylated ganciclovir metabolites to neighboring cells. In vivo this is further enhanced by the release of tumor antigens which can be taken up by antigen presenting cells and elicit an anti-tumor immune response. Other commonly used genes that activate prodrugs include: the *Escherichia coli* cytosine deaminase (cd) gene which activates 5-fluorocytosine to the cytotoxic agent 5-fluorouracil, the *E. coli* guanine phosphoribosyltransferase (gpt) gene which activates 6-thioxanthine and 6-thioguanine, the *E. coli* nitroreductase gene, the mammalian deoxycytidine kinase gene which activates cytosine arabinoside, the rat cytochrome P450 2B1 gene which activates cyclophosphamide/ifosfamide, and the bacterial carboxypeptidase G2 gene which activates 4-(*N,N*-bis(2-iodoethyl) amino) phenoxycarbonyl L-glutamic acid (CMDA) (reviewed by [12,16,60]).

The process of neovascularisation of pituitary adenomas offers another attractive target for experimental gene therapy approaches based on inhibiting angiogenesis. Several growth factors and their respective receptors are involved in the process of angiogenesis and have been shown to be critical for tumor progression. These include vascular endothelial growth factor (VEGF) [72] and its receptor VEGFR-1 or flt-1 and VEGFR-2 or flk-1 [56], transforming growth factor  $\alpha$  (TGF $\alpha$ ) and its receptor, epidermal growth factor (EGF) and its receptor, TGF $\beta$ , human platelet factor 4 (PF4) and its receptor [53] and basic fibroblast growth factor (bFGF) and its receptor [63].

It has been shown the EGF and its receptor (EGFR) are present in human pituitary adenomas, and the levels of EGFR correlate with tumor aggressiveness [47]. High molecular mass forms of bFGF have also been identified and characterized in human pituitary adenomas [49]. Normal human pituitary tissue and secreting and non-secreting adenomas can also express TGF $\alpha$  [19,22]. Moreover a lactotroph-targeted TGF-overexpressing transgenic mouse exhibited hyperplasia of lactotrophic cells and they developed prolactinomas [55]. Gene therapy approaches including anti-angiogenic genes may provide an effective in vivo therapy, i.e., expression of antisense cDNAs for bFGF, EGF or VEGF, or the expression of dominant negative mutants of their respective receptors. These strategies have been successfully used in vivo for treatment of brain tumors in animal models [10,56,70,71].

Although nerve growth factor (NGF) is known for promoting the growth, differentiation and survival of

neurons of the peripheral sympathetic nervous system, the ascending cholinergic neurons of the basal forebrain and sensory nerve cells derived from the neural crest [34,48], endocrine cells are also responsive to NGF [57]. Human prolactinomas that were totally resistant to receptor-mediated pharmacological therapy because they lacked D-2 receptors for dopamine, expressed NGF receptors [58]. When treated with NGF, the tumor cells decreased their proliferation rate, lost their capacity to form colonies in soft agar, lost their tumorigenic properties in nude mice and were immunoreactive for the D-2 receptor. These results suggest that short-term treatment with NGF may induce the reversion of human prolactinomas to a more differentiated less malignant dopamine-sensitive state [58]. Therefore, it should be possible to develop a gene therapy strategy, entailing the delivery of NGF to the tumor in situ, which may restore the susceptibility of refractory tumors to conventional receptor-mediated pharmacological treatment using D-2 agonists.

Adrenocorticotrophic hormone (ACTH)-secreting pituitary tumors are associated with high morbidity due to excess glucocorticoid production. No suitable drug therapies are currently available, and resection cannot always provide a cure. It has recently been demonstrated that immunoreactive expression of the nuclear hormone receptor peroxisome proliferator-activated receptor- $\gamma$  (PPAR- $\gamma$ ) is exclusively located within normal ACTH-secreting human anterior pituitary cells; PPAR- $\gamma$  was also abundantly expressed in human ACTH-secreting pituitary tumors [33]. These authors also showed that PPAR- $\gamma$  activators induced G(0)/G(1) cell-cycle arrest and apoptosis and suppressed ACTH secretion in human and murine corticotroph tumor cells and in animal models of ACTH secreting tumors [33]. These findings have important implications for the pharmacological treatment of ACTH secreting pituitary tumors, and also provide a novel gene therapy strategy which could be implemented by overexpressing the PPAR- $\gamma$  within ACTH producing cells.

#### 4. Preclinical models for implementing gene therapy for pituitary tumors

One of the most broadly used strategies for the treatment of pituitary tumors in preclinical animal models (Fig. 2), is the expression of cytotoxic genes from adenoviral vectors to target specific tumor cells. The HSV1-TK/GCV system is shown to be an effective strategy and is commonly used to develop cytotoxic gene therapy for cancer. One of the therapeutically useful aspects of this treatment combination is the ‘‘bystander effect’’ phenomenon as previously described. This is particularly helpful in terms of therapy, as it means that not all tumor cells need to be transduced for

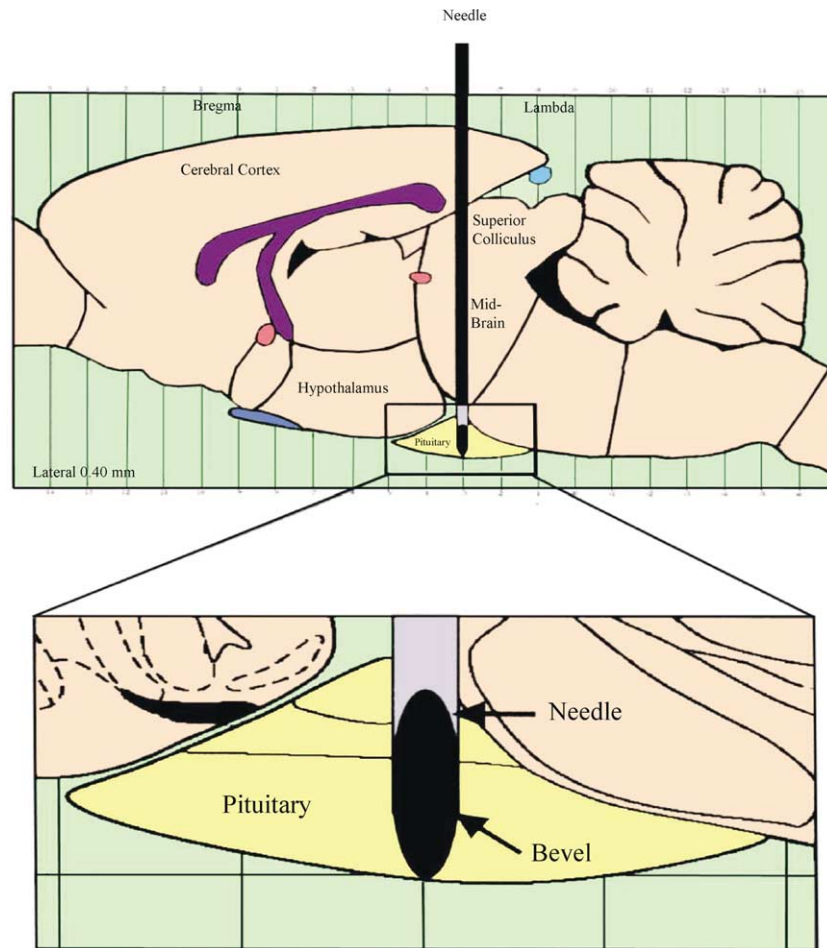


Fig. 2. Delivery of recombinant adenovirus to the anterior pituitary gland in vivo. Following anesthesia with halothane and placement of the rat in a stereotaxic frame, the skull is exposed and a hole is drilled posterior to Bregma revealing the superior sagittal sinus, and surrounding brain. Intrapituitary injections are made using a 26 gauge modified Hamilton syringe needle, with its tip previously ground until the opening of the needle is at the base of the tip. Injections are made at the following coordinates: antero-posterior from Bregma,  $-0.57$ ,  $-0.60$ , and  $0.63$  cm, and lateral on each side of the midline at  $0.05$  cm. A total of six injections per pituitary gland are made. The needle is lowered at each coordinate until touching the sphenoidal bone, and making contact with the bottom of the rat equivalent of the sella turcica. This leaves the opening of the needle within the pituitary gland and adequate amounts of recombinant vector are then injected. Under these conditions of injection, the pituitary can be transduced by recombinant adenovirus in 100% of surgical attempts. At each of these six coordinates  $1 \mu\text{l}$  of the recombinant vector,  $2 \times 10^7$  pfu, is then delivered over 1 min per injection site. Animals are then given 10 ml of saline subcutaneously and allowed to recover.

the therapy to be effective. Both in vitro and in vivo studies have demonstrated that targeted expression of conditionally cytotoxic genes such as HSV1-TK to specific anterior pituitary tumor cells, using hormone specific promoters, is an effective therapeutic strategy (Fig. 3). Adenoviral vectors encoding HSV1-TK under the control of the GH promoter have been shown to result in noticeable reduction in tumor size of GH secreting tumors [42]. Similarly, using an in vivo model, injection of adenoviral-mediated targeted expression of HSV1-TK under the control of the POMC (proopiomelanocortin) promoter was shown to significantly induce regression of transplantable anterior pituitary tumors [44]. AtT20 cells synthesizing ACTH transplanted into nude mice induced Cushing's syndrome like features and were used as an in vivo model of ACTH-producing tu-

mors. The administration of a conditional cytotoxic gene under the regulation of pituitary hormone specific promoters via adenoviral mediated transfer, had an effective therapeutic outcome with regression of anterior pituitary tumors.

Suicide gene therapy with HSV1-TK has been shown to be effective and nontoxic to the pituitary gland in situ. Adenoviral vector encoding HSV1-TK in the presence of GCV causes cell death in pituitary tumor cells, i.e., AtT20 Ad GH3 cells. In the in vivo model of sulpride- and estrogen induced lactotroph hyperplasia, intrapituitary delivery (Fig. 2) of RAd-HSV1-TK and consequent treatment with GCV decreased circulating prolactin levels and reduced the tumor mass without causing adverse side effects on other pituitary hormone levels [95].

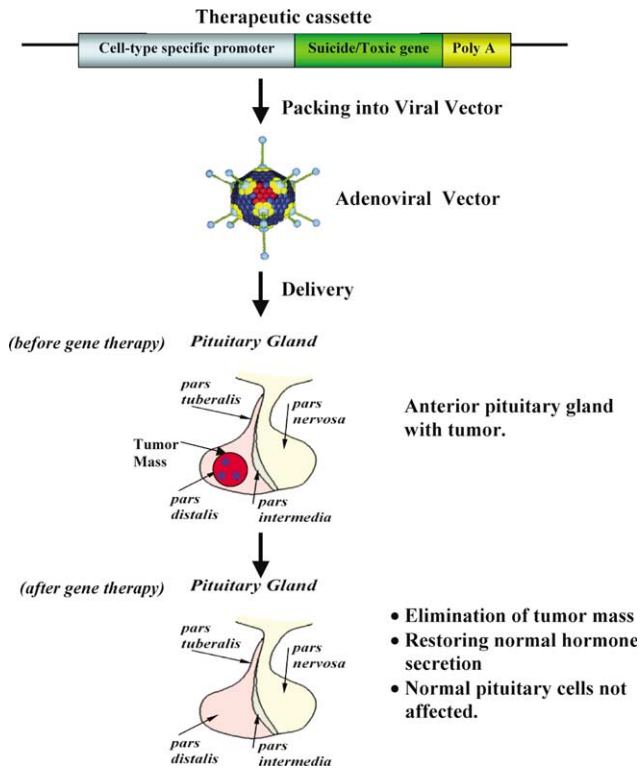


Fig. 3. Diagrammatic representation of a transcriptional targeted gene therapy approach for the treatment of pituitary tumors. Viral vectors encoding the therapeutic cassette can be delivered directly to the pituitary tumor mass by stereotaxic injection. Expression of therapeutic transgene will be under control of a tumor cell-type specific promoter. This will result in the specific elimination of the tumor mass, with restoration of normal hormone levels in the periphery. This type of targeted gene therapy should minimize any putative adverse side effects in the normal surrounding areas within the anterior pituitary gland.

As well as the HSV1-TK gene, other suicide genes such as the diphtheria toxin, a potent cellular toxin, was proven to be effective for targeted-toxin gene therapy in the anterior pituitary gland. The diphtheria toxin has often been utilized for the management of cancers that are of malignant nature [45]. In spite of its potent toxicity, the usefulness of DT was demonstrated for targeted suicide gene therapy for GH secreting adenomas [45]. The co-infection of a dual-component recombinant adenoviral system to GH secreting tumor cells in vitro and in vivo, using the Cre-recombinase integrase system, allowed controlled and efficient targeted transgene expression to GH secreting tumor cells. Cre-recombinase, a topoisomerase from bacteriophage initiates the site-specific recombination of DNA between unique sites called the loxP sites. The first component encodes sequences for the inhibition of transcription of the suicide/marker gene edged with loxP sites. The second component carries the Cre-recombinase construct under the control of cell-specific (GH) promoter. An adenovirus carrying the DT gene and Cre recombinase was shown to cause a significant regression of GH secreting so-

matotropic tumor cells [45]. These findings demonstrate the potential application of Cre-mediated activation of a loxP-inactive form of the DT gene as an effective therapeutic strategy for localized suicide gene therapy.

It has also been possible to achieve transcriptional targeting of transgenes to lactotrophic cells within the normal anterior pituitary gland in vivo and in an estrogen/sulpiride-induced hyperplasia model [18,79]. In in vitro studies, the use of the human prolactin (Prl) promoter within adenoviral vectors, targeted transgene expression to lactotrophic tumor cell lines. Targeting the expression of the HSV1-TK conditional cytotoxic gene, to lactotrophic hyperplastic cells in situ was not effective to reduce either the mass of the pituitary gland or circulating prolactin levels in the in vivo model of lactotroph induced hyperplasia [78]. In contrast, the human cytomegalovirus promote (hCMV) promoter driving the expression of HSV1-TK was shown to be effective in causing reduction of the pituitary mass and normalization of circulating hormone levels. This could be due to the lower levels of transgene expression elicited by the Prl promoter when compared to the hCMV promoter [78].

#### 4.1. Cell-type specific and regulatable transcriptional targeting of transgene expression

An effective and a promising molecular strategy for pituitary tumor management is the ability to not only restrict, but also be able to regulate the levels of transgene expression within a specific pre-determined cell type. Pituitary cells, due to their unique expression of distinct hormone gene, epitomize a perfect model for developing targeted expression of specific genes using cell-type specific promoters.

The tetracycline (tet)-dependent regulatory system is a popular and widely studied transcriptional activator system that enables one to regulate transcription of certain gene(s). Tet-off and novel tet-on systems allow us to induce desired gene expression in the absence and presence of an inducer (doxycycline) respectively. The regulatory system is composed of two essential components, the transactivator under the control of either a ubiquitous or cell-type specific promoter and a tet-response element directing the expression of the transgene. The capacity to control transgene expression will be essential to the successful gene therapy treatment of anterior pituitary tumors. Occasionally surgical resection of pituitary tumors can bring about hormone deficiencies, the ability to induce transgene expression in hyposecretion related pituitary disorders would be of great importance for treatment of pituitary tumors.

Under the regulation of the tet-off system, controlled expression of tyrosine hydrolyase (TH) via adenoviral vectors has been shown to be an effective strategy in

the treatment of prolactinomas [93]. These frequent kind of pituitary adenomas cause enhanced synthesis and secretion of prolactin levels and the existing foremost line of treatment is the utilization of dopamine D<sub>2</sub>-receptors agonists. TH is the essential rate-limiting enzyme in dopamine production. Dopamine is recognized to exert an inhibitory effect on the release of prolactin via inhibition of the transcriptional activation of the prolactin gene. In a model of estrogen-induced pituitary tumors, regulated RAd-mediated delivery of the TH gene resulted in enhanced dopamine synthesis locally and consequential regression of induced prolactinomas within the anterior pituitary in situ [93]. Expression of TH was observed in all different hormonal cell types in the absence of tetracycline. These data illustrate that the effective regulation of dopamine production can be attained and would be of enormous advantage therapeutically for the successful treatment of prolactinomas. This gene therapy approach can be a valuable and effective alternative for those prolactinomas which do not respond to classical treatment modalities.

A further improvement to this approach involves the development of gene delivery systems which can not only provide a molecular switch to turn gene expression “on” and “off,” but can also restrict transgene expression to pre-determined cell-types. Dual adenoviral vector systems carrying cell type specific and transcriptional regulatory units have been engineered and tested in both in vitro and in vivo to attain the desired cell specific transgene expression and their regulation. Adenoviral vectors encoding cell specific promoters such as glial derived fibrillary protein (GFAP) and neuronal specific enolase (NSE) promoters have been shown to confine tet dependent transgene expression to glial and neural cells in vitro and in vivo respectively [76]. Development of such systems with inducible transcriptional elements using adenoviral vectors are of great potential to not only restrict therapeutic transgene expression within brain but also successful transgenesis and gene therapy approaches for pituitary diseases. The tet dependent tet-off system enabled the switching “on” and “off” of transgene expression within specific cells of the anterior pituitary gland in vivo. Using the tet-off system, under the control of the human prolactin promoter, the inducibility, lactotrophic cell-type specificity and levels of transgene expression were characterized in both in vivo and in vitro [77]. The absence of doxycycline induced effective transgene expression within lactotrophic cells and the presence of doxycycline inhibited transgene expression. The use of combined cell specific and regulatable transgenesis first accounted with these studies have important implications for gene therapy and can permit timed and sequential transgene expression within the specific desired site tumor cell-type [77,93].

#### 4.2. Long term transgene expression within the anterior pituitary

The time span of transgene expression is another important issue for gene therapy. Current crucial concerns associated with long term stable transgene expression is cytotoxicity and immune responses [51]. Stable expression of 3–12 months is considered long term expression. No profound cytotoxicity and disturbances of anterior pituitary hormonal functions were reported in some studies during long term transgene expression [78]. Long term adenoviral vector mediated delivery carrying the HSV1-TK gene with human prolactin promoter to the anterior pituitary in situ was evaluated with the intent to evaluate the safety of this approach [78]. Virus induced inflammation was induced after the initial viral injection but it subsided within one month after the initial infection. Pituitary hormone levels were impervious with the exclusion of ACTH, during long-term transgene expression mediated by adenoviral vector gene delivery. This could be due to the initial stress induced by the surgical interventions. Work performed in the sheep pituitary gland in situ, has recently shown that when very high doses of recombinant adenovirus vectors ( $>10^9$  pfu/gland) were used, inflammatory responses, prigliandular fibrosis, lymphocytic infiltration and renulitis were observed [18]. Focal necrosis and/or apoptosis were also noted in six out of nine cases. These results highlight the critical importance of evaluating both the efficacy and also the putative side effects of these gene transfer systems employed. In this case, it highlights that the dose of vector employed in a critical consideration when assessing the safety of these approaches and the use of strong promoter systems should be critical to ensure the use of lower and safer doses of vectors [25]. Additional developments need to be made to examine the efficiency of long-term expression observed over time in non-human primates and eventually in humans.

#### 4.3. Adenoviral mediated delivery of hormonal receptor genes for hypoplastic pituitary disorders

Hormonal deficiencies within the pituitary possibly as a consequence of cell receptor mutations can lead to hypoplasia. Also, hypopituitarism can arise as a consequence of tumor development and/or treatment within the anterior pituitary gland. The use of adenoviral vectors to express the hormonal specific receptor gene within the pituitary is an effective way of restoring hormonal function of the pituitary gland. Studies with GH secreting cells and breast cancer cells were performed to evaluate the effectiveness of receptor transfer via adenoviral vectors. Adenoviral vector encoding the growth hormone releasing hormone (GHRH) receptor with the cytomegalovirus (CMV) promoter infected in

GH3 cells showed GHRH receptor expression on cell membranes and revealed GHRH binding resulting in enhanced cellular proliferation and second messenger components for receptor mediated activation [44]. Estrogen receptors are expressed in human breast cancer tissue and are regarded as an essential pharmaceutical target for the management of breast cancer. Delivery of mutants of the dominant negative type of the estrogen receptor that interrupt endoplasmic reticulum function, within breast cancer cells, *in vitro* and *in vivo*, via adenoviral vectors, inhibited tumor formation. Also, administration of adenoviral vectors carrying these mutants to pre-established tumors, triggered tumor regression and apoptosis [44]. The use of estrogen receptor mutants may be an essential molecular tool for targeted therapy of breast cancer. Extensive studies need to be conducted to establish the safety, efficacy and possibly examine the stability and response to regulatable and long-term receptor gene expression within the pituitary gland. Cell-type specific hormonal receptor transfer via adenoviral vectors mediated gene therapy is an attractive approach for the management of hypoplastic pituitary disorders.

### 5. Clinical implementation of gene therapy for pituitary tumors

Gene therapy is a new medical technology, and as such, it can be associated with unknown risks and adverse side effects. It is therefore of critical importance that these new therapeutic strategies are extensively tested both in terms of their efficiency and of their safety in preclinical animal models before embarking in clinical trials. Clinical trials of gene therapy have been conducted in patients suffering from cystic fibrosis and adenosine deaminase (ADA) deficiency, which, although incurable, can be treated with pharmacological therapies. Although most clinical trials in gene therapy have been for the treatment of cancer, many others have been performed in patients who do not have a terminal disease.

As the technology to develop safe gene transfer vectors improves, gene therapy applications will increase and gain clinical consensus for the treatment of chronic diseases [8,52]. Novel vectors have been generated that

do not elicit strong inflammatory responses, do not express viral genes that can be recognized by the adaptive immune response, and allow long term transgene expression. For clinical applications, one year of transgene expression does not appear to be a very long time, nevertheless, this represents 30–50% of the total life span of a rodent and, in pre-clinical models, stable expression for a period of 6–12 months is considered stable, long-term expression. Nevertheless, it will be critical to assess the effectiveness of long-term expression and long-term treatment over several years in clinical trials. This is currently the case for implementation of novel pharmacological treatments.

The implementation of gene therapy approaches for non-life-threatening diseases needs to be considered very carefully (Table 4). Pituitary tumors can be an excellent but the same time, very challenging therapeutic target. Many pituitary tumors are treated successfully and are generally not life-threatening, in the short term at least. In spite of this, there are several tumors which are resistant to treatment and that do not respond to currently available therapies, [6,14]. Tumor recurrences are also often encountered, and also there are patients who do not tolerate pharmacological treatments [69]. Surgery also presents complications, the most common of which are permanent diabetes insipidus or hypopituitarism. The incidence of these complications is low, and usually this is dependent on the expertise within the different clinical centers [87,94]. One also needs to take into account that a small proportion of pituitary tumors either cannot be respected completely or can be invasive. This allows tumors to recur and disease to progress after the implementation of classic therapies. Radiotherapy is also used for the treatment of some pituitary tumors. Potential complications after radiotherapy include damage to the optic nerves, hypopituitarism and, in the long term, the development of secondary brain tumors [26,37]. The incidence of such side effects is currently low, and therefore it does not represent an important clinical limitation.

Due to the limitations discussed earlier, there is a need to develop novel treatments that could complement those which already exist. Gene therapy could constitute a very attractive therapeutic modality to treat pituitary tumors. The pituitary gland is an attractive organ for attempting gene therapy because its biology is reason-

Table 4  
Considerations for the implementation of clinical gene therapy

Effective	Efficient delivery and expression of therapeutic transgenes which should enable reversion of the disease phenotype. Adequate persistence of transgene expression. Transgene expression should be turned “on” and “off” as and when it is needed. Cell-type specific expression of therapeutic genes
Safety	Elimination of adverse immune responses and other cytotoxic reactions, both local and/or systemic, within the patient. Limited therapeutic vector spread <i>in vivo</i>
Clinical implementation	Large-scale production and quality control of vectors. Compliance with local and national gene therapy advisory committee’s regulations. The cost of the therapy versus size of patient population to be treated. Benefits to health-care provision. Effects on long term survival and overall quality of life

ably well understood and many advances in our understanding of the molecular basis of several pituitary disease states have now been made. However, there are several issues that need to be addressed before successful therapy can be implemented. These include the characteristics and stage of the disease to be treated, the therapeutic target genes that are most appropriate, the levels and long-term persistence of transgene expression, and the specific cell type, which needs to be transduced. All these considerations will all play a crucial role in designing and developing a gene therapy vector for clinical use. It will also be critical to evaluate the therapeutic effectiveness and potential adverse side effects of the proposed therapeutic approach in preclinical animal models, such as rodents. The toxicity will also need to be evaluated in non-human primates. Once these issues have been extensively investigated and evaluated, one could plan, and then implement, clinical trials (Table 5).

Gene delivery to the pituitary gland in humans will initially make use of current neurosurgical approaches, and gene therapy will be performed concomitantly with current neurosurgical treatments. Gene therapy could be used either to eliminate an otherwise intractable pitui-

tary tumor. It could also be used to engineer cells to secrete pituitary hormones under inducible promoters to insure adequate levels of hormonal secretion. In the case of scant normal pituitary tissue remaining after surgery, it should be possible to engineer the remaining cells using gene therapy so that they will secrete the appropriate hormones, at the appropriate levels (see Figs. 1–3).

Realistically, the first pituitary gene therapy trials will be conducted in combination with current surgical treatments. We envisage a scenario in which the neurosurgeon removes a pituitary tumor by transsphenoidal surgery. In the case of complete removal, the neurosurgeon might not need to administer additional gene therapy. However, in some cases of partial tumor removal, the surgeon might choose to deliver the vector into the tumor bed with the aim of transducing any tumor cells remaining within the pituitary gland (Fig. 4). Also, taking into account that many pituitary tumors recur, the surgeon could transduce the remaining pituitary tissue with gene therapy vectors and, should the tumor recur, expression of the vector could be reactivated. Such treatment modality would limit the number

Table 5  
How to improve existing gene therapy vectors for use in clinical trails

- Increase efficiency of expression to allow lower doses of vector as required
- Prolong the duration of transgene expression
- Generate vectors with reproducible high titres and no contaminants.
- Increase the area of transduction
- Target infection and transgene expression to allow systemic delivery
- Generate transcriptional switches to allow to turn therapeutic transgene expression “on” or “off”

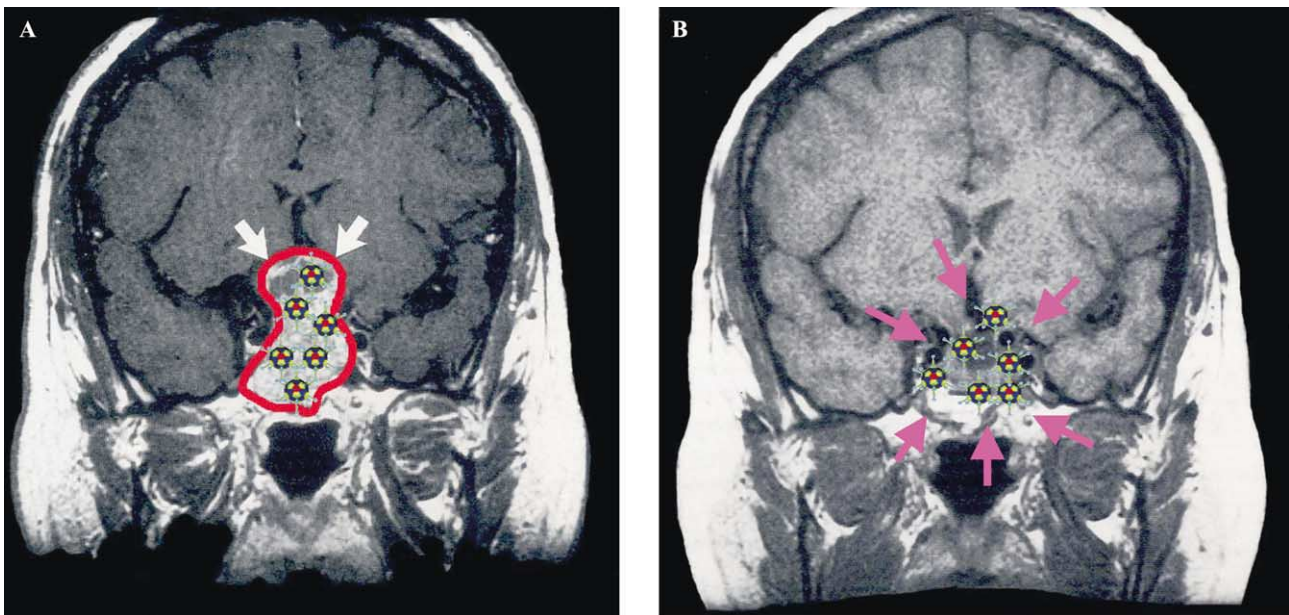



Fig. 4. Gene therapy strategy to treat pituitary tumors in humans. MRI scan shows a lobulated gonadotroph adenoma before surgery (A) and the sellar appearance three months following transsphenoidal surgery (B). Gene therapy can be delivered intratumorally or in the tumor bed at the time of surgical resection.  symbolize the gene therapy vector within the tumor mass itself (A) or the tumor bed (B).

of interventions to which the patient would need to be exposed and would also combine two complementary treatment modalities.

In the case of inoperable tumors, the gene therapy vector could be delivered within the tumor mass itself. In addition, gene therapy would be aimed at reducing tumor mass or normalizing hormone secretion. Thus, combining dopamine agonists with gene therapy might provide a long-term cure for macroprolactinomas that do not respond well to dopamine agonists alone, or when patients develop dopamine intolerance.

Ideally, pituitary gene therapy would be administered via a vector that could be targeted directly to specific pituitary cells; systemic administration would be preferable. However, at present, no such system has been developed for any organ. The main limitation to this development is that all vectors are taken up by non-specific, widely distributed mechanisms. Nevertheless, novel retargeting methods are proving instrumental in removing this non-specific viral vector entry [83,91].

## 6. Conclusions

Gene therapy offers many potential strategies for the treatment of pituitary tumors and other pituitary diseases. Approaches that can ablate tumor cells, inhibit their growth and/or restore normal hormone secretion could be implemented. Taking into account that pituitary function can be easily monitored by using hormone assays in peripheral blood, the availability of excellent imaging techniques and the easy surgical access to the gland, it would be realistic to assume that clinical trials to treat these disorders could become a reality within the next 5–10 years.

The critical issues, which need to be addressed, are the stability of transgene expression, the capability to regulate very accurately the levels of therapeutic transgenes and even, the ability to switch transgene expression “off” and “on” as and when needed. Also, and of critical importance is to develop gene delivery vectors which exert minimal or no adverse side effects and preserve normal hormone secretion from the cell populations which are not affected by the disease. This will need the concerted efforts of basic scientists developing the tools for the gene therapies, endocrinologists, neurosurgeons, and imaging specialists. This will ensure not only the crucial basic science developments, but equally important, the best clinical provision.

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