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Molecular therapy in a model neuroendocrine disease: developing clinical gene therapy for pituitary tumours

Maria G. Castro, Thomas Southgate and Pedro R. Lowenstein

The main objectives of pituitary tumour treatment are to restore normal function of the pituitary gland and prevent tumour recurrences. In spite of the success of current therapies in the treatment of relatively small tumours, new therapeutic alternatives need to be explored for large invasive tumours, tumour recurrences postsurgery, and when intolerance to drug treatment develops. Gene therapy, which uses nucleic acids as drugs, is a very attractive alternative to classic therapeutic modalities. With the development of efficient gene delivery vectors, which allow widespread distribution and long-term transgene expression with limited side effects, the clinical implementation of gene therapy for the treatment of pituitary tumours will become a reality within the next five to ten years.

The development of gene therapy has created a large enterprise dedicated to the production of vectors capable of delivering therapeutic nucleic acid constructs to the appropriate target cells, organs or tissues. The development of potential gene therapies for the treatment of many different types of diseases needs underpinning by efficient and safe transgene delivery systems.

Vectors in clinical and experimental use are either viral derived or non-viral[1]. Initial experimental and clinical gene therapy was based on the use of retrovirus-derived vectors[2], although almost any virus can be adapted to perform as a gene therapy vector. The robustness of retroviruses is exemplified by their selection as the vectors of choice in the recent successful gene therapy clinical trial for human severe combined immunodeficiency X1 (SCID-X1) disease[3].

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Box 1. Advantages and disadvantages of different gene therapy vectors

Advantages of different vector systems

- Adenoviruses can be grown to very high titres
- 'Gutless' adenoviruses allow cloning of up to 28 kbp of foreign DNA and can sustain very long-term transgene expression even in the presence of peripheral immunization[a]
- Murine retroviruses integrate into dividing cells only
- Lentiviruses integrate into both dividing and nondividing cells
- Adeno-associated viruses are minimally proinflammatory and exhibit minimal immunogenicity
- HSV-1-derived vectors can establish latency in dorsal root ganglion neurons and possibly in neurons of the central nervous system
- Liposomes are devoid of the potential toxic side effects associated with viral gene products
- RNA virus-derived vectors (e.g. vaccinia- and Semliki forest virus-derived vectors) can express transgenes at very high levels[b,c]

Disadvantages of different vector systems

- Adenovirus capsids and genome-encoded proteins are inflammatory and immunogenic
- Expression from retroviruses becomes silenced despite the insertion of their genome into that of target cells
- Lentiviruses can only carry up to 10 kbp of foreign DNA
- HSV-1-derived vectors could potentially reactivate latent wild-type HSV-1
- RNA virus-derived vectors only allow transient transgene expression

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Box 2. Improvements needed for existing gene therapy vectors

- Increase efficiency so lower doses of vector are required
- Decrease immunogenicity so readministration is possible
- Increase levels and duration of transgene expression
- Generate vectors with reproducible high titres
- Increase transduction areas
- Target infection and transgene expression

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One limitation of retroviruses, to their use as gene therapy vectors, is their lack of integration into quiescent cells. This failing led to the development of lentiviral vectors[4,5], which can infect both actively dividing and quiescent cells. Other commonly used vectors are those derived from human and animal adenoviruses, human adeno-associated viruses (AAVs) and human herpes simplex virus type I (HSV-1)[1].

Each vector system has both advantages and shortcomings, vis-à-vis individual gene therapy applications (Box 1). To take advantage of the best features of each individual vector system, there is an intense effort to construct hybrid vector systems in which the strengths of individual vector systems are combined (Box 2). An example of this is the construction of adenoviruses[6,7] or HSV-1-derived vectors[8,9], which are used to deliver retroviruses and AAVs into target cells, respectively. Such chimaeric systems allow the production of vectors that can be grown to the high titres achievable with adenoviruses, and have the capacity of retrovirus- or AAV-derived vectors to integrate their genomes into those of the host cell, thereby enabling long-term transgene expression.

The likelihood of developing the ideal universal vector is comparable with the development of the 'ideal' antibiotic that treats all infectious diseases. Just as small chemical drugs each have their own niche of appropriate application, so do the viral vectors. Consequently, the aim is to develop tailored vectors that are ideal for particular applications (Box 2). For example, the ideal vector for persistent, high-level gene expression in the liver will probably differ from that which is most suitable in the pituitary or hypothalamus.

Side effects: which therapy does not have one?

Another issue that needs to be considered is the immune response that can be elicited by viral delivery systems, especially if readministration of the therapy is required. Moreover, circulating anti-viral antibodies (pre-existing or generated after infection with viral vectors) could hamper not only the infection but also transgene expression. The effects of such antibodies have been investigated in both preclinical animal models and human patients treated with adenovirus gene delivery vectors[10–14]. Although circulating antibodies were able to inhibit the systemic administration of a viral vector, they did not inhibit the transduction of tumour cells if vectors were injected directly into the tumours[12].

Another consideration that is important in developing an ideal vector system is the proliferative status of the target organ. Target cells can be actively proliferating (tumours), slowly proliferating (pituitary gland, some pituitary tumours or liver) or postmitotic (brain). The permanent transduction of cells that are slowly proliferating or postmitotic could be achieved by the use of lentiviral vectors, which have proved to be very successful in transducing cells of the central nervous system (CNS)[4].

Adenoviruses, herpes viruses and AAVs are also suitable for the infection of postmitotic or slowly proliferating cells, even though the genomes of adenoviruses and herpesviruses fail to integrate into the genome of the target cell[15–22]. By contrast, wild-type AAVs integrate specifically into the genome of the target cell, at a site located within chromosome 19

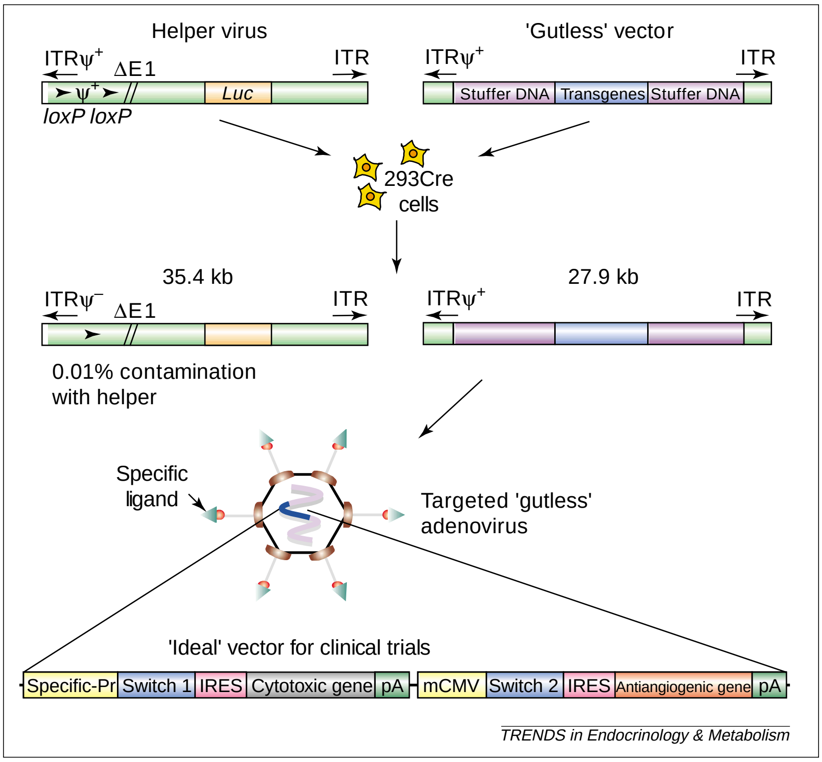

Fig. 1. 'Ideal' gene therapy vector for the treatment of pituitary tumours. Diagram of an ideal gene delivery system for gene therapy strategies using the 'gutless' adenoviral vector to produce a targeted vector that is also regulatable in its ability to express both a cytotoxic and an antiangiogenic gene. The packaging signal (ψ) of the helper virus is flanked by *loxP* sites, and this signal is excised after passaging in HEK293 cells expressing Cre recombinase. The expression of the two therapeutic genes can be regulated independently of one another because they are under the inducible control of two different regulatable switches. In addition, the expression of the cytotoxic gene is under the control of a cell type-specific promoter (Pr) to prevent damage to normal anterior pituitary cell types. A specific ligand (green triangles) engineered on to the fibre (knob) would mediate binding of viral vectors to receptors on the cell surface of predetermined anterior pituitary cells. Abbreviations: IRES, internal ribosome entry site; ITR, inverted terminal repeat; *Luc*, gene encoding luciferase; mCMV, murine cytomegalovirus promoter; pA, polyadenylation signal.

(Ref. 23), and can also provide long-term transgene expression[24]. However, whether AAV-derived vectors integrate into the genome of target cells, and whether this is the molecular basis that allows the long-term expression of these vectors remains to be shown experimentally.

Despite inducing acute inflammatory and immune responses, adenoviruses can sustain expression in the pituitary gland for up to three months[25], and in the CNS for at least 12 months[19,21]. With the development of new generation adenovirus vectors – 'helper-dependent' or gutless – long-term expression has been improved further[26], even after the injection of vectors into tissues that tend to develop immune responses[26] (e.g. the liver) and even after immunization against adenovirus.

Turning the gene tap 'on' and 'off'

A theoretical difference between gene therapy and pharmacology is that gene therapy delivers a vector that will produce the pharmacological agent (the therapeutic transgene product). This makes the regulation of the levels of transgene expression crucial to the success of gene therapy in the treatment of human disease. This is especially true for endocrine disorders (such as diabetes, acromegaly, hypopituitarism and hyperprolactinaemia), in which it will be very important that levels of transgene expression can be regulated, either by the physician or the patient.

Recently, there have been efforts to engineer transcriptional activators that can be turned on and off by small molecules such as the antibiotics tetracycline or rapamycin[27,28]. If the transgene is under the control of such an activator, administration of the antibiotics will tightly regulate the levels of the therapeutic gene product[29–31]. Such tight transcriptional regulation has recently been combined with cell type-specific promoters, thereby enabling regulation of both the levels and location of transcription.

The trade-off for using these cell type-specific transcriptional targeting approaches is the lower level of expression that usually accompanies cell type-specific promoters (compared with that driven by promiscuous viral promoters)[32]. Thus, the need to engineer strong, cell type-specific transcriptional control elements will play a crucial role in the design of gene therapy vectors for use in human clinical trials because levels of transgene expression are crucial for achieving a beneficial therapeutic outcome (Fig. 1). The use of naturally occurring cell type-specific promoters will necessitate increasing the dose of the viral vector used, and this will probably be accompanied by cytotoxic side effects known to be elicited by high doses of vectors[33].

Using an oestrogen-sulpiride-induced pituitary prolactinoma *in situ*, it has been shown that if the conditional cytotoxic gene, HSV thymidine kinase (HSV-*tk*), is driven by the strong human cytomegalovirus promoter, in combination with the prodrug ganciclovir, the levels of circulating prolactin are four times lower than those of nontreated animals, and the pituitary mass is reduced by 30% (Ref. 34). By contrast, if the same transgene was driven by the human prolactin promoter, the prolactinoma phenotype could not be reversed – prolactin levels remained several times higher than basal levels and the mass of the pituitary gland was unchanged after treatment[32]. Nevertheless, the human prolactin promoter allowed lactotroph-specific expression within the pituitary gland *in situ*[32].

Overcoming 'once bitten, forever smitten'

Gene therapy systems will also need to overcome immune responses to vectors, which currently limit vector readministration and cause the gradual reduction of transgene expression. New generations of vectors (such as HSV-derived amplicons, high-capacity adenoviruses and lentiviruses) and novel regulatory elements, combined with pharmacological manipulations of the immune response, will make gene therapy safer, long-lasting and more effective in the future.

Box 3. Crucial issues to be considered before the implementation of gene therapy trials to treat pituitary disease

- The disease: benign versus malignant
- When to treat
- How to treat – what are the molecular targets?
- For how long will the treatment be needed?
- What levels of transgene expression will be needed?
- Which cells need to be targeted?
- How severe are the side effects of therapy?
- Clinical implementation: feasibility, ethical aspects and cost implications

Gene therapy for non-life-threatening diseases? Will pituitary tumours ever be treated with gene therapy?

Because gene therapy is a new technology, it is associated with unknown risks and potential side effects. There has thus been a perception that it should only be applied in terminal cases, where no other treatments are available. However, this perception is incorrect. 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 various drugs. Although most clinical trials in gene therapy have been for the treatment of cancer, many others have been performed in patients who are not terminally ill.

Nonetheless, the currently available technology is better suited to the treatment of cancer than to the treatment of chronic diseases[35]. However, as the technology improves, gene therapy applications will increase and gain clinical acceptance for the treatment of non-life-threatening diseases. During the past two years, new vectors have been generated that induce fewer inflammatory responses, do not express epitopes that can be recognized by the adaptive immune response, and allow transgene expression over long time periods. From a clinical standpoint, 12 months of expression does not appear to be a very long time, although this represents 30–50% of the total life span of a rodent and, experimentally, stable expression for a period of 6–12 months is considered long-term expression. However, it will be necessary to test the effectiveness of long-term expression and long-term treatment over many years in human patients, as is currently the case during the implementation of novel pharmacological treatments.

The implementation of gene therapy approaches for non-life-threatening diseases needs to be planned. Why will pituitary tumours be an excellent and, at the same time, challenging therapeutic target? Although many pituitary tumours are treated successfully and are generally not life-threatening, in the short term at least, there are several treatment-resistant tumours that do not respond to currently available therapies.

In some cases, patients do not tolerate pharmacological therapy[36], and/or the tumours do not respond to such treatment[37,38]. Surgery also presents complications, the most common of which is permanent diabetes insipidus or hypopituitarism. Although the incidence of these complications is low, its extent varies among clinical centres[39,40]. In addition, a small proportion of pituitary tumours either cannot be resected completely or are truly invasive. This allows tumours to recur and disease to progress after the implementation of classic therapies. Radiotherapy is also used for the treatment of some pituitary tumours and, despite having potential complications, which include damage to the optic nerves, hypopituitarism and, in the long term, the development of secondary brain tumours[41–43], the low incidence of such side effects might not represent an important clinical limitation. However, there is a need to develop alternative treatment modalities that could complement those which already exist and gene therapy constitutes a novel and attractive therapeutic approach.

The pituitary gland is an attractive organ for attempting gene therapy because its biology is reasonably 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 tackled before successful therapy can be implemented (Box 3). The nature and stage of the disease to be treated, which therapeutic transgenes are appropriate to use, the duration and levels of transgene expression required, and which cell type will need to be transduced will all play a crucial role in designing and choosing a gene therapy vector for clinical use. In addition, it will be crucial to assess the effectiveness and side effects of the proposed therapeutic approach in relevant animal models such as rodents, and also to assess its toxicity in non-human primates. Only after these issues have been extensively assessed could one plan, and then implement, clinical trials.

Therefore, the implementation of clinical trials will depend on the careful evaluation of the natural history of the disease to be treated versus the efficacy and side effects of the treatment to be used. As side effects are reduced and vectors become safer, gene therapy will be applied increasingly to treat diseases currently treated with available drugs, radiotherapy or surgery.

There are clinical cases of pituitary tumours for which available treatments are inadequate. Gene delivery to the pituitary gland in humans will initially make use of current neurosurgical approaches, and gene therapy will certainly be an adjunct to current neurosurgical treatments. Gene therapy could be used either to eliminate an otherwise intractable pituitary tumour, or to engineer cells to secrete pituitary hormones under regulatable promoters.

In the case of scant normal pituitary tissue remaining after surgery, gene transfer might enable the remaining cells to secrete the necessary hormones. Even though this type of cellular engineering might not sound feasible at this time, it is not unlikely that such clinical trials will be under way within the first decade of the new millennium. Thus, a central challenge of the treatment of endocrine diseases – the capacity to replicate artificially the tightly regulated secretion of hormones in response to physiological stimuli – will be attempted through cellular engineering using gene transfer technology.

Gene therapy strategies for tumour treatment

The most widely used approaches of gene therapy for tumour treatment include either conditional cytotoxic, directly cytotoxic or immune-stimulatory transgenes[44,45]. Therapy with conditional cytotoxic genes, such as HSV-*tk*, combined with ganciclovir, has shown that, in some cases, tumour mass can be reduced and an antitumour immune response can be generated[46,47]. Experimental studies of pituitary tumours have used conditional cytotoxic genes in both the pituitary gland *in situ* and transplantable anterior pituitary tumours[32,34,48,49]. The results from these approaches have been very encouraging. In the case of the *in situ* models of pituitary adenomas, the reduction in tumour mass was 30%, which is considered a good therapeutic outcome[34]. In addition, circulating prolactin levels were four times lower than those found in the adenoma-bearing animals[34]. In the case of transplantable tumours implanted subcutaneously into nude mice, there was a significant reduction of the tumour mass and excellent survival of the animals[48]. These results indicate that this approach might be suitable for the treatment of pituitary disease in humans, although one must take into account that HSV-*tk* harnesses the division potential of tumour cells to achieve tumour cell killing, and pituitary tumours or adenomas are known to have a very low mitotic index[50]. Thus, HSV-*tk* might need to be used in combination with other strategies, including approaches that would kill both actively dividing and non-dividing or quiescent cells. Examples of cytotoxic or conditional cytotoxic approaches that could be used to kill quiescent or slowly proliferating cells include *Pseudomonas* exotoxin A, diphtheria toxin and the bacterial enzyme carboxypeptidase G2, which can convert prodrugs into alkylating agents that become incorporated into the DNA and consequently cause cell death[44].

Gene therapy in general, and cancer gene therapy in particular, illustrate that gene therapy will only work in combination with the best classic therapeutic approaches – chemotherapy, radiotherapy and surgery[51].

Clinical implementation

Pituitary diseases subjected to gene therapy will be those that are caused by either hypopituitarism or hypersecretion of particular hormones. Gene therapy would allow the regulated expression of the missing hormones in predetermined cell types such as remaining pituitary tissue, or in peripheral cellular implants. Hypersecretion is usually caused by tumours, and so would be treated either by the destruction of tumour mass, or through specific neurotransmitters and regulatory hormones known to reduce specific hormone secretion (e.g. dopamine to inhibit prolactinomas, and somatostatin to inhibit growth hormone-secreting tumours). In addition, because the surgical treatment of pituitary tumours sometimes leads to hormone deficiencies, using a gene therapy approach is a very attractive alternative.

The ideal vector

The ideal vector for human gene therapy trials would be a vector of minimal toxicity and good efficiency of transduction, which would allow long-term transgene expression (e.g. a gutless adenovirus vector, an AAV vector or a lentivirus vector). For the treatment of tumours, the vectors could encode a cytotoxic gene, the expression of which should preferentially be driven by a tumour- or cell type-specific promoter so that expression from the vector would be transcriptionally targeted. In addition, the vector could encode a second transcription unit/therapeutic transgene encoding an antiangiogenic protein[52,53] driven by the strong murine cytomegalovirus promoter (Fig. 1). Furthermore, therapeutic genes should be under the control of regulatable switches that would allow gene expression to be turned on and off, as needed, with the regulation of their expression driven individually (Fig. 1). The vector should also be targeted; it should have a specific binding site on its surface to allow its binding and internalization into predetermined cell types. Most of these features are available although, to date, they have not all been combined in one single vector system. Such a vector would be safe because once the treatment is completed, any gene expression from the vector would be rendered silent; however, vector DNA would remain within the transduced cells. Alternatively, systems could be developed to allow the elimination of the vector from the transduced cells. The elimination of any remaining, therapeutically unnecessary foreign DNA could be of importance from a safety perspective.

How to administer pituitary gene therapy

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[54].

Combination with neurosurgery

Realistically, the first pituitary gene therapy trials will be conducted in combination with current

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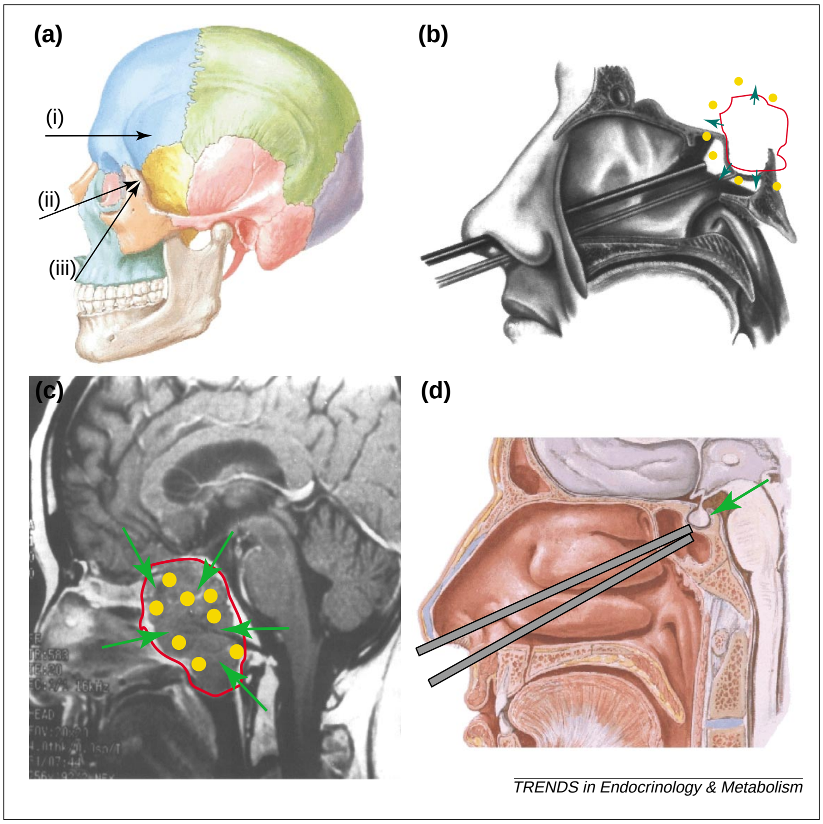

Fig. 2. Delivery of gene therapy vectors to pituitary tumours in humans. (a) Surgical approaches to the pituitary gland. Arrows illustrate the three main routes of access to the pituitary: (i) subfrontal, (ii) trans-sphenoidal and (iii) sublabial. (b) Delivery of the recombinant adenoviral vectors to the pituitary tumour bed after surgical removal of the tumour mass. Blue arrows and yellow circles illustrate the dispersion of the virus throughout the surrounding tissue. (c) Illustration using a magnetic resonance imaging scan of a patient with a pituitary tumour. A proposal for the delivery of recombinant therapeutic vectors to the tumour *in situ* (green arrows and yellow circles). (d) Delivery of the recombinant viral vectors to the remaining anterior pituitary gland for the treatment of putative side effects arising after current treatment of pituitary tumours (i.e. hypopituitarism).

surgical treatments. We envisage a scenario in which the neurosurgeon removes a pituitary tumour by trans-sphlenoidal surgery (Fig. 2a). In the case of complete removal, the neurosurgeon might not need to administer additional gene therapy. However, in some cases of partial tumour removal, the surgeon might choose to deliver the vector into the tumour bed (Fig. 2b) in the hope of transducing any tumour cells remaining within the pituitary. Alternatively, given that many pituitary tumours grow back, the surgeon could transduce the remaining pituitary tissue with vectors and, should the tumour recur, expression of the vector could be reactivated. Even though this might not sound practical at the present time, it is possible to devise such systems. Such a combined treatment would limit the number of interventions to which the patient would need be subjected and would also combine two complementary treatment modalities.

In the case of inoperable tumours, the gene therapy vector could be delivered within the tumour mass itself (Fig. 2c). In addition, gene therapy could be applied in combination with drug therapy aimed at reducing tumour 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 a dopamine intolerance.

Hormone replacement

Hypopituitarism is often a side effect of the treatment of pituitary tumours, either because of the removal of most of the pituitary tissue or because of damage caused by pituitary irradiation. In such cases, gene therapy could be implemented to replace the missing hormones. If enough pituitary cells remained viable, gene therapy might enable pituitary hormones to be expressed in the appropriate cells (Fig. 2d). In the absence of sufficient pituitary tissue, gene therapy strategies could be used either to express pituitary hormones from other cells in the body, or to provide an adequate and specific growth factor environment for pituitary stem cells to replace, at least partially, the lost cells.

Conclusions

In summary, gene therapy offers many potential approaches to the treatment of pituitary disease and other endocrine diseases, in which either tumour cells need to be ablated or hormone secretion needs to be replaced while the physiological patterns of secretion are maintained. Given its relatively small size, easy monitoring of functional status through the levels of peripheral hormones, and effective neurosurgical approaches, it is probable that the pituitary will be targeted by gene therapy within the first decade of the new millennium. What is crucially needed is a close-knit collaborative effort between the scientists developing the basic technologies and the clinicians dealing with the clinical challenges. When the first clinical gene therapy trial was implemented in 1990 (Ref. 55), the 1972 Friedmann and Roblin paper[56] took on an almost prophetic quality. What this paper described in theory was technically impossible to achieve with the technology available in 1972; however, from the perspective of a reader in 1990, it looked like a promising blueprint because restriction enzymes had just been discovered, and the 'cutting and pasting of DNA' still remained to be achieved.

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