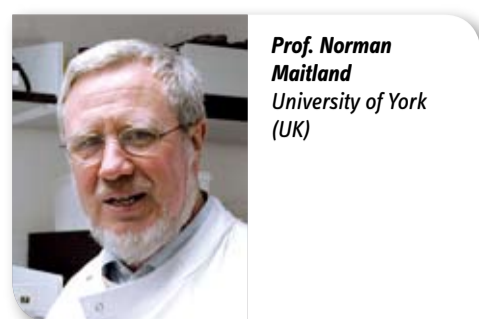


Getting DNA into cells



Prof. Norman Maitland
University of York
(UK)

OK, so I'm a Urologist...why on earth should I be interested in getting DNA into cells? It won't help me to treat patients...or will it?

In fact, according to the aims of the EU funded GIANT programme (Gene therapy: an Integrated Approach to Neoplastic Therapy) it will provide a useful backup to the more conventional treatments for prostate cancer, and may provide a good example of 'personalised treatments'. After all, new drugs directed against genetic defects and fusions found in cancer such as Imatinib are beginning to change clinical practice in other types of human cancer. However decisions about prostate cancer treatment will not be taken by scientists, but by clinicians, and it is important that those who take the decisions are both aware of the technology and the evidence that has been accumulated which could result in a new therapy.

Why introduce DNA into cells?

The most obvious reason in a urological context is indeed gene therapy, which has promised much but delivered relatively little since its first uses in the 1980's. In fact there have been recent successes, particularly with gene augmentation in children suffering from Severe Combined Immunodeficiency Disorder (SCID). The problem is that, despite many successful treatments, there were also some unfortunate side effects (Kohl et al, 2003). However improved treatment regimes, with lessons learned from the initial pioneering studies have resulted in a safer and more effective treatment. If you compare the current situation with SCID to the constant and ongoing improvements in surgical techniques for prostate disorders, then there is little real difference. New treatments take time to optimise, and we should not be unduly critical of gene therapy.

Gene therapy for cancer has been the principal use of DNA transfer for almost as long as the technology has been practical. It is easier to accomplish than the more exacting gene replacement or augmentation (for SCID) – the end point is not living functioning cells but dead tumour cells! It is one of the aims of the GIANT project to make gene therapy both acceptable and feasible as a treatment for prostate cancer. This necessitates safety and efficacy studies, and much more importantly, taking the major step from the laboratory, via relevant animal models, into actual patients. Furthermore, by adopting standard testing schemes throughout Europe, we hope to facilitate the clinical testing in several countries, having surmounted the inevitable obstacles of ethical and regulatory authorities.

In the decade after the Sequencing of the Human Genome there is now an equally important role for gene transfer in medicine: confirming the function of the 28,000 genes which are essential for the correct operation of the human 'machine'. By expressing a

gene in a cell, which normally doesn't express it, we can see how gene expression alters cell behaviour. Equally by 'knocking down' expression of the same gene (by introduction of a blocking or interfering short RNA molecule to block translation of messenger RNA into proteins) in a cell which normally expresses it a high levels, we can decide both the normal and disease related functions of the gene. If such a gene is essential for the growth or perhaps better the survival of a cancer cell (relative to normal equivalents) then it represents a novel target for therapy.

How is it done?

DNA itself is quite a stable molecule, and can be added directly to cells in the laboratory (see figure 1). However cells present barriers to the uptake of many molecules and are unlikely to take up genes with any efficiency, even in a laboratory setting. There are a number of ways to enhance the uptake of naked DNA into cells. The simplest to envisage is **microinjection**, which can either introduce genes into the cytoplasm (where DNA will normally be degraded) or directly into the nucleus (harder, but more efficient). Sophistications of the pricking method (a non specific injection which damages the cell membrane to encourage DNA uptake) such as giving the cells an enormous electric charge (electroporation) and the use of accelerated gold particles as a bullet (gene gun) are more recent and efficient developments. Other protective measures, such as carrier precipitates of calcium phosphate or DEAE dextran, have been used for more than 30 years but are really only feasible in the laboratory. These methods still have a very real relevance for what is known as *ex vivo gene therapy*, where cells are removed from a patient, modified, and then returned autologously. This is particularly useful for modifications of immune system and stem cells.

For direct introduction of genes into cells within the human body, the barriers and protection are even more formidable. Therefore to carry out efficient gene transfer, we need to use a *carrier molecule or vector*. **Non viral vectors** such as *polyplexes* and *liposomes* are a systematic and sophisticated enhancement of the original carrier precipitates and are now beginning to show efficacy *in vivo* as well as in the laboratory. The most evolved vectors are still **viruses** of human and non-human origin, although many of us retain immunity to such virus infections. Some of the common vectors and gene transfer techniques with a brief discussion of their strengths and weaknesses are listed in Table 1.

The need to target

The use of a vector also introduces the concept of *gene targeting*. This is frequently overlooked in the design of therapies, and just like a drug which accumulates in the wrong tissue, **expression of a gene in the wrong location can result in a more severe disorder than that to be treated**. The initial (and frequently disastrous) efforts at gene therapy (in the 1980's) can be compared to 19th century surgery, as they were both poorly targeted. One of the main aims of the GIANT programme is to achieve prostate targeted therapy (reviewed in Stanbridge et al, 2004). What all of the vector systems shown have in common is the *protection* they offer the genes outside of the cell. This is traditionally a role played by viruses, and indeed many of the most effective vectors are disabled or modified human and animal viruses.

Recent advances in synthetic chemistry have allowed non-viral liposomal and nanoparticle vectors to begin to rival viruses in their ability to get DNA into cells. There is a big difference in the structures of the viruses and non-virus DNA complexes, although they are about the same size (50-100nm). The DNA is generally confined within virus particles, imposing a size restriction on the number and type of genes to be transferred, whereas some non-viral

particles seem to wrap the DNA around themselves and can change their size to accommodate more DNA.



In the case of both viral and non-viral vectors, targeting molecules, which allow the vectors to attach to specific cell types, such as ligands (for cell surface receptors), antibodies (against cell surface proteins and glycoproteins) and specific but sometimes empirical binding proteins such as affibodies or aptamers can be attached to the surface of the vectors, changing the cellular tropism of the vector molecule.

Getting Genes TO cells is only the first step

In practice, most gene transfer vectors have equivalent efficiency in getting genes to the surface of the target cell. When the genes get into the cells there is a further restriction. Most genes are targeted to *endosomes*, where viruses have evolved mechanisms to exploit the acidic environment to release and further target their genes to the correct compartment within the cell (DNA to the nucleus and RNA to cytoplasmic organelles). If they fail to do this then the genes are retargeted for destruction. (see figure 1). Non-viral vectors have to be further modified to accomplish this targeting and clearly do not benefit from the millions of years of evolution that viruses have undergone! The various degrees of targeting and ultimate fate of the DNA payload are summarised in figure 1.

The RNA Lifestyle

As implied earlier, the type of nucleic acid within the vector also affects the efficiency of transfer of genes into cells. It is no accident that there are many times more types of viruses with RNA as their genome, compared to the more conventional DNA (like humans!). Many of these RNA viruses are the most successful pathogens in nature, such as polio, 'flu as well as more sinister agents such as Ebola and Marburg viruses. Most of these RNA viruses complete their entire life cycles in the cell cytoplasm. There is now a trend to exploit this efficiency, and to use the selectivity of interfering RNA for therapeutic purposes (Dorsett and Tuschl, 2004).



(ECAM members are: Chris Bangma, Anders Bjartell, Zoran Culig, Freddie Hamdy, Norman Maitland, Jack Schalken and George Thalmann)

Uncontrolled gene expression is as bad as no expression at all

The more conventional DNA viruses must get their DNA (intact) into the cell nucleus where they hijack cell enzymes (often with higher efficiency) to express their own, and in the case of gene therapy, the therapeutic genes. Once again, to ensure specificity, expression of therapeutic genes as mRNA are frequently controlled by synthetic *promoters* (gene switches) such as that for PSA in the case of prostate cancers. Again knowledge of gene expression and the complete human genome sequence has taken some of the mystery out of this targeting process. Such control sequences can now be excised from their normal position in the human chromosome (for example for PSA on chromosome 19) copied, enhanced and linked to a therapeutic gene, whose expression pattern now mimics that of PSA in appropriate cell types.

To integrate or not?

There now remains one further decision to be made. In the case of cytotoxic gene therapy (for cancer) the aim is generally to express the therapeutic gene at high levels in the cancer cells, to the exclusion of all other cell types. But once DNA has reached the cell nucleus, there is a further possible fate for genes to undergo. Under normal circumstances DNA introduced by a vector will remain as a mini-

Continued on page 24

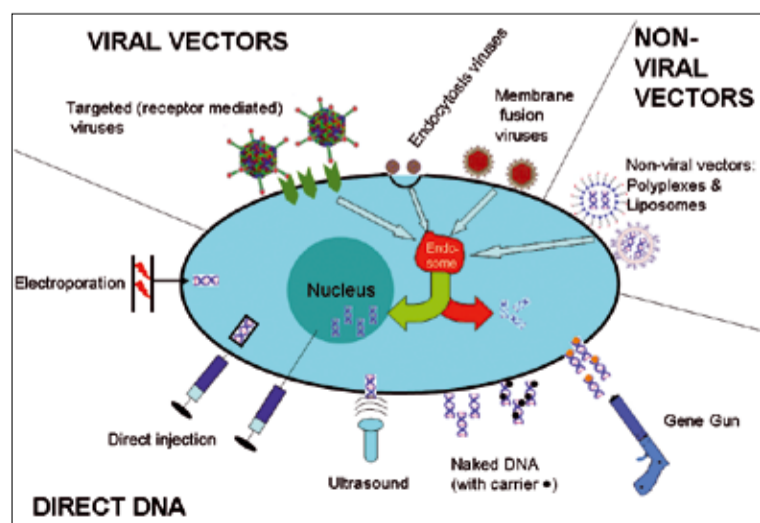


Figure 1: Gene Transfer Technologies

Viral Vectors	Advantages	Disadvantages
Targeted Viruses (receptor mediated entry)	<ul style="list-style-type: none"> Efficient (pathogens have been developed over millions of years. Excellent intracellular trafficking of DNA to nucleus. Can be cell-type specific. 	<ul style="list-style-type: none"> Some limitation on size of gene transferred. Recombination with pre existing viral infections in the human host. Pre-existing re induced immune response. Ease of production and use as a mass market medicine.
Non-specific Viruses (endocytosis mediated entry)	<ul style="list-style-type: none"> Easy to produce. Possible tumour specific growth (oncolytic). 	<ul style="list-style-type: none"> Not-specific. Probably need dividing cells. General virus concerns above.
Membrane fusion viruses	<ul style="list-style-type: none"> Highly efficient. Can be targeted to specific cell types. Retroviruses require permanent association with target cell chromosomes. 	<ul style="list-style-type: none"> Insertional mutagenesis (retroviruses) Immune response.
Non-Viral Vectors		
Polyplexes	<ul style="list-style-type: none"> Chemically defined composition. Ease of bulk production under GLP conditions. Ease of use, particularly <i>in vitro</i>. Can be retargeted. 	<ul style="list-style-type: none"> Relatively poor <i>in vivo</i> performance. Immuno-stimulation. Normally requires plasmid DNA (instability). Poor endosome escape.
Liposomes	<ul style="list-style-type: none"> Chemically defined composition. Ease of bulk production under GLP conditions. Particularly easy to generate. Effective in a wide range of cell types <i>in vitro</i>. Can be targeted. 	<ul style="list-style-type: none"> Relatively poor <i>in vivo</i> performance. Immuno-stimulation. Normally requires plasmid DNA (instability). Poor endosome escape.
Direct DNA Introduction		
Naked DNA	<ul style="list-style-type: none"> Cheap. Works <i>in vitro</i>. 	<ul style="list-style-type: none"> Inefficient. Untargeted.
Direct Injection	<ul style="list-style-type: none"> Cheap. Can be targeted to particular tissues (DNA immunisation in skin). <i>In vitro</i> DNA can be directly injected into the nucleus. 	<ul style="list-style-type: none"> Limited targeting in Urology (skin best) Needs large amounts of pure DNA. Targeting limited but possible (promoters)
Ultrasound	<ul style="list-style-type: none"> Works <i>in vivo</i>. Moderate targeting capacity. Familiar technology in Urology. 	<ul style="list-style-type: none"> Moderate targeting capacity.
Electroporation	<ul style="list-style-type: none"> Works better <i>in vitro</i>, but can boost effectiveness of injection <i>in vivo</i>. Boosts vaccine effectiveness in skin. 	<ul style="list-style-type: none"> Risks in clinical practice (high voltage). Urology applications more difficult.
Gene Gun	<ul style="list-style-type: none"> Works mainly <i>in vitro</i> and possibly <i>in vivo</i>. Area targeting possible. 	<ul style="list-style-type: none"> Expensive (gold particles and equipment) Urology applications could be difficult

Table 1: Methods of getting DNA into human cells

Continued from page 23

chromosome in the nucleus, but some genes can also become integrated into one of the human cell chromosomes. The latter situation is good if your aim is to achieve long term expression of a 'curative' gene, but can be something of a nuisance if it occurs in the wrong place – imagine if a gene which was meant to render a tumour susceptible to a cytotoxic drug was also incorporated into brain cells. When a therapeutic gene package does get integrated into the cell chromosome, the new environment within the human genome can also *silence* the therapeutic gene, or alternatively destroy or override the sophisticated control mechanism which we may have attached to our therapeutic gene. Again we can exploit functions from viruses called episomal maintenance proteins

and sequences to keep therapeutic genes OUT of the human chromosome, and allow them to replicate efficiently within the cell nucleus for many generations.

Conclusions

As described here, getting genes into cells is really quite routine in the molecular biology laboratory. We have access to a toolkit of useful genes and molecules, which should allow us to target the expression of new genes to any particular cell. What is at issue remains the *clinical use* of these sophisticated tools. It is perfectly acceptable to ask questions such as: Are they safe? Will gene therapy work, and if it does can it really replace the techniques with which urologists are so familiar? Should we bother if our current technologies are working? As a proponent of the technology I would

of course answer Yes to these questions. If you really want to know more, then please access the GIANT programme website at www.giant.eu.com.

Some further reading:

- **siRNAs: Applications in functional genomics and potential as therapeutics.** Yair Dorsett & Thomas Tuschl *Nature Reviews Drug Discovery* 3, 318-329 (2004)
- **Occurrence of leukaemia following gene therapy of x-linked SCID.** Kohn DB, Sadelain M, Glorioso JC. *Nat Rev Cancer*. 2003 Jul;3(7):477-88.
- **Targeting gene therapy for prostate cancer.** Maitland NJ, Stanbridge LJ, Dussupt V. *Curr Pharm Des*. 2004;10(5):531-55.