

## **SLIDES SERIES 'GENE DOPING'**

**S Rusconi**

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**«Sports doping: is there a realistic application of gene transfer?»**

### **slide 1; Technical remarks**

This slide show was prepared with Power Point 98 for Macintosh, Therefore, 'thank' to the limited portability it may halt or improperly display on windows-based machines. This is not due to our bad will but to the sloppiness of software suppliers who are apparently not able to produce a platform that is genuinely interchangeable in spite we have reached the third millenium !

The movie clips listed below accompany the full size slide show and require the installation of Quicktime player or a compatible application. You are welcome to write to sandro.rusconi@unifr.ch if you want to have a full size slide show (over 100 Mb)

Movie clips list: sruSki2.mov; genet walk on DNA.mov; sport boxe 01.mov; aa getting oldcomp2.mov; molecular\_therapycardio1.mov; sport GIRO98.MOV; sport football 01.mov; sport maradona 01.mov; sport motorbike 01.mov; sport snowboard slalom.mov; sport hunter jones.mov; sport weigh women 2.mov; sport sydney2000.mov; sport weights advert.mov; iceage.sub.mov;

### **slide 2 CV Rusconi**

My CV implies that I am is not a medical doctor but I have a certain experience in gene transfer. Most of the experience was accumulated during the period where he was acting as scientific director of the Swiss national research program 37 'somatic gene therapy'.

### **slide 3: Schedule**

this slide lists the topics discussed in this talk

### **slide 4: Genetics has been used since millennia...**

contrary to popular belief, empirical and practical genetics has accompanied our process of civilisation since thousands of years. It is only since thirty years that we can understand and get a direct handling on the molecular components of heredity.

### **slide 5 1 gene -> one or more functions**

Lets first disclaim another myth that many of us have learned in the school, that is that one gene represents one function. In fact this is a 'creationistic' view, since neither genes nor their products were 'designed' to serve a particular purpose, but rather have 'carved out' their purpose in an evolving system, where no positive advantage means disappearance. As a consequence, genes and their products have invariably more than one function. This comes about through differential processing of the mRNA or the final proteins, but also, the s exactly same protein can have much different functions when interacting with different partners.

### **slide 6: what is in fact a gene?**

in this animation I bring out the concept that a gene is a segment of DNA consisting of regulatory (red) sequences, coding (blue) sequences, and flanking (brown) sequences. In the current view, regulatory sequences attract specific transcription factors (different shapes) which in turn attract the enzyme that produces a copy of one DNA strand into an RNA strand (RNA polymerase). The RNA is then processed, transported out of the nucleus and used to direct protein synthesis.

**slide 7: one organism -> more than 10 exp5 functions**

In this sequence we go from visible to invisible. Let's remind that in one cubic centimetre of soft tissue there are around 1 billion cells. This notion will be important when willing to understand the difficulties in somatic gene transfer.

**slide 8: Reductionist molecular biology paradigm**

In the everyday's life molecular biologists take a rather reductionist approach to consider that when a gene is in its 'normal' sequence configuration (so called wild type) the corresponding function(s) will be available, whereas when the gene is defective, the corresponding functions will be also defective, and, most importantly when we transfer a gene (including its own or foreign regulatory regions) we in fact transfer one or more functions to a cell.

**slide 9: gene amplification / manipulation...**

The techniques of gene isolation, splicing into plasmid vectors, amplification in bacterial hosts and further cut-and-paste events is illustrated. The necessary enzymes are available for cutting (restriction enzymes) and for re-ligating (ligases). Also artificial sequences can be synthesised. The technology is easy to recapitulate and requires little investment. So it is easy to produce milligrams or grams of science grade recombinant genes. However, it remains tricky and extremely expensive to produce clinical-grade recombinant DNA. This distinction between science-grade and clinical grade material will be very important when we will speak of possible abuses.

**slide 10: the four eras of molecular medicine**

In molecular medicine, the capacity of detecting, splicing and transferring genes has gone through four major periods: in the eighties (genes as probes) we started using gene detection to prenatally diagnose hereditary diseases. Today this technology has very much progressed and is routinely used in the clinic. In the nineties (start of the period 'genes as factories'), the first biopharmaceuticals derived from genetically altered cells/yeast/bacteria, entered the market, today in Switzerland there are over 90 registered such pharmaceuticals. This includes some doping-relevant factors such as erythropoietin. Towards the end of the last century (beginning of the period that I call 'genes as drugs') the scientists started considering to use directly gene transfer into humans to correct or heal various kinds of diseases. The sequencing and global functional characterisation of genomes (initiated in 2000) has marked the genomic and post-genomic era in which all the preceding activities of molecular medicine will be consequently altered (hopefully ameliorated)

**slide 11: The major disease of the 21st century: ageing**

taken at face value ageing is indeed the major public health concern of this century. this owes to the dramatic increase of life expectancy (first panel) and to the fact that many diseases such as cancer (second panel) have a hyper-exponential incidence curve when plotted against ageing. Also in Alzheimer's disease we note a dramatic fall of percentage healthy persons after the age of 60 (third panel). Genetic traits can shift these curves to predisposition (red curves) or towards protection (green curves) but cannot stop the progression of incidence of these degenerative diseases. if we live long enough we all get those diseases. the major challenge of today's medicine will be to find some solutions that ameliorate the life quality after the threshold ages. Many of the treatments destined to this amelioration may turn out to generate concepts that can be used to boost physical performances in young adults, therefore are prone to become novel doping sources.

**slide 12: Gene Therapy**

in this slide I define GT as the use of gene transfer in somatic cells to heal or treat disorders. this strategy can be applied to chronic, acute or preventive treatments and to hereditary or acquired diseases.

### **slide 13: GT four fundamental questions**

To be clinically successful, somatic gene transfer technologies must answer a number of questions. 1: the efficiency of the transfer must be adapted to the disease we intend to treat (remember the 1 billion cells per cubic centimetre). Not all diseases require a 100% transfer in a particular tissue. 2. The gene transfer must be reasonably specific. We intend to cure one particular type of tissue and not another one. Thus, we have to find strategies by which the gene will be finally expressed in the desired tissue and only there. This can be achieved either by targeting the delivery or by constructing gene recombinants that have the regulatory sequences that render them tissue-selective. 3: when willing to treat chronic disease we may be interested in maintaining the gene and the transformed cells for a long time. There are no simple ways to guarantee this. 4: Finally, depending on dose, specificity and persistence, we may have to cope with either acute, short term or mid/long term toxicity of the approach. These toxicities largely depend on the type of vector and delivery type. Some kinds of toxicities, such as insertional mutagenesis are intrinsic to randomly integrating vectors and represent a major risk, that justifies momentarily the use of gene transfer only for deadly or severe incurable diseases.

### **slide 14 why 'somatic'?**

A mammalian body is comprised of a majority of somatic cells (those that form our muscles, bones, skin, blood, brain etc) and a few germ line cells (those that give rise to sperms and ova). If gene transfer occurs in somatic cells, this will have no repercussion in the progeny, since these cells do not contribute to the germ line. However if the transfer occurs also in germ cells, then the alteration will be transmitted to the progeny. In case of randomly inserting vectors (see 'toxicity' point slide 13) this corresponds to a random mutation of the genome, and this has unpredictable consequences. Therefore, there is a wide consensus among all specialists and interested parties that in the light of the current technology, gene therapy must be confined to the somatic cells.

### **slide 15: Pharmacological considerations**

Here I compare gene transfer to classical drugs (first panel) and protein drugs (second panel). The biggest issues in gene transfer are the intrinsic difficulties in delivery since there is no specific natural transport that we could exploit like in the case of conventional low molecular weight drugs.

### **slide 16: Three classes of physiological gene delivery**

Anatomically, gene transfer can be performed in three distinct manners: (a) ex vivo (first animation) in which cells are first explanted and briefly cultured (example bone marrow cells), exposed to the gene transfer, possibly selected for positively transformed, and then re-infused into the patient. This is the technique that has so far given the most promising results. One big advantage is that the transfer vector does not directly enter in contact with the immune system of the host, and therefore, part of the toxicity is not appearing. However, this method is very laborious and time consuming, and certainly not adapted for all types of disease. (b) in vivo local transfer (second animation). This includes intramuscular, intra-joint, intra-cerebral, intraocular, intratumoral etc.. delivery of the gene vector. The methods are appropriate for many localised diseases and limit both the toxicity of the gene transfer and the possible contamination of the germline. (c) in vivo systemic delivery (third animation). This includes intravenous, intra-arterial, intra-vascular, intra-peritoneal deliveries. There are not yet good vectors that justify this delivery system. The major obstacles are: 1) non specific trapping in capillary systems, non-specific trapping by the liver, non-specific delivery, destruction of transfer particles by serum and blood components, insufficient accumulation in relevant tissues due to lack of understanding of the vascular molecular zip-code etc. besides that this method does not guarantee sparing of the germ line.

### **slide 17: two classes of gene transfer vehicles: nonviral and viral**

Today many nonviral ways to introduce genes into cells are available. However, and contrary to the common belief) the major difficulty in such transfer is not the barrier of the

cell membrane but the lack of transport into the cell nucleus. nonviral gene transfer is extremely inefficient, when you divide the number of DNA molecules that reach the nucleus by the number of DNA molecules used for the transfer. Viruses have made of gene transfer their way of life, and this since ever. I say to my students that viruses are the best molecular biologists of the world and the absolute champions in gene transfer. the major reason is that viruses have evolved all the necessary strategies to a) dock onto cells, b) get internalised, c) remain protected from degrading cytoplasmic enzymes, d) migrate in proximity of the cell nucleus, e) manage to translocate their genetic load into the nucleus. Thus, it is not a surprise, if scientists turned to viruses to get the best vehicles to transfer their preferred genes into cells. This is achieved by constructing so called 'recombinant viruses'.

#### **slide 18: Transfection versus infection**

In this slide I bring examples of a cell culture that has been transfected (nonviral transfer) with a so-called 'reporter gene'. When this gene has reached the cell nucleus, it is expressed and generate an enzyme that is capable of converting a colourless substance into a blue pigment that precipitates in the cell. The number of blue cells indicates the number of 'transfectants'. With this nonviral transfer we transfect in the best cases 5-10% of the cells and exposing the for over 12 hours to a concentration of about one million DNA molecules per cell. If we engineer a recombinant adenovirus (second panel) to carry the same reporter. it is sufficient to expose the cells to 3 adeno-particles per cell for 30 min to obtain a 100% gene transfer. This illustrates the several logarithms of difference in efficiency between nonviral and viral gene transfer.

#### **slide 18: Parade of popular vectors/methods**

This slide lists the vectors that I will briefly comment in the following slides. Since this was not intended as a specific lecture in gene therapy I will only point to the essential features of each delivery method.

#### **slide 20: Recombinant adenoviruses**

These vectors are easy to engineer and are very popular in basic research. The recent generations of such vectors can accommodate up to 30'000 base pairs of recombinant DNA. Clinically they have been used primarily in anti tumoral strategies. Their disadvantage is that the recombinant genome is not integrated in the host genome (less persistence). However this is at the same time an advantage because the approach is not altering the host genome (not genotoxic).

#### **slide 21: recombinant adeno-associated viruses (AAV)**

AAV recombinant vectors are also easy to assemble but have the main disadvantage to be able to accommodate only 4500 base pairs of foreign DNA. this limits their use to the transfer of small-sized genes. Nevertheless, they allowed some interesting clinical results in the case of Hemophilia B (transfer of gene for factor VIII in the muscles). One advantage of AAV vectors is that they integrate into the host genome. This can cause insertional mutagenesis (risk of cancer).

#### **slide 22: recombinant retroviruses**

Retroviruses transmitted their genome RNA form which is converted into cDNA and then integrated in the genome. Since they randomly integrate, they also bring with themselves the risk of insertional mutagenesis that can result in enhanced cancer risk. Nevertheless these vectors are the ones that brought the best clinical results (see the example of Prof. Alain Fischer with the SCID disease). In recent years retroviral vectors with the properties of lentiviruses (the class including HIV virus) have been developed. they should enter in clinical testing this year. their advantage is that they can infect non dividing cells, a favourable situation when willing to treat tissues that have no or low turnover (example brain)..

**slide 23: Naked /complexed DNA**

With the exception of muscle, where naked DNA is apparently taken up at considerably good rate by the cells, the nonviral transfer in other tissues requires complex formulations into liposomal particles. The main advantage is that they are less complex to assemble than viruses and are probably less toxic. the major disadvantage is their intrinsic low efficiency (see slide 18).

**slide 24: oligonucleotides**

The palette of gene-specific treatment includes the use of very small gene fragments (called oligo-nucleotides) that can be designed to have several interesting properties: a) antisense blockers, hammerhead ribozymes (can specifically degrade matching sequences); gene-repair inducer oligos (can prompt repair of specific resident sequences); decoy oligonucleotides (swamp away pathological factors that abnormally bind DNA and activate undesired genes) etcetera. Except for gene-repair oligonucleotides, the other approaches cannot be taken as bona fide gene therapy. The characteristic of oligonucleotide treatments is their short persistence (except for gene-repair oligo). This makes them good candidates in transient treatments in the doping arena.

**slide 25: current limitations of popular gene transfer vectors**

This slides summarizes the disadvantages of each system commented before.

**slide 26: The most feared potential side-effects of gene transfer**

Depending on the used vector, delivery route, or transferred gene, gene transfer presents a certain number of risks. 1) The use of viral vectors can stimulate the immune system, if incorrect doses are used, an acute shock can occur. this was the case of the death of Jesse Gelsinger in 1999. 2. The newly expressed gene product could be recognised as non-self in individuals that have no matching sequences expressed in their own genome. This has proved to happen for instance after transfer of the gene for factor VII in hemophiliac dogs. 3. viral vectors are by themselves generally toxic., For instance adenoviral capsid proteins can kill cells at certain dosage. If the preparation of recombinant viruses is accompanied by a vast majority of non-functional particles, this can result in acute toxic shock. 4. Recombinant viruses preparation can contain particles that reverted to the original virus, by picking up the helper genes. If non-clinical grade material is used, this is a major source of risk. 5. Random integration in the genome (such as provided by AAV or retroviral vectors) is genotoxic and increases the mid term risk to cancer. 6. If inappropriately delivered, gene transfer could contaminate the germ line cells, thus resulting in randomly mutated chromosomes that will be inherited to the progeny.

**slide 27: Gene therapy in the clinics (trials world-wide)**

This slide summarises clinical trials and number of treated patients along the years. The curves are representing categories of diseases. The pie-graph in the panel represents the fraction of GT trials that have reached different phases of clinical testing. Phase I (test of side effects only) has still the majority of trails, even 12 years after the start of gene therapy). only 13% of trials have reached phase II (test of efficacy), and 1% have reached phase III (none of them terminated yet). This means that we will have to wait still several years before seeing GT used routinely in the clinics.

**slide 28: gene therapy milestones**

In spite of all the difficulties GT can count many successes, these are summarized in this slide, starting with the first trial of F Anderson in 1990 which for several years was considered as unsuccessful, but turned out to be perfectly successful.

**slide 29: GT adverse events**

Three major adverse events (one resulting in the death of the patient directly caused by the treatment) are mentioned in this slide. in fact this also demonstrates that the experimentation as in general been done with the necessary care. only one death out of 4000 treated patients. Not many experimental treatments can proudly recount such a honourable score. This score could shift rapidly and dramatically to many more negative

events if GT is getting out of the hands of experts and careful clinicians that practice it under full GCP compliance (for instance this could happen if GT would be practiced in some illegal procedure like doping)

### **slide 30: ups and downs of gene therapy**

The fate of gene therapy has been always marked by strong hypes and strong downs in the general 'mood' of the involved scientists. The major adverse events generated controversies, halting of trials, enquiries, debates etcetera. The hypes were in part artificially boosted by reckless scientists of company executives that made statements containing unrealisable promises. There is nothing like the unjustified raising of hopes that can react in a dramatically opposite manner when the reality becomes manifest. The bottom line of this slide is to say that in spite of its relatively long existence (almost 13 years) gene therapy is still in its infancy and still produces more questions than answers.

### **slide 31: the three levels of doping**

In my simplified view there are three levels at which doping procedures can apply: before the event (preparatory doping); during the event (performance doping); after the event (recovery doping). Molecular medicine can provide assistance at all these three levels.

### **slide 32: which gene transfer approaches could be compatible with doping**

we can imagine gene transfer being adopted at different manners: a) ex vivo on the hematopoietic tissue, the genes for special factors like erythropoietin receptors or other bystanders could be transferred to ameliorate erythropoiesis. (b) in vivo local delivery in the muscle is certainly the most attractive and straightforward approach, since it could be done with naked DNA. Genes encoding metabolic enhancers, growth factors or muscular fiber shift inducers could be envisaged for this transfer. The expected result is probably meager, at the current state of vectorology and certainly not justified in light of the associated risks. c) another target for in vivo local delivery could be joints and tendons: in that case we could imagine the transfer of pain reducers (like proenkephalins), inflammation inhibitors, recovery and repair factors (treatments analogous to anti rheumatoid arthritis approaches). Finally we could even imagine some systemic delivery of genes encoding endocrine anabolic factors, pain killers or anti inflammatory agents. These are all science-fiction if taken under GCP vision. However, it may be that some reckless medical doctors could indulge in such practices even without any documentable effect.

### **slide 33: which are the current limitations in gene-based doping**

The current status of research allows us to identify several limitations that render gene transfer an objectively risky and inefficient procedure for doping purposes. If viral gene transfer ought to be used (first panel) this procedure is linked to moderate to severe immune reaction that certainly impair subsequent administration and could lead to general toxicity (see slide 26). In the long term the use of randomly integrating viral vectors could lead to genotoxic consequences and potentially carcinogenic insertional mutagenesis. In nonviral gene transfer (second panel) the major issue is its intrinsic inefficiency that implies repetitive use and relatively high amounts of formulation. It is known that chronic exposure to DNA could cause auto-immune conditions, and this remains the major potential side effect of this procedure. In more general terms (third panel) we shall acknowledge that gene transfer technology is rather laborious and not necessarily available. the regulation of transferred genes is not yet mastered and this could become a serious risk factor when powerful endocrine or paracrine factors are expressed beyond control. Finally, gene transfer is bound to leave a long term trace (tagging) of the tissue, and this could facilitate detection by anti-doping officials. Thus it would make it a procedure that is rather difficult to disguise.

### **slide 34: which side effects could be feared in gene-based doping strategies?**

Concerning the side effects we can recapitulate the short term ones that are the more or less severe immune shock and toxic shock (depending on the used vector, delivery

method). In the long term the insertional mutagenesis- induced cancer risk would be a factor that shifts the balance towards the negative side. In my opinion the factor that one should fear mostly is linked to general malpractice by reckless medical doctors and even more reckless athletes. Lack of proper knowledge could lead to unsuitable administration in terms of doses and or vector used; secondly, due to the illegality level we could fear the circulation of non-clinical grade materials (either viral vectors contaminated with replication competent particles or DNA preparations containing adventitious pathogenic micro-organisms or endotoxins (typically present in high amounts in poorly purified DNA).

**slide 34: which detection methods would be (or not) envisageable for gene doping strategies?**

Foreign genes are relatively easy to detect in a biopsy, when the suspected tissue is known. Recombinant vectors remnants are certainly also detectable up to few days after delivery in blood if they have been administered systemically (which is unlikely in the today's practice). However, in absence of strong suspicion it may be difficult to have a rapid and generalised screening. Recombinant proteins produced from transferred genes could only be detected if they have some detectable difference with endogenous proteins either in their sequence or in their post-translational modification pattern (such differences can happen when the genes are expressed in tissues where they are normally not expressed, and therefore the final product is processed in a slightly different manner).

**slide 35: final side-by-side comparison: gene-based versus drug- or protein-based doping**

In this slide I have tried to put myself in the mind of a pro-doping rational athlete and establish a common-sense comparison of the odds in favour /disfavour of conventional doping (drug or protein-based) and gene based procedures. In terms of rapidity of the effects and reversibility of the manifestation the conventional doping is certainly superior. In terms of dosage, gene-delivery also has the big disadvantage that it would be cumbersome to adjust to the exact needs. Also in terms of complexity of the procedure and of associated risks, gene delivery does not presents advantages, and there are also chances that it could be relatively straightforward to detect in case of suspicion. This means that objectively and rationally speaking, gene-based doping would not offer any particular advantage compared to conventional doping, at least in the light of today's knowledge. However, these considerations apply to common-sense thinking, and this does not seem to be an aspect that can be guaranteed in the doping arena.

**slide 36: thanks!**

With this slide I thank the organisers for a very interesting meeting (that has given me the occasion to learn a lot of novel concepts), my laboratory collaborators, the SNF for having offered me the chance to learn so much about gene therapy in directing the NFP37, and the audience, who seemed to appreciate very much the talk, at least judging from the lively discussion. All those who may have *a posteriori* queries in the field of gene transfer are welcome to contact us at [sandro.rusconi@unifr.ch](mailto:sandro.rusconi@unifr.ch), or to visit the WEB site for the gene therapy program: <http://www.unifr.ch/nfp37>