Review

Drug Testing and Analysis

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Detection of *EPO* gene doping in blood

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Gene doping – or the abuse of gene therapy – will continue to threaten the sports world. History has shown that progress in medical research is likely to be abused in order to enhance human performance. In this review, we critically discuss the progress and the risks associated with the field of erythropoietin (EPO) gene therapy and its applicability to EPO gene doping. We present typical vector systems that are employed in *ex vivo* and *in vivo* gene therapy trials. Due to associated risks, gene doping is not a feasible alternative to conventional EPO or blood doping at this time. Nevertheless, it is well described that about half of the elite athlete population is in principle willing to risk its health to gain a competitive advantage. This includes the use of technologies that lack safety approval. Sophisticated detection approaches are a prerequisite for prevention of unapproved and uncontrolled use of gene therapy technology. In this review, we present current detection approaches for EPO gene doping, with a focus on blood-based direct and indirect approaches. Gene doping is detectable in principle, and recent DNA-based detection strategies enable long-term detection of transgenic DNA (tDNA) following *in vivo* gene transfer.

Keywords: gene doping; gene therapy; detection; EPO; erythropoietin

Introduction

More than 1700 gene therapy clinical trials have been completed since the concept of gene therapy emerged in the 1970s.[1] Conceptually, gene therapy is based on the strategy of introducing genetic material into an organism in an effort to treat an underlying pathology. Gene therapy can be further distinguished into *ex vivo* and *in vivo* gene transfer. *Ex vivo* gene transfer approaches are based on the ability to isolate cells from a donor and to expand them in culture with or without genetic modification. The cells can be subsequently transplanted back into the donor.[2] In contrast, *in vivo* gene transfer aims to directly introduce genetic material into the individual.[3] Most commonly, viral vectors[4] and non-viral vectors such as plasmids[5] are utilized to transfer genetic material. Transgenes are assigned to either replace a defective gene, modulate the activity of an existing gene, or to enhance gene expression as an additional gene copy. Currently, gene addition is the most commonly attempted approach in preclinical and clinical studies.[3] In this strategy, target cells are transduced with a protein-coding DNA sequence in order to supply a sufficient level of the protein to patients who suffer from a loss of function mutation.

Gene therapy was initially conceived to treat hereditary genetic diseases by correcting or curing the underlying genetic defect[6] and number of effective gene therapy studies have been conducted to cure severe diseases such as x-linked severe combined immunodeficiency disease (SCID),[7] x-linked granulomatous disease,[8,9] and hemophilia B.[10] Currently, more than 80%[11] of gene therapy research aims to cure diseases such as cancer,[12] cardiovascular disease,[13] neurodegenerative disorders,[14] and infectious diseases such as AIDS.[15] As reviewed by Friedman, the success of early gene therapy studies demonstrates that the introduction of genetic material into humans is efficient and stable enough to ameliorate the status of life-threatening diseases and to alleviate suffering.[16]

While the concept of gene transfer sounds quite simple, history clearly shows that adverse events are difficult to predict. In 1999, an 18 year old boy who suffered from a partial deficiency of ornithine transcarbamylase died in response to viral gene transfer that was part of a gene therapy trial. The injection of 3.8×10^{13} particles of a second generation E1- and E4-deleted adenovirus vector led to a massive inflammatory response and the patient died approximately 98h after gene transfer due to Systemic Inflammatory Response Syndrome.[17] It was discovered that the patient's immune system was sensitized by a previous exposure to a wild type virus. A second patient tolerated a similar dose of viral vector and only exhibited mild fever.[18] Beginning in 1999, several young patients received *ex vivo* gene therapy to cure X-linked SCID-XI disease. Despite initial success, some of the patients developed leukemia caused by insertion of retroviral DNA into critical human DNA elements.[19]

In spite of such fatal setbacks, the misuse of gene technology is becoming a real threat to the world of sports. A number of animal studies have demonstrated the possible performance-enhancing capacity of genetic manipulation. This fact, coupled with the availability of gene technology makes it amenable to abuse with the intention to modulate normal human traits and to enhance athletic performance.[16] Certain people are willing to try anything to achieve their ambitions and past experience demonstrates that doping substances and methods find application in sports before safety and efficiency is demonstrated.[16] It is not

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uncommon for an athlete to risk his or her life for success,[20] and at times the coach is at fault for obtaining and introducing poorly evaluated drugs.[21]

As reviewed by Haisma and de Hon, concern with the abuse of gene technologies, commonly referred as 'gene doping' emerged over a decade ago.[22] As a result, the Medical Commission of the International Olympic Committee held a meeting in 2001 to discuss gene therapy and its potential impact on sports.[22] In 2003, gene doping was included in the World Anti-Doping Agency's (WADA) list of banned substances and methods. The 2012 WADA Prohibited List defines gene doping as 'the transfer of nucleic acids or nucleic acid sequences' and/or, 'the use of normal or genetically modified cells' with the intention to enhance sport performance.[23] A number of potential targets have been shown to enhance strength or endurance performance. These include growth hormone (*GH*),* insulin-like growth-factor-I (*IGF-I*), myostatin (*MSTN*), follistatin (*FST*), vascular endothelial growth factors (*VEGFs*), and erythropoietin (*EPO*).[25,26]

All of the above-mentioned entities are endogenously produced proteins with various effects on growth. Due to their anabolic function, the recombinant versions of GH and IGF-1 have been used as conventional doping agents in sports.[27] In 2004, Lee *et al*. demonstrated that intra-muscular (i.m.) injection of a recombinant adeno-associated virus (rAAV) vector containing the *Igf-I* gene resulted in a 14.8% increase in muscle mass in rats after 8 weeks without training. A combination of vector-mediated gene over-expression and exercise doubled this effect.[28] MSTN is a protein produced in the muscle itself and acts as a negative regulator of muscle growth. Inherited inability to produce MSTN leads to enlarged muscle mass,[29] whereas the inhibition of MSTN action, e.g. by increased FST levels, leads to a rapid increase in muscle mass and strength.[30] In addition, recombinant AAV-mediated *Vegf* gene transfer improved muscle regeneration and vascularization after muscle damage in ischemic mice.[31]

In this review, we will critically discuss the applicability of *EPO* gene transfer technology for the enhancement of sports performance. There have been no recent reports suggesting that gene transfer technology has been misused in sports. However, it was feared that a virus-based drug containing the human *EPO* gene (Repoxygen™) might be misused for gene doping. The plausibility of this concern came to light in an email correspondence between the German athletics trainer T. Springstein and his drug dealer.[21] For some time, it was speculated that gene doping remains undetectable and that this lack of detectability would encourage athletes to abuse gene therapy to enhance sports performance.

In the following discussion, we will describe and discuss the potentials of gene delivery strategies, including viral and non-viral gene transfer, applied *in vivo* or *ex vivo*. We will then highlight potential blood-based *EPO* gene doping detection approaches based on highly sensitive long-term detection of transgenic DNA (tDNA) following *in vivo* gene transfer. Finally, safety concerns that emerge from gene doping for both the individual as well as the surrounding population will be presented.

*According to the guidelines for gene nomenclature, human and non-human primate gene symbols are italicized, with all letters in uppercase. Gene symbols for mouse and rat are italicized, with only the first letter in uppercase and the remaining in lowercase letters. Protein symbols are represented in standard fonts. Please find details in.[24]

EPO physiology and its therapeutic use

EPO is a hypoxia-induced cytokine that is a major regulator of erythropoiesis. The kidney is the main site of EPO production in adult mammals, although it is also synthesized at lower levels in several other tissues such as liver and brain.[32] The human *EPO* gene is located on chromosome 7 (7q22)[33] and it contains 5 exons and 4 introns. The transcription product is a 165 amino acid glycoprotein containing 3 N-linked and 1 O-linked carbohydrate chains, which make up approximately 40% of the molecule's weight.[34] *EPO* gene expression increases with decreasing O_2-pressure in tissue. EPO protein circulates in blood and stimulates proliferation and differentiation of erythroid precursor cells in bone marrow,[35] leading to an increase in the red blood cell count and enhanced O_2 transport capacity. A large number of EPO stimulating agents (ESAs) including recombinant EPO (rEPO), rEPO biosimilars, and EPO-mimetics have been developed to treat anemia.[36]

Anemia is associated with a number of chronic and heredity diseases such as progressive renal failure and AIDS, and it can occur as a result of chemotherapy and radiation treatment in cancer patients. Due to the short half-life of EPO, patients require repeated injections of rEPO with a frequency of up to 3 times a week. Despite its effectiveness, this kind of therapy is expensive and inconvenient.[5] Gene therapy protocols aim to facilitate the treatment of anemic diseases, by enabling sufficient and sustained production of functional EPO.[37] Gene therapy could reduce costs and hospital visits, improving quality of life.[5] A large number of *EPO* gene therapy trials have been completed and efficiency has been demonstrated in a number of rodent and non-human primate studies.

Viral gene transfer with EPO

Viruses are highly evolved biological machines that can gain access to host cells and deliver their genetic material with high efficiency. This property led to the development of viruses as vectors for gene transfer.[4] By removal of distinct viral genes, viral replication can be hindered and genes of therapeutic relevance can be inserted. Viruses show different features depending on the therapeutic aim and type of target cell (see Table 1). More than 68% of all gene therapy studies used viral vectors.[1] The major pitfall for sustained transgene expression has been the host's immune response, which can be targeted against the viral vector system or the transgene product.[3]

Following the initial success of virus-mediated *EPO* gene transfer in immune-deficient mice,[38] a large number of studies were conducted using Ad-,[38–40] AAV-,[41–43] and lentiviral[44] vectors in mice and non-human primates (see Table 2).

Due to the significantly reduced immune reaction against rAAV[45] and its high efficiency in transducing muscle tissue,[46] almost all studies employed rAAV vector systems for *EPO* gene delivery. In 2000, Chirmule *et al*. showed prolonged transgene expression and elevated hematocrit over the course of 600 days in non-human primates after a single i.m. injection of rAAV carrying the rhesus monkey *EPO* gene.[47] Nevertheless, a number of studies reported immune responses targeting the endogenous EPO protein after i.m. gene transfer with rAAV[42,48,49] leading to severe anemia and the necessity for animals to be euthanized.[42] The reasons for autoimmune reaction against endogenous EPO are not completely understood, but they are likely to be associated

Table 1. Features of viral vector systems.

	Adenovirus	Adeno-associated virus	Oncoretrovirus	Lentivirus
Features of wild type virus	Non-enveloped double stranded DNA virus	Small non-enveloped single stranded DNA virus	Enveloped single stranded RNA virus (two copies)	Enveloped single stranded RNA virus (two copies)
Packaging capacity of the recombinant vector	~30 kb (3rd generation)	~4.5 kb	~8–10 kb	~8 kb
Chromosomal integration	Remains mainly episomal	Small part integrates into host cell genome (~1%)	Integrates into the host cell genome of dividing cells	Integrates into the host cell genome of dividing and non-dividing cells
Risk of insertional oncogenesis	Small	Small	High risk	Lower when compared with oncoretrovirus (non-integrating lentivirus vectors are under development)
Long term gene expression	No	Yes	Yes	Yes
Advantages for EPO gene transfer	- 3rd generation shows less immunogenicity compared to further generations - Can be produced at high titres	- Low immunogenicity	- Shows long term gene expression	- Transduces dividing and non-dividing cells
Disadvantage for *in vivo* EPO gene transfer	Short term gene expression due to its remaining immunogenicity [124],[125]	- High efficiency to transduce muscle cells Difficult to produce at high titres [45]	- Usually used *ex vivo* Inability to infect non-dividing cells such as muscle cells. [126]	- Usually used *ex vivo* Low efficiency to transduce muscle cells [126]
More comprehensive overview and details in				

with high level production of the therapeutic protein at ectopic sites,[48] along with the efficiency of rAAV in transducing antigen presenting cells.[49,50]

Regulation of *EPO* transgene expression

A number of regulatory systems have been developed to control or adjust transgene expression in an effort to avoid the adverse side effects of transgene overexpression. Some examples include rapamycin,[43] doxycyclin,[51] mifepristone,[52] and a hypoxia inducible regulatory system.[53] In 2002, Binley *et al.* described a hypoxia regulatory system containing a hypoxia response-element called 'Oxford Biomedica hypoxia response element' (OBHRE).[53] OBHRE serves as a promoter element that requires hypoxia inducible factor-1 (HIF-1) to stimulate transcription. I.m. injection of rAAV-2 vector systems containing murine *Epo* under control of OBHRE was tested in healthy and anemic Epo-Tag^h mice. In anemic Epo-Tag^h mice, the hematocrit reached normal physiological levels for the duration of the 7 month time course of the study.[53] This physiological O_2 dependent regulatory system was further utilized by the drug Repoxygen™ (Oxford BioMedica, Oxford, UK). However, the development of the drug was stopped and never continued beyond experiments in mice.[53] It was speculated that i.m. delivery of the drug leads to physiological hematocrit levels because the OBHRE should cease EPO production in muscle cells under normoxic conditions. Nevertheless, misuse of this kind of vector system could lead to unpredictable consequences in healthy and physically active athletes. O_2-tissue levels decrease in muscle cells as a result of endurance training, or any kind of prolonged muscle activity.[54] An acute bout of exercise induces HIF-1 mediated pathways in healthy human muscle cells, including an increase in EPO mRNA levels.[55] This may lead to production of supra-physiologic levels of EPO. Hence, the influence of exercise has to be considered before using HREs in muscle cells.

Only tetracycline and rapamycin regulatory systems have found application in large animal studies.[56] Favre *et al.* used a Tet-On regulatory system in non-human primates wherein administration of doxycycline (a tetracycline derivate) led to high EPO secretion and increased hematocrit. 1 out of 6 rhesus macaques showed adjustable EPO regulation for the entire experimental period of 13 months. In 5 out of 6 animals, the efficiency of EPO secretion vanished over time and expression was diminished after 2 to 5 doxycycline administrations.[51] The investigators found that a humoral immune response was directed against regulatory system proteins, leading to a cell-mediated immune response against genetically modified myofibers.[51] At least one cynomologus macaque showed sustained and adjustable transgene expression for at least 5 years.[57] As reviewed by Le Guiner *et al.*, in contrast to the tetracycline dependent systems, the rapamycin regulated system contains exclusively human proteins, which should somewhat reduce the likelihood of an immune reaction.[56] In 2005, Rivera *et al.* demonstrated long term expression of the EPO transgene in non-human primates over a period of at least 6 years and 26 induction cycles in the longest non-human primate study. 100% long-term persistence with no apparent immune response was reported using a 'next-generation rapamycin-regulated rAAV vector.[43] Overall, the investigators described persistent regulation of gene expression in 21 rhesus monkeys and 5 cynomolgus monkeys.[43] However, the degree of i.m. *EPO* gene transfer safety is still unclear and systematic administration is not yet possible.

Table 2. Selected *EPO* gene transfer studies using viral vector systems.

Viral *EPO* gene transfer	Rodents	Non-human primates	Duration of the study	Related citations
rAd		Cynomolgus macaques	84 days	[40]
rLV	Fischer 344 rats	-	14 month	[44]
rAAV		Rhesus macaques	>600 days	[47]
rAAV + HRE regulated	Epo-Tag^{h} mice	-	7 month	[53]
rAAV + Tet-regulated		Cynomolgus macaques	>5 years	[51],[57]
rAAV + rapamycin regulated		Rhesus macaques	>6 years	[43]

Non-viral gene transfer

The typical vector system for non-viral gene delivery is plasmid DNA (pDNA). This kind of gene delivery offers some advantages compared to viral gene delivery. pDNA vectors are less immunogenic and do not provoke pre-existing antigen-dependent immune reactions.[58] Furthermore, pDNA vectors can be produced in large quantities with greater ease and at a reduced cost when compared to viral vectors.[59] In addition, the safety of application is not limited by potential replication effectiveness, which has to be critically evaluated and controlled in viral vector production.[59] However, the major limitation of *in vivo* use of pDNA is poor transfection efficiency and short duration of successful transgene expression.[3] (See Table 3). Only approximately 19% of all gene therapy studies to date utilized naked DNA or plasmid vectors, whereas viral mediated gene transfer was used in a comparatively larger number of studies.[1] Alongside the injection of 'naked' pDNA which is most inefficient, methods such as electro transfer have been used, showing very poor transfection efficiency in non-human primates.[60]

The most impressive results in the field of non-viral *EPO* gene transfer are presented by Sebestyén *et al.*[5] The authors used the hydrodynamic limb vein (HLV) procedure for pDNA delivery; this is a simple and effective technique for rapid injection of a large volume of DNA-containing fluid.[61] Repeated injection of small doses of pDNA led to sustained EPO protein expression over a period of 450 days in non-human primates. Nevertheless, high inter-individual variability was observed and at least a 20–30 fold higher pDNA dose was needed in non-human primates compared to rodents to reach a similar effect.[5] Sebestyén *et al.* treated at least 22 rhesus monkeys and more than 200 anemic healthy rats. None of the animals developed lower than 'normal' red blood cell counts,[5] which is indicative of a lack of autoimmune reaction against endogenous EPO.

Development in the field of plasmid vector construction led to production of 'minicircle' DNA vectors, which are characterized by improved transfection efficiency.[62] However, it currently remains unclear if the problems associated with delivery efficacy can be overcome and whether *in vivo* application of pDNA can reach clinical significance.[3]

In addition to DNA based gene delivery, the use of modified mRNA for EPO production has recently been reported. In 2011, Kormann *et al.* developed nucleotide-modified EPO mRNA. This modification led to decreased mRNA interaction with various Toll-like receptors and reduced activation of the innate immune system. In addition, the stability of mRNA increased *in vivo*. A single i.m. injection of 5 μg dual-modified mEpo mRNA in BALB/c mice increased the hematocrit from ~51% to ~64% during the 4 week time course of the experiment.[63] Nevertheless, the efficiency of modified mRNA in non-human primates remains to be proven.

Ex vivo gene transfer

Apart from *in vivo* gene transfer, a number of *ex vivo* approaches have been developed. They all share the same strategy of retrieving cells from a donor and genetically modifying these cells in culture.[2] The first *ex vivo* gene therapy approaches for *EPO* gene transfer were based on the transplantation of myoblasts, smooth muscle cells,[64] and fibroblasts.[65] In spite of some success in mouse models, the approach is generally hampered by a number of limitations, including immune response and poor survival of the transduced cells.[66,67] In 2005, Lippin *et al.* used human dermal cores for *ex vivo* transduction with Ad-5 viral vector. The dermal samples had a diameter of about 1.5-2.5 mm and were 3–3.5 cm long. 10 human participants with moderate chronic renal failure received subcutaneous 'Biopump-hEPO' implants. Depending on the pre-determined *ex vivo* EPO secretion rate of the 'Biopump', between 1 and 7 'Biopumps' were reimplanted under the abdominal skin. Following transplantation, EPO levels peaked on day 3 in most cases and declined until day 10, due to immune response. No increase in hematocrit was observed.[68] In 2011, Medgenics Company reported successful Phase I/II clinical trials and to date, 14 patients showed safe and sustained EPO production after a single Biopump administration.[69] However, this information has not been published in a scientific journal and the blood parameters have not been reviewed by the scientific community.

More than 40 years ago, the idea to encapsulate cells in microcapsules prior to implantation emerged.[70] The membrane surrounding the cells allows inward diffusion of nutrients and outward release of therapeutic protein, simultaneously preventing cell

Table 3. Selected *EPO* gene transfer studies using non-viral gene transfer.

Non-viral *EPO* gene transfer	Rodents	Non-human primates	Duration of the study	Related citation
Naked pDNA	Balb/c mice	-	90 days	[127]
pDNA + electroporation		Cynomolgus macaques	41 days	[60]
pDNA + HLV		Rhesus monkeys	>450 days	[5]
Modified mRNA	Balb/c mice	-	4 weeks	[63]

contact with immune competent host cells. In addition, the implant is removable and the pharmacological effect can be reversed.[71] A number of various cell types have been utilized for *EPO* transgene delivery in mouse models, including fibroblasts,[65] encapsulated myoblasts,[72] and bone marrow stroma cells.[71] While a number of materials such as collagen have been used for cell encapsulation, the most frequently used material is alginate.[73] Nevertheless, as reviewed by Rabanel *et al*., far from their early promise, microcapsules have failed to achieve clinical significance.[74] Problems include an inflammatory response against the capsule material, the decreased viability of the encapsulated cells, and insufficient mechanical resistance of the microcapsules.[74]

Detection of *EPO* gene doping in blood

Suitable gene doping detection methods are a major prerequisite for preventing athletes from using gene doping technologies.[75] The detection method must be sensitive and sufficiently robust to withstand legal scrutiny. Furthermore, samples must be easily accessible with minimally invasive techniques and the method should be cost effective and permit high-throughput. A number of potential strategies and concepts have been suggested to detect the abuse of gene transfer techniques.

Detection of transgenic EPO protein

The first approach for detection of *EPO* gene doping in blood was reported by Lasne *et al*. in 2004. The investigators discovered that isoelectric focusing patterns of EPO proteins derived from genomic DNA (gDNA) and proteins artificially encoded by tDNA in muscle cells discriminate in serum and can be distinguished by a conventional EPO test consisting of double blotting and isoelectric focusing.[76] EPO molecules differ in their glycosylation pattern due to different post-translational modifications in various tissues.[76,77] However, detection of gene doping on the protein level is hampered by the possibility that post transcriptional modifications may differ depending on the gene transfer protocol, the route of vector administration, the vector used, the target tissue and finally of course, the target species.[78] This must be determined in humans because it is questionable whether findings related to posttranslational modifications in non-human primates can be extrapolated to humans. Additionally, this detection method would not be applicable for the detection of transgene derived proteins that are naturally expressed in muscle tissue, such as muscle specific isoforms of IGF, or proteins without carbohydrate chains such as GH.[79]

Detection of immune response specific for *EPO* gene transfer

A number of indirect strategies have been suggested for the detection of gene doping. These include the detection of specific immune responses to the vector system, the regulatory systems or the transgene protein.[79] However, screening for innate, adaptive cell-mediated and adaptive humoral immune responses seems to be of limited value. Memory T-cells and antibodies against vector particles could be detected easily over an extended period of time by the use of specific ELISA or Western blot. However, the approach is hampered by the possibility of natural (non-doping related) viral infection. Unfortunately, the most frequently utilized viral vectors are the ones for which prevalence and incidence of natural infections is very high. Accordingly, such procedures would be unacceptable and unspecific, causing false positive test results far too often. Such detection procedures would also have limited use as non-viral mediated gene transfer is unlikely to produce antibodies or other detectable adaptations of the immune system and would not be detectable by such a procedure.[79]

Detection of EPO gene transfer using transcriptomics

Since technical progress has enabled scientists to investigate genome-wide gene expression patterns, it has been suggested that the abuse of gene doping can be detected by screening the blood's transcriptome.[80] In general, large scale screening techniques such as Serial Analysis of Gene Expression (SAGE) or microarrays are utilized to define specific biomarker or gene expression patterns that respond to distinct influences such as diseases,[81] exercise,[82] or the abuse of doping substances.[83] Due to the superior sensitivity and reproducibility of reverse transcription quantitative PCR (RT-qPCR), it is generally used to validate potential candidate genes identified by approaches such as SAGE or microarrays.[84] In 2009, Varlet-Marie *et al*. screened the blood transcriptome before, during, and after rEPO administration in humans. 5 genes were identified to be slightly deregulated for at least 1 week following the last administration. A potential advantage of the transcriptomic approach would be the ability to detect a spectrum of different EPO doping procedures, including all kinds of ESAs and gene doping, because all share a common downstream pathway following EPO-receptor activation.[85] However, the inter-individual variation poses a major challenge of indirect detection approaches along with the determination of normal levels and levels providing evidence for doping.[25] To circumvent these problems, early indirect detection approaches rely on a Bayesian statistic that is based on a large number of population-derived reference intervals, thereby correcting for inter-individual levels.[86] Gene expression levels show distinct inter-individual variation according to biological traits such as ethnicity,[87] sex, age, gender,[88] and health status.[89] Furthermore, external influences such as exercise,[90] medication,[91] nutrition[92] and natural stimulants[93] influence gene expression patterns. In addition, sample retrieval, storage and processing[94] are likely to influence the outcomes of gene expression analysis.[95] To validate potential biomarkers and to establish reference levels in the future, a large number of blood samples will have to be collected following strict standardized conditions. A major problem with mRNA profiling, in particular utilizing the blood transcripome, seems to be that the impact of doping on gene expression does not seem to be pronounced enough to distinguish from naturally occurring intra-individual and technical variation.[83,95] Conceivably, screening for micro RNA (miRNA) in serum or plasma leads to more reliable tests. miRNAs are short (~22 nucleotides) non-coding RNA molecules, that modulate gene expression post transcriptionally and have important functions in various biological processes.[96] The contribution of miRNAs to various diseases and their stability in plasma and serum samples raised the possibility that miRNAs serve as reliable biomarkers for the diagnosis of cancer and other diseases.[97] Recently, McDonald *et al*. described preanalytical and analytical parameters which vigorously influence the outcome of miRNA quantification.[98] It remains to be shown whether the abuse of doping substances induces a distinct up- or down regulation of biomarkers that can be distinguished from naturally occurring intra-individual and technical variation. Following identification, potential biomarkers must be validated against external

influences to avoid false positive results. It is important keep in mind that knowledge about the transcritpome, its regulation, and confounding variables is still very limited, and a contemporary validation of reliable biomarkers seems to be unlikely.[95]

Screening for blood parameters

In 2009, WADA approved the Athlete Biological Passport (ABP).[99] The ABP is based on the concept of monitoring athletes' biological variables over time to facilitate indirect detection of doping.[99] The hematological module of the ABP is based on a global interference approach for the detection of abnormal blood parameters.[100] Over the course of the past decade, much effort has been placed on improving the efficiency and reliability of this indirect detection method. The implementation of the WADA Operation Guidelines was a major step towards reducing the risk of false positive results, as the guidelines serve as a protocol for standardized sample collection, processing and results management.[101] In the event that blood doping is suspected as a result of unusual blood parameters, 3 specialists have to evaluate the probability of doping.[101] A unique advantage is that any method that aims to increase red cell mass and enhances oxygen transport capacity, including gene doping is detectable in principle.[102] However, sophisticated doping protocols enable athletes to continuously dope below the detectable threshold.[103] Gene doping protocols utilizing regulatory systems in combination with blood draws to lower hematocrit and blood infusions when reticulocyte count needs to be lowered could also warrant to remain undetectable. Given this situation, direct detection methods that allow unequivocal identification of doping agents are preferable over indirect detection methods and should be the method of choice whenever possible.

Detection of transgenic DNA in spiked samples

In 2008, Beiter *et al.* described a procedure that enables direct detection of gene doping through tDNA detection in blood.[78] As a basis for diagnostic discrimination, the investigators considered the fact that gene transfer protocols do not use 100% homologous human gDNA, but tDNA that lacks intronic sequences (see Figure 1).

Since intron-less DNA does not occur naturally, the investigators developed 'single-copy primer-internal intron–spanning PCR' (spiPCR). Primers were chosen with respect to the gene-specific exon-exon structure. The primer pairs bind only at the exon-junctions of intron-less tDNA (see Figure 2).

A major advantage of this approach is that a clear 'yes' or 'no' answer is possible when assessing the presence or absence of tDNA. The principle of this detection approach was introduced in 2006 by Simon *et al.* as a general method to detect the fate of tDNA in blood following gene transfer, and it is currently patent pending.[104] In 2008, the spiPCR protocol was tested for *EPO* and *VEGF-D*. Using HotStart and nested PCR protocols, detection of 1 copy of *EPO* tDNA in the presence of 300 ng gDNA was reported. In addition, whole blood samples were spiked with *VEGF-D* transduced cells and a calculated number of 2.25 cells/μl could be detected in blood DNA preparations.[78] In 2010, Baoutina *et al.* evaluated the intron-spanning detection protocol to detect pDNA-EPO in the background of gDNA. Using intron-spanning hydrolysis probes (see Figure 3) the investigators developed various assays to optimize test specificity and sensitivity. Performing serial dilution standard curve analyses, investigators could detect the presence of ~1 copy of tDNA in a background of 27000 copies of the endogenous *EPO* gene with 100% specificity and no false-positive results. This corresponds to ~11 copies of tDNA in 1 μg human gDNA in the *in vitro* system.[105]

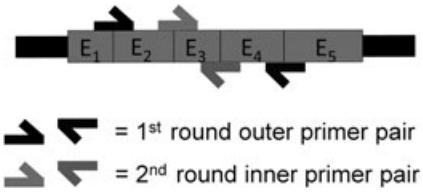

Figure 2. spiPCR is based on a nested PCR using inner and outer intron-spanning primer pairs.

Recently, Scarano *et al.* suggested surface plasmon resonance imaging (SPRi) for the detection of tDNA following gene doping.[106] SPRi is an optical surface sensitive technique that can be used to simultaneously study a large number of biological interactions.[107] To prove the principle of SPRi for the detection of tDNA, the investigators developed an *in vitro* approach to detect PCR amplicons of enhanced green fluorescence protein (EGFP) and CMV promoter, which serve as markers for transgenosis.[106] An injection loop of 50 μl was used for each SPRi measurement. The experimental detection limits for the various targets were 12 nano molar (nM) for EGFP1, 0.2 nM for EGFP2 and 3.5 nM for CMV.[106] Although this approach represents an innovative tool for simultaneous tDNA detection, it seems unlikely that it is sufficiently sensitive to detect tDNA after a single gene transfer without previous amplification in 3 ml whole blood.

Detection of transgenic DNA in animal models

For the simple reason that sensitivity and specificity of *in vitro* model systems may not be reproducible under field conditions, detection approaches must first be validated in *in vivo* animal models. Ideally, they would be validated in humans, but this would be completely unethical. In 2011, Beiter *et al.* developed a sample preparation and processing protocol that enables simultaneous detection of 6 gene doping candidates (*EPO, IGF-1, VEGF-A, VEGF-D, GH-1, FST*) in a single blood sample. The detection protocol includes a two-round, nested PCR assay using outer and inner intron-spanning primer pairs. As a first step, the reliability, sensitivity and specificity of the detection protocol was confirmed *in vitro*. A background of 750 ng gDNA was spiked with 1 kb cDNA standards containing known numbers of transgene copies. For each candidate, the detectability of 2 tDNA copies was reported. To reduce reagent consumption and preparation

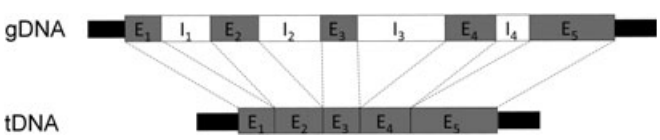

Figure 1. Genomic DNA (gDNA) contains exons (EN) and introns (IN), whereas transgenic DNA (tDNA) contains only exons.

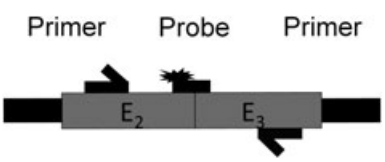

Figure 3. Real-time PCR setup using intron spanning probe.

time, the investigators developed a multiplex PCR assay. At least 4 candidate genes (*GH-1*, *VEGF-A*, *VEGF-D* and *EPO*) could be included in a first-round multiplex PCR. The second round of the multiplex nested PCR assay is separate for each candidate.

To validate the detection protocol *in vivo*, C57BL/6N mice were treated by i.m. injection with 10 μg plasmid vectors, or 3.3×10^{11} viral genomes of rAAV2/1 containing transgenic human *VEGF-A*. 20 μl blood samples were taken at days 1, 2, 7, 14, 28, 56. All rAAV treated animals tested positive until day 28. In 4 out of 6 animals, the tDNA could be detected until the end of the experiment at day 56. The presence of tDNA after plasmid gene transfer was only detected through the second day following gene transfer. In this context, it is notable that plasmid gene transfer was unsuccessful when compared with rAAV mediated gene transfer.[108]

Ni *et al*. evaluated the detectability of *EPO* gene transfer in non-human primates.[49] The experiments were conducted in 6 cynomolgus macaques. The primates received i.m. injection of either a high dose of plasmid vectors (pKanaORI/PGFcmEPO) or a very low dose of rAAV vectors (rAAV-1-MD2-cmEPO or rAAV-8-MD2-cmEPO). Plasmid and rAAV vectors contained transgenic cynomolgus macaque *EPO* cDNA under control of different promoters (PGK or CMV, respectively). In order to detect the biodistribution of the injected tDNA sequences and to distinguish them from endogenous DNA, the investigators designed a Taqman primer-probe set spanning the 'EPO Exon 2-3' boundary with the probe hybridizing to the exon-exon junction (see Figure 3). This assay can detect transgenic *EPO* DNA regardless of other genetic elements incorporated into the vector. To test the efficiency of the 'EPO Exon 2-3', the investigators developed assays to detect SV40pA along with the kanamycin resistance cassette, because these targets have no competition with endogenous sequences. In the presence of 500 ng gDNA, the efficiency of the 'EPO Exon 2-3' assay was inhibited by approximately 10% because of competition with endogenous gDNA sequences. *In vitro* testing for the lower limit of detection demonstrated that 3 copies of target plasmid could be detected in a background of 500 ng gDNA.

For *in vivo* detection of tDNA, the investigators utilized DNA that was extracted from white blood cells (WBC) in 3 ml of whole blood. To determine the detectability of plasmid vector tDNA, 2 cynomolgus macaques received i.m. injection of a high dose (10 mg) of pKanaORI/pGKcmEPO plasmid vector. The determination of hematological parameters showed that only 1 of the 2 macaques experienced a slight increase in the number of reticulocytes, but no effect on hematocrit was detected. In the second macaque, no significant effect on reticulocytes or hematocrit was observed. Using the 'EPO Exon 2-3' detection assay, the results emphasize that plasmid gene transfer is detectable for 3 weeks in WBC. To detect viral gene transfer, 4 macaques were injected with low doses (5×10^9–2.5×10^{11} vector genomes/kg body weight) of rAAV-1 or rAAV-8. Using the EPO Exon 2–3 assay, the vector genome could be detected through the final blood collection. rAAV8 vector DNA was detected for 57 weeks and rAAV1 DNA for 26 weeks.[49]

Principle consideration for gene doping detection approaches

The applicability of the presented *in vivo* detection approaches as standardized doping tests depends on sufficient sensitivity, reliability, and specificity of each approach. Following virus mediated *in vivo* gene transfer, sensitive detection of tDNA has been reported up to 57 weeks from 3 ml whole blood in non-human primates,[49] and up to 56 days from 20μl blood samples in mice.[108] A number of studies confirm that viral genomes can be detected over an extended period of time. Recombinant AAV-1 vector sequences were detected for 90 days after i.m. gene transfer in humans[109] and intravenous injection of 4.4×10^8 transducing units of retroviral vector genomes could be detected for more than 1 year in human blood.[110]

To confirm the reliability of the detection approach, Ni *et al*. validated the stability of DNA samples during storage and shipping over a time period of <72 h.[49] Subsequently, the specificity of the test procedure needs to be determined in a sufficiently large number of samples taken from individuals who did not undergo gene transfer. If, for example the specificity of a gene doping procedure is as high as 99.0%, it has to be accepted that 10 out of 1000 tested athletes will be detected as false positives for gene doping. In contrast to clinical diagnostics, where it is more crucial to detect a disease in a sensitive way then to achieve 100% specificity, such a test procedure would not be acceptable. Beiter *et al*. tested the specificity of their *in vivo* detection approach by screening 327 blood samples taken under field conditions from professional and recreational athletes.[111] No false positive results were detected in screening the 327 samples for 6 gene doping candidates, representing 100% specificity of the test procedure.[111]

Plasmid gene transfer could be detected for at least 3 weeks in non-human primates by Ni *et al*.[49] However, the efficiency of plasmid vectors to transfect cells with sustained production of transgene protein is significantly lower compared to virus-mediated gene transfer, especially in larger animals. Repeated injection would be necessary for sustained transgene expression in humans, which would in turn increase the likelihood of positive detection. Abuse of chemically modified RNA is not detectable by the intron-spanning PCR assay. Nevertheless, detection might be possible via mass spectrometry as it has been shown that mass spectrometry is capable of detecting siRNA doping *in vitro*.[112]

It might not be possible to detect *ex vivo* gene transfer including 'Biopumps' or encapsulated cells with current technologies because tDNA is more restricted to transplanted cells and is unlikely to spread to other cells or even other tissues, as in the case of viral gene transfer. Presence of tDNA in plasma or blood cells is impossible if cells are properly encapsulated. In the event that cells are transplanted without encapsulation, occurrence of tDNA in blood will depend on death of the transplanted cells, and the presence of just a few thousand transgene molecules within the total blood circulation of about 5 liters would be almost impossible to detect with current technologies. However, due to the sustained EPO release by transduced cells, the conventional EPO detection approach presented by Lasne *et al*.[76] may be a useful tool for the detection of gene doping following *ex vivo* gene transfer. The glycosylation pattern of transgene EPO (which might be produced in encapsulated myoblasts) should differ from the 'normal' glycosylation pattern. However, the assumption that the approach by Lasne *et al*. leads to a robust test system remains highly speculative for the reasons presented above. This gene doping approach does not seem to be feasible at the moment due to a lack of prolonged efficiency and mechanical stability, side effects, and visibility (if implanted subcutaneously).

In addition to the blood based detection strategies, Segura *et al*. suggested a non-invasive *in vivo* profiling approach to detect EPO mRNA expression at ectopic sites, which is indicative of gene transfer.[113] The most sensitive techniques might be positron

emission tomography or single photon emission computerized tomography.[113] However, this technology is unlikely to be used as a large scale detection approach and harbors an additional major disadvantage in the necessity to administer radioactively labeled probes, thereby restricting the application to severely ill patients.[79] Nevertheless, it could be an applicable approach for athletes who are afraid of the possibility of having received gene doping without their knowledge and/or explicitly wanting the transgene to be detected and if possible, removed from their body.

Safety concerns related to EPO gene doping

The risks of gene doping depend on many different points including, but not limited to, the gene transfer protocol, the type of vector, and the type of delivered transgenes including the kinetics of protein expression in the ectopic cells. Furthermore, the potential risks are not only limited to the gene-doped athletes but can additionally extend to offspring, relatives, or even to the surrounding population.

Safety concerns for the individual

In vivo gene transfer and especially use of viral vector systems, harbors significant safety concerns for potential gene dopers. Adenovirus vectors induce the strongest immune response and the intensity of the immune response highly depends on vector dose and administration route, but also varies considerably between individuals for reasons that are not yet fully understood.[114] As mentioned before, the preexisting immunity against a 'first generation' rAd-5 virus led to the death of a patient.[17] In spite of the fact that the latest generation of Ad-based vectors show less toxicity due to removal of most viral DNA sequences, the immune response against the capsid protein remains to be addressed. A large number of people have a pre-existing humoral immunity against Ad and AAV due to former wild-type virus infection.[115] Therefore, it is important that patients in gene therapy trials are initially screened for presence of virus-specific antibodies.[116] Unsuspected 'gene dopers' who ignore this concern have to anticipate immune responses including inflammation and destruction of transduced cells.[117] The immune response against rAAV vectors is significantly less pronounced when compared to Ad vectors and thus far only low immune responses were reported in humans.[109] The same applies for plasmid vector systems, which induce only modest immune reactions depending on the type of plasmid and the route of administration.[59]

Despite the advantages of rAAV, i.m. administration is generally associated with the risk of triggering an anti-transgene immune response. Severe autoimmune responses against transgene and endogenous EPO have been noted in non-human primate studies. Subsequent occurrence of severe anemia required the animals to be sacrificed.[42,48,49]

Aside from the immune reactions caused by the vector system, the unnatural boost in EPO levels and hematocrit leads to a thickening of the blood and can hamper normal blood flow, subsequently increasing the risk for stroke, heart attack, and other complications associated with abnormal blood clotting. This requires regulation of transgene expression through the use of regulatory systems or small doses of tDNA. The latter possibility is difficult because transgene expression differs between individuals receiving equal amounts of the transgene.[49] The use of encapsulated cells is potentially easier to adjust because EPO production can be determined *in vitro* before microcapsules are transplanted.[72]

A further advantage of encapsulated cells is the protection of transduced cells from immune cells. Subcutaneous implantation of such systems is a non-invasive procedure that can easily be revoked. However, a major drawback of the subcutaneous site is that the superficial location of the implant is associated with a high risk of mechanical stress, which can lead to damage of the graft.[73] Furthermore, subcutaneous implants will be visible in athletes with typically very low levels of subcutaneous body fat.

Safety concerns for the surrounding population and the offspring of gene dopers

In addition to safety concerns for the individual, it must be taken into account that unapproved and uncontrolled use of gene transfer protocols bears safety risks for the athlete's surrounding population. Following rAAV-mediated gene transfer into skeletal muscle, transient viremia and persistence of rAAV particles in lymph nodes and liver can be detected for approximately 6 days.[118] This suggests the ability of viruses to spread into the environment.[118] As reviewed by Haisma and de Hon, controlled gene therapy trials require patient monitoring for shedding vectors.[22] Before patients are allowed to leave the hospital, no detectable viral particles should be detected in blood, stool, urine, semen, or salvia.[22] The possibility of virus spread cannot be excluded in uncontrolled doping scenarios. Additional concerns arise as the viral vector production and purification process may be faulty in non-controlled laboratories and the production of potential replication-competent viruses that carry the transgene is a major safety concern.[119] Furthermore, the use of rAAV bears the risk of vector mobilization.[120] This could lead to the spread of recombinant transgenic material into the population causing a pandemia.[120] Finally, viral based *in vivo* approaches could lead to integration of viral genomes and of the transgene into the host germ cell genome; as a result, subsequent generations could carry the genetic modifications within their genome. This is rather unlikely for rAAV because it usually remains at the site of injection and only $<1\%$ of tDNA integrates into the host genome.[121] Nevertheless, despite the low probability, it cannot be excluded that viral vectors could integrate into the genome of germ cells.[122] Following retroviral gene transfer in humans, vector genomes were detected in semen of a patient.[110] Furthermore, it has been reported that Ad and plasmid vectors, which remain in the episome, may integrate into ovarian cells.[123] The expression of a transgene could at least induce epigenetic changes, whether or not the transgene is integrated into the germline.[123]

Conclusion

Currently, there is no systematic and controlled application of *EPO* gene transfer that would warrant both efficiency and safety. The promising results originating from animal studies do not properly reflect the application of this technique in healthy human beings. Currently, it remains speculative whether the hurdles associated with *EPO* gene transfer can be overcome and if gene therapy will offer an alternative to conventional EPO therapy. Due to the high costs and the time involved in transitioning from preclinical studies in small animals to clinical studies in humans, it is unlikely that *EPO* gene therapy will reach clinical relevance soon. Hence, it is unlikely that athletes or coaches could acquire an evaluated drug.

EPO gene doping does not yet offer an alternative to conventional EPO or blood doping and it is highly unlikely that EPO gene transfer techniques can be abused in a systematic way with the intention to enhance sports performance. Nevertheless, it cannot be excluded that people might try to gain benefits from gene transfer technology, even though it has not been proven to be successful or safe. Long-term detectability for several months or even years is a major prerequisite for preventing athletes and trainers from using gene transfer technology for doping purposes. As discussed here, the abuse of *EPO* gene doping is detectable by a long term detection approach which could find application as a standardized doping test. The high sensitivity of this approach solicits special attention, as it is the basic requirement for a successful detection approach and warrants the long term detectability.

Finally, it is notable that gene doping bears a risk for the surrounding population. This is a unique difference to conventional doping practices and should find consideration in the field of anti-doping policy.

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The University of Tübingen, Germany has a patent pending for the 'Detection of transgenic DNA' (PCT/EP2007/003385; http://www.wipo.int/pctdb/en/wo.jsp?WO=2007124861) that relates to the detection of transgenic DNA in a living being and to a kit for performing such a method. A free use without charge of the patent pending procedure has been granted to the World Anti-Doping Agency for the purpose of doping analysis in sports.

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