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The hunt for gene dopers

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Gene doping, the abuse of gene therapy for illicit athletic enhancement, is perceived as a coming threat and is a prime concern to the anti-doping community. This doping technique represents a significant ethical challenge and there are concerns regarding its safety for athletes. This article presents the basics of gene doping, potential strategies for its detection and the role of promising new technologies in aiding detection efforts. These include the use of lab-on-a-chip techniques as well as nanoparticles to enhance the performance of current analytical methods and to develop new doping detection strategies.

Keywords: gene doping; gene therapy; doping detection; athletic enhancement; lab-on-a-chip

Introduction

In 2003, the term 'gene doping' found its way into the official list of prohibited substances, materializing the concerns that were raised a few years earlier in the sports anti-doping community. Gene doping is defined as any manipulation of genes or their expression or delivery of genes or genetically modified cells into humans. This also includes the use of agonists of gene functioning such as GW1516; an agonist for peroxisome proliferator-activated receptor delta (PPAR δ).[1] Gene doping represents the worst nightmare of the anti-doping community because it has an immense and versatile performance-enhancement potential but there is no conclusive detection method to date.[2–4]

Gene therapy aims to rectify a medical problem and restore a specific function to it's normal state by introduction of genetic materials or modifying the expression of existing ones. On the other hand, gene doping aims to enhance the abilities of a normal healthy individual using the same principles of gene therapy.[5] The gene expression product (a protein) would be essentially the same as the natural endogenous one; however, the *in vivo* expression would avoid the pharmacodynamic ups and downs associated with repeated injection of a recombinant protein.[6] Conventional doping methods generally involve the introduction of a synthetic compound that has an enhancing effect – for example, anabolic steroids, or amphetamines. These can also have severe side effects. In response to the development of highly sensitive methods for detection of synthetic doping compounds, dopers resorted to abusing the more sophisticated doping agents generated by biotechnology: recombinant proteins such as recombinant erythropoietin (EPO), which has been shown to closely mimic its endogenous counterpart.[7] The availability of such compounds, primarily for medical purposes, is known to the anti-doping community and, although challenging, detection methods for recombinant proteins are available.[2,7] Consequently, it is anticipated that dopers in modern competitive sport will now seek out better doping strategies with more subtle manifestations (gene doping).

The case of Belgian Blue cattle or the so-called double-muscled cattle, which have a natural mutation in its myostatin gene causing it to be substantially down-expressed, is a striking example of how alterations in genetic makeup can lead to enhanced muscle mass.[8] Alarm about the possibility of gene doping began to develop in 2001.[6] The origin of concern was the advances in gene therapy trials along with a number of animal studies in which genetic manipulation led to significant physical performance enhancement.[6,9] These concerns have not diminished although there is no direct evidence yet that gene doping has actually been practised.[6,10,11] The Chinese approval of a gene therapy for head and neck sarcomas in 2004 has fuelled concerns about gene doping.[12] At the same time, several companies have gene therapy clinical trials in phase II and III for treatment of different types of cancers.[12,13] Although these trials may not be of direct relevance to athletic enhancement, they are an indicator of the general trend in the field, especially given that the basic tools for gene therapy and doping are essentially the same. It must also be noted that dopers are not expected to await official approval of gene therapy in order to abuse it.

The number of doping cases found in the 2008 Olympic Games in Beijing (nine cases, with a few more pending) was substantially less than that reported in the Games of 2004 (26 cases). The decline was contrary to the expectations of the International Olympic Committee (IOC), which had anticipated that the number would actually increase significantly in 2008 due to the use of more extensive and improved anti-doping testing.[1,14] What could be the origin of the decline? Did the increased and improved testing and severe sanctions deter dopers or is there an increased respect for the spirit of fair play? Or could it be that the dopers are using new substances and/or methods that are either unknown or undetectable by the available anti-doping measures?

There are at least a hundred chromosomal loci in the human genome that are related to athletic performance[15] and may be exploited as potential gene doping targets. However, a number of genes are generally regarded as the top candidates for gene doping and are closely investigated, including EPO and insulin-like growth factor-1 (IGF-1). This article will introduce the basics of gene doping and review the current and future detection strategies and their challenges.

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Gene Doping: from Gene to Performance Enhancement

Gene doping candidates

Athletes may desire various performance enhancements depending on the sport they practise. For example, boxers would be interested in increasing muscle strength and pain tolerance, weightlifters would value strength, and for marathon runners and cyclists endurance is critical. This makes for a long list of potential genes that can be targeted for gene doping. EPO may be overexpressed to enhance oxygen-carrying capacity and thus endurance. Phosphoenolpyruvate carboxykinase (PEPCK), an important metabolic gene, has also been shown to enhance endurance. Endorphin genes may be targeted for increased pain tolerance, in addition to the downstream regulatory element antagonistic modulator (DREAM) inhibitor genes. Inhibition of the myostatin gene or increasing the expression of dystrophin would result in increased muscle size. Overexpression of vascular endothelial growth factor (VEGF) would increase blood supply to muscles and thus improve their performance.[4,7,10,16–18] The main candidates for gene doping are presented in Table 1. Examples of human studies involving the top candidates for gene doping are summarized in Table 2.

Gene delivery

As in gene therapy, gene doping involves the modulation of expression of endogenous genes.[4,5,11] Expression can be modulated using different approaches, for example by introducing extra copies of the gene into the host, or decreasing the expression of the endogenous gene by delivering specific antisense oligonucleotides, or short interfering RNA (siRNA).[18,19] One of the most critical and challenging steps in gene doping/therapy is the delivery of target gene into specific host cells. The delivery can be done using either *in vivo* or *ex vivo* methods. *In vivo* gene doping involves the direct introduction of the target gene into the body via intramuscular injection of gene delivery vehicles including viral vectors or liposomes encapsulating plasmid DNA. On the other hand, the *ex vivo* methods involve collecting cells from the host and culturing them under appropriate conditions, gene transfer to cultured cells by a variety of methods such as electroporation or gene gun, and finally reintroduction of the genetically modified cells into the host.[4,11,20]

The use of viral vectors as gene delivery vehicles has proven to be the most successful method for gene therapy/doping. These vectors are derived from retroviruses, adenoviruses, or lentiviruses which were made replication-deficient and with low immunogenicity (tamed viruses).[11] Adenoviruses, the most popular, are incapable of integration into cellular genome and thus do not carry the risk of insertional mutagenesis. However, gene doping using adenovirus leads to gradual loss of encoded genetic information and the need for repetitive administration of the transgene.[21]

Non-viral vectors are also gaining favour and advances are being achieved in their use. Their primary advantage is circumvention of the risks involved in the handling and taming of the viral vectors.[22,23] These include infection risk to the technicians preparing and manipulating the viral vectors and the recipients, as well as the risk of adverse immune response against the viral vectors.

Methods of introducing the naked DNA or recombinant vectors (delivery vector containing a transgene) into the body include hydrodynamic limb vein delivery. This method has shown efficiency and safety for delivery of plasmid DNA into skeletal muscles.[23] Another promising strategy is the use of liposomal bubbles. In this method, the gene of interest was encapsulated into polyethyleneglycol-modified bubble liposome, which are then injected into the femoral artery of the host. Finally ultrasound waves are applied to cause the liposomes to burst and release their genetic load at specific target tissues. Suzuki *et al*.[24] showed that delivery of plasmid DNA into mice using bubble liposomes gave better results than classical biochemical lipofection methods. Although the bubble liposomes were injected into the blood stream, the expression of the plasmid cargo was only limited to the areas subjected to sonication. Another trend is the use of extrachromosomal vectors, both viral and non-viral, such as papillomavirus-derived episomal systems and artificial bacterial chromosomes. These have the advantages of high insertion capacity (>300 Kbp) as well as eliminating the risk of damage to the host genome due to insertion of DNA sequences.[22] Table 3 presents a comparison of selected gene delivery vectors and their properties.

Gene expression modulation

In addition to the conventional approach of genetic modification, which involves the introduction of additional copies of the target gene, new tools for modulation of gene expression including siRNAs, zinc finger proteins (ZFP) and transcription factor mimics have been developed.

Short-interfering RNAs

siRNAs are promising tools for specific down-regulation of gene expression. They are double-stranded RNA molecules consisting typically of 21 nucleotides and have 3′ overhangs of two nucleotides. After being processed by cellular machinery (known

Table 1. Animal gene transfer studies using leading gene doping candidates

Target gene	Physiological role	Expected performance enhancement	Selected animal studies
EPO	Promotes production of red blood cells and enhances oxygen delivery[7,62]	Endurance	EPO in an adenovirus is introduced by intramuscular injection in mice (n = 7) and monkeys (n = 2). *Main findings* – Increase in hematocrit from 49% to 81% (mice) and from 40% to ≥70% (monkeys) – Effects lasted for 12 weeks in monkeys and more than a year in mice – Elevated serum EPO level determined using radio-immunoassay[63]

Table 1. Animal gene transfer studies using leading gene doping candidates

Target gene	Physiological role	Expected performance enhancement	Selected animal studies
hGH	Increase in muscle mass and size[62]	Strength	*Notes* – Critical dependence of persistent gene expression on the dose of viral vector, in case of immunocompetent animals (threshold dose of vector is $2.5-8 \times 10^7$ pfu/g of body weight) – Some highly elevated hematocrit levels ($\geq$65%) made it necessary to carry out weekly phlebotomy for animals to avoid cerebral thrombosis hGH in AAV serotype 2 was introduced via ductal delivery to salivary glands of male Balb/c mice (n = 5) *Main findings* – An increase in salivary hGH levels of 1084 $\pm$ 102 pg/mL and serum levels of 151.5 $\pm$ 17.3 pg/mL, 4 weeks post gene administration. Serum and saliva hGH levels were undetectable prior to hGH gene delivery and by week 8[64]
IGF-1	Promotes muscle growth and increase in power and recovery from damage[7,19]	Strength	Gene insertion in rats (n = 24) using AAV vector *Main findings* – Muscle mass increased by 15%, while peak tetanic tension of FHL increased by 17% in the transgenic rats without any resistance training. Rats which received resistance training had 32% increase in muscle mass and 28% increase in peak tetanic tension after 8 weeks – IGF-1 expression was detected in rats injected with the viral vector but not in controls[65]
PPAR δ	Promotes formation of slow-twitch muscle fibers, and also enhances metabolism of fats[7]	Endurance and resistance to obesity	Injection of an activated form of the gene (PPAR δ gene fused to an activation domain) into mice zygotes (4 transgenic and 4 wild type controls) *Main Findings* – Running time improved by 67% while the distance improved by 92% – Resistance to obesity even in lack of exercise and on fat-rich diet. – Activation of PPAR δ is needed to switch the muscle type to slow-twitch. Gene overexpression in absence of the activation domain did not lead to significant functional changes[66]
Myostatin	Negative regulator of muscle growth[4,7]	Strength	Limb-girdle muscular dystrophy mouse models (one with mutations in α-sarcoglycan gene, and the other with mutations in calpain 3 gene, n = 6) received intramuscular injections of an AAV encoding a mutated myostatin propeptide *Main Findings* – Muscle mass and absolute force of EDL and soleus muscles increased in the calpain 3-deficient model. The increase in mass was 31%, while the force of EDL and soleus muscles increased by 60% and 85% as compared to untreated mice, respectively – Normal mice that received the mutated propeptide transgene showed a 15% increase in mass of contralateral muscle over the controls (n = 4)[67] *Notes* – No measurements of biochemical markers, for example myostatin levels or related regulatory proteins, were performed
PEPCK	Catalysis of the conversion of oxaloacetate to phosphoenolpyruvat, via GTP hydrolysis[68]	Endurance	Transgenic mice (n = 9) and controls (n = 10) received cytosolic PEPCK cDNA. *Main Findings* – Transgenic mice ran a distance of about 4.9 km as compared to control, which stopped at 0.2 km – The transgenic mice outlived the controls by almost 2 years, and were reproductively active at age 21 months, as opposed to normal mice which could not reproduce after 12–18 months – Mice up to an age of 2.5 years ran twice as fast as 6–12-month-old controls. – Transgenic mice ate 60% more than controls but had half the body weight and 10% the body fat[17,68]

AAV: adeno-associated virus. EDL: extensor digitorum longus. EPO: erythropoietin. FHL: flexor hallucis longus. hGH: human growth hormone. IGF-1: insulin-like growth factor-1. PPAR-δ: peroxisome proliferator-activated receptor-delta. PEPCK: phosphoenolpyruvate carboxykinase.

Table 2. Human gene therapy trials involving candidate doping genes

Gene target	Indicated medical condition	Study description	Main results/comments	Reference(s)
EPO	Chronic renal failure	Dermal tissues from ten patients were harvested and transduced with an adenovirus encoding EPO; genetically modified tissue was then reimplanted	– Increased EPO expression – Increased reticulocyte levels – Transgenic EPO expression lasted for two weeks *Notes* – The decline in EPO expression was possibly due to immunological reaction against the dermal implant – No significant change in hemoglobin levels was detected following implantation	[69]
VEGF	Coronary artery disease	Patients received adenoviral vector (n = 37) or a plasmid/liposome vector (n = 28), both encoding VEGF, via intracoronary delivery using perfusion-infusion catheter (right after coronary angioplasty but before stent implantation)	– Myocardial perfusion increased significantly in patients treated with the adenoviral vector – Eight-year follow up of 82% of patients showed the treatment to be well tolerated and safe *Notes* – The transgene was undetectable (using specific PCR) in serum or urine two days following the gene transfer – There was no increase in serum VEGF levels of treated patients	[70,71]
Dystrophin	Duchenne or Becker muscular dystrophy	Patients (n = 9) were injected intramuscularly with a plasmid containing full-length human dystrophin gene	– Low expression of dystrophin was found in six out of nine patients (in biopsies) – No side effects or anti-dystrophin immune response were observed	[72]

Table 3. Characteristics of gene delivery vectors

Delivery vector	Insert capacity	Genome integration	Tissue persistence	References
Adenovirus	30 Kb	No	Days in blood	[3,22]
Adeno-associated virus	4.5 Kb	No	Days in blood	[3,6]
Herpes virus	40–150 Kb	Stays episomal/latent	Hours in blood Days in urine	[3,6,22]
Lentivirus	8–10 Kb	Yes	Months in muscle	[3,6,37]
Plasmid	Typically up to 10 Kb	No	Hours in plasma if injected intravenously About a week if injected intramuscularly	[3,22]
Retrovirus	8 Kb	Yes	Months in PBMC	[3,6]

PBMC: peripheral blood mononuclear cells

as RNA interference machinery), siRNAs bind to mRNAs with complementary sequences and lead to their degradation. This way, siRNAs can be used for target-specific post-transcriptional down-regulation of gene expression.[25,26] They have the capability to decrease the expression level of their target down to 10-40% of its initial value.[25] The efficiency of delivering siRNAs is a prime challenge to their use that varies according to the used method. They can be delivered to the desired tissue using plasmid vectors, lipid-based agents, polyethyleneimine carriers, hydrodynamic injection, local injection, as well as some modified viral vectors (the safest and most advanced of which are the non-replicating lentiviral vectors).[25,27] Another challenge in using siRNA is their short-lived effect, which typically fades off within two weeks, which means that for long-term treatments repeated siRNA administrations are needed.[25] Nevertheless, several groups investigated the utility of siRNA in gene-expression regulation. In a study by Magee *et al.*,[28] plasmids encoding myostatin-specific siRNA were introduced into rats. After four weeks, myostatin mRNA levels were found to have decreased by 27% while the protein's expression level fell by 48%. siRNAs for down-regulation of VEGF expression are also currently being investigated as therapy for the neovascular disease age-related macular degeneration. The results of animal studies are promising in terms of treatment efficacy and tolerability of siRNAs.[26]

Zinc finger proteins (ZFPs)

There is another innovative approach that carries potential for gene expression modulation applications: the use of ZFP transcription factors and zinc finger nucleases (ZFN).[18,19] ZFPs are DNA-binding

proteins that have a characteristic finger structure formed by a short amino acid sequence around a zinc atom. One group of ZFPs that has been extensively investigated is known as the His_2/Cys_2 zinc fingers. Each finger consists of about 30 amino acids and is structured as a $\beta\beta\alpha$-fold held by the zinc atom. The α-helix has two histidine residues while the β-sheet has two cysteine residues that interact with the zinc atom.[29] ZFPs have the ability to recognize and bind to specific 3–4 bp DNA sequences, and the binding specificity is conferred by the amino acid residues sequence in the binding region, rather than the shape of the region.[29,30] The combination of multiple zinc finger domains allows the recognition of specific stretches of DNA sequences, which can be combined with chosen effector domains (repressors or activators) to selectively up or down-regulate expression of the target gene.[29] ZFPs can also be coupled to the DNA cleavage domain of a type IIS restriction enzyme (such as *Fok* I; these enzymes cleave DNA at a defined distance from an asymmetric sequence they recognize), to form ZFN that can be used to selectively disrupt DNA sequences by creating double-stranded breaks at particular sites.[30,31] The coupling can be done by cloning the ZFP in plasmid as an N-terminal fusion to the catalytic domain of the restriction enzyme.[30] Xie *et al*.[32] induced angiogenesis in hypercholesterolemic mice using viral vectors encoding zinc-finger DNA-binding transcription factor designed to increase the expression of all VEGF isoforms.

Synthetic transcription factor mimics

These synthetic molecules are composed of a sequence-specific DNA binding molecule and a molecule that can recruit the transcriptional machinery. Xiao *et al*.[33] illustrated the use of this approach in living cells and made a chimera composed of cell-permeable coactivator-binding peptoid (oligo N-substituted glycine) fused to a DNA-binding hairpin polyamide. This molecule was capable of increasing expression of 45 genes in HeLa cells by 3-folds.

Proposed Detection Strategies

Several detection strategies for gene doping are currently under investigation. These can be divided into direct and indirect methods. Direct methods involve the detection of the gene or delivery vector, or the recombinant protein expressed by the transgene. Indirect strategies aim at the detection of consequences of the gene delivery and/or the expression of the introduced gene, for example host immune response, or change in expression pattern of other genes.[2,3,10] Figure 1 presents an overview of gene doping and possible detection approaches. The development of a gene doping test incorporates a number of challenges other than its analytical performance. Non-invasive (urine or saliva) or minimally invasive (blood) specimens would be favoured, whereas a muscle biopsy may never be applicable in a sports setting). This is a challenge since the transgene as well as the transgenic protein may be only locally expressed in the injection site, for instance IGF-1 is locally expressed in muscle tissues.[3,4,6,11] There is a possibility in this case of using a much less invasive fine needle aspiration from the tissue followed by real-time PCR analysis (this technique allows real-time multiplex quantification of expression of different genes from a starting RNA material as low as 10 pg).[34] Table 4 presents the potential detection strategies and their advantages and expected challenges. There are also legal and logistical considerations since the conviction of a doping offense entails extensive legal ramifications. This would make indirect detection methods more difficult to apply or admit as evidence in a court of law. The detection method also has to be time and cost effective to be suitable for use in screening large numbers of athletes at major sporting events within an acceptable timeframe.[2,6,19]

Direct detection methods

Expressed transgenic proteins

Direct approaches are based on identification of structural differences between the recombinant protein and its endogenous counterpart. These differences are most likely due to variation in post-translational modifications, which vary with the location of protein expression.[2,3,35] Lasne *et al*. used an adeno-associated virus to transfer EPO gene to macaques.[35] Isolelectric focusing was used to differentiate between the recombinant EPO protein and the endogenous one in the macaque serum.[35] In a related study, Stieger *et al*.[36] administered an AAV vector carrying an EPO gene by subretinal injection. Recombinant EPO isoforms expressed in the retina were different from the endogenous ones found in serum as well as those expressed in skeletal muscles to which the same recombinant vector was delivered. This approach may not be applicable to other targets, which do not show significant post-translational modifications between the recombinant and endogenous protein.

Transgenes

Introns. Synthetic gene sequences typically lack the non-coding regions or introns that are typically present in endogenous human gene sequences. Therefore, detecting the genes lacking introns would be evidence of the presence of a transgene and of gene doping.[3] Recently, Beiter *et al*.[16] developed a PCR-based test, termed single copy primer-internal intron-spanning PCR (spiPCR), for detection of genes without introns. The test is a nested PCR, which employs primers that span the intronic sequences. In this case, the first bases of the primer hybridize to the 3′ end of one exon whereas the last bases of the primer hybridize to the 5′ end of the following exon. In the presence of the transgene, which has only the coding sequences (exons), the primers will be able to bind and target amplification will occur. In the presence of only the endogenous genomic DNA, the presence of the introns will prevent the primers from binding and no amplification will occur. The study reported detection of one copy of transgenic DNA in 300 ng of genomic DNA using samples spiked with both EPO and vascular endothelial growth factor-D (VEGF-D) genes.[16] This work remains to be tested on real serum samples. Additionally, designing primers for different targets remains a difficult task.

Tissue-specific and inducible promoters. Expression of the introduced gene may be controlled by incorporation of elements such as transcription regulators or tissue-specific promoters. For example, a transcriptional regulation sequence may be incorporated in the vector to regulate the gene expression in response to environmental stimuli, for example hypoxia.[19] Ligand-inducible promoters have been incorporated into retroviral and lentiviral vectors.[37] These promoters can be non-human in origin, which makes them targetable for detection. Viral promoters are the most

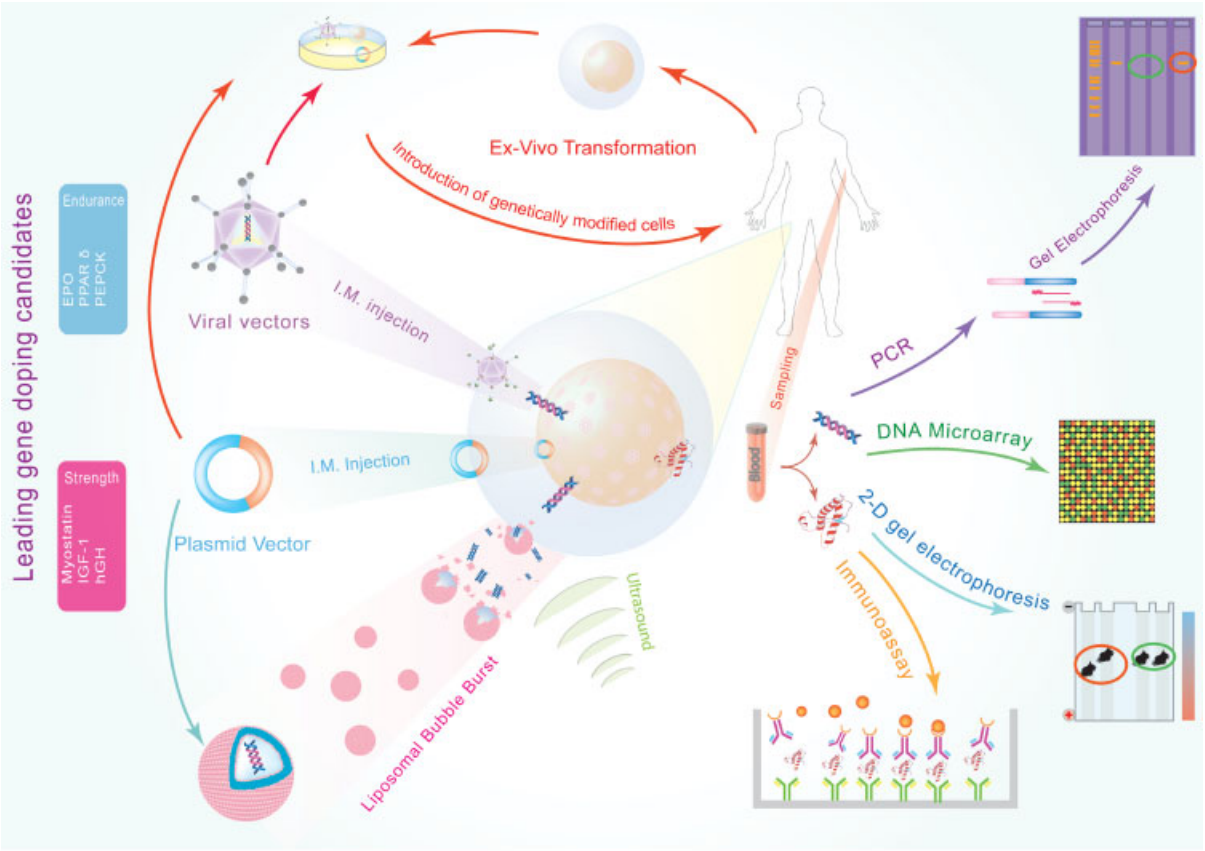

Figure 1. Overview of gene doping methods and detection strategies. In *in vivo* gene doping the transgene can be inserted in a viral vector or a plasmid and injected directly into the athlete. Alternatively, the recombinant vector can also be encapsulated within a liposome to be injected into the body; the liposome will be burst open by ultrasound waves to release the transgenic cargo in the target tissue. When the transgene enters the athlete's cells it is expressed producing the recombinant protein. Gene doping can be performed *ex vivo* as well, by obtaining cells from the athlete and genetically modifying them in culture by introduction of the recombinant vector or plasmid using a gene gun or electroporation. The modified cells will then be reintroduced into the athlete. For detection, whole blood would be the most suitable specimen. Detection strategies can either target the transgenic protein by immunoassays and/or 2D gel electrophoresis, or the DNA. Direct detection of the transgene or the viral vector can be done by PCR whereas indirect detection of gene expression can be achieved by monitoring changes in expression profile of host genes using microarrays.

commonly used in gene therapy studies, with cytomegalovirus (CMV) promoter being particularly popular.[38,39] Other common promoters include the Rous sarcoma virus (RSV) long terminal repeat and Simian virus 40 (SV40) promoters.[39] The CMV immediate early (CMV-IE) promoter and enhancer has shown the capability of driving gene expression in almost all tissue types. On the other hand, RSV enhancer-promoter showed tissue-specific expression favouring bone, muscle and tendons.[38,40] Other tissue-specific promoters include the muscle creatine kinase promoter, which induces sustained gene expression and has been used in gene therapy of Duchene muscular dystrophy.[38] Custom composite promoters are made by fusing two or more enhancer elements to a promoter, such as the CAG promoter, which is a fusion between the CMV enhancer and first intron of chicken or human skeletal h-actin.[39,40]

It is also important to highlight the potential of inducible regulatable promoters. The most commonly used of these is the tetracycline (tet) regulated system, which works in both viral and non-viral vectors. The system contains tet transactivater and tet repressor fused to a transcription factor activation domain (VP16) from herpes virus. The desired transgene is under the control of the tet operator. The transactivater binds to the tet operator and drives the expression. In the presence of tetracycline or its doxycycline derivative, the transactivater cannot bind and expression is halted. This is known as the tet-Off system, while in the tet-On system, the design makes expression occur only in presence of tetracycline or doxycycline (by use of a mutated transactivater that would only bind to the operator when tetracycline is available).[38–40] All the abovementioned systems contain known non-human genetic elements that can be targeted for detection. In the case of use of some variants of the tet-regulatable system the transactivater is constitutively expressed and can be assayed for, and it can also induce a detectable immune reaction.[3,40,41]

In vivo mRNA imaging. A recent report by Segura *et al.*[42] proposed a strategy for monitoring of gene therapy by imaging of mRNA of transgene. They injected mice with fluorescently and radioactively labelled peptide nucleic acids (PNAs) capable of binding to murine EPO mRNA. The labels allowed *in vivo* imaging and tracking of the PNAs bound to their targets.[42] The authors proposed this as a non-invasive technique that could be adapted to gene doping detection. However, this may be a significant overestimation of the method's potential. The method would require knowledge of the exact target sequence and its ability to discriminate between endogenous and recombinant RNA targets is questionable. There is also a significant level of invasiveness,

although it is not as invasive as a muscle biopsy, because this technique involves the introduction of labelled antisense molecules. Additionally, the antisense RNA could adversely affect the normal gene expression of the tested athlete. Finally, the clearance mechanism of the probes from the body is not entirely clear.

Delivery vector

Of course, it may be possible to detect the viral delivery vector itself; however, this approach also has its challenges. First, the individual may have been infected with the virus via other routes. Adenoviruses have many serotypes that are endemic in humans. The various viral vectors also persist in bodily fluids for variable times and are usually only measurable shortly after administration, which may give a very limited detection window. The same applies to plasmid DNA directly injected into the host.[3]

Gene function agonists

As mentioned earlier, the use of gene function agonists is formally prohibited and is within the scope of gene doping. Liquid chromatography tandem mass spectrometry (MS) can be used to detect the presence of PPAR δ agonist GW1516. Another agonist, known as AICAR, is trickier to detect since it is a natural intermediate in purine biosynthesis. Although conclusive results are not yet available, isotope ratio MS may help in the discrimination between natural and exogenous AICAR.[43]

Indirect detection methods

Immune response assessment

Assessment of the host's immune response against viral vectors, the introduced DNA sequence, or even the transgenic protein itself, could be used for detection of gene doping.[3,11] Nevertheless, the downside of this approach is that there is a possibility that the tested athlete could have been infected with the virus via natural non-doping routes and therefore the detection of antibodies in blood would generate false positive results. This is in addition to the fact that viral vectors are modified to be less immunogenic. However, this method may be useful for detection of specific viral vectors. For example, type C retroviruses are not known to infect humans,[3] the presence of antibodies against them in an athlete would be strongly indicative of doping.

An immune response can also be generated against plasmid vectors and siRNAs, as well as the transgene protein product.[3,15] The high content of unmethylated CpG motifs in plasmids can cause them to be recognized as foreign by the host immune system and plays a role in the development of an inflammatory response following the administration of cationic lipid-plasmid DNA complexes.[39] A sequence-specific immune response can also be generated against siRNAs, where siRNAs longer than 22 base pairs invoke an interferon response.[3,25]

Expression, proteomic, and metabolic profiling

Expression and proteomic profiling using DNA and protein microarrays may allow for monitoring the change in expression of endogenous genes or the corresponding proteomic profile following the introduction and expression of a foreign gene. The same principle may be applied to the metabolic profile associated with exposure to certain stimuli.[2–4] For example, it is known that exposure of erythroid progenitor cells to EPO results in the up-regulation of 54 genes and down-regulation of 36 other genes.[44] Protein profiling is already used in a similar manner in cancer diagnosis.[10] MS analysis of serum from healthy individuals and cancer patients generated different proteomic profiles.[45] Koomen

Table 4. Summary of gene doping detection strategies

Detection strategy	Technique(s)	Possible sample(s)	Challenges	Chances of application	References
Determination of structural differences between the endogenous protein and the one encoded by the transgene	Immunoassays to detect the expressed transgenic protein Electrophoresis (isoelectric focusing or 2D gel electrophoresis) to determine the difference in migration patterns between the endogenous and transgenic protein	Blood or urine Muscle (if the protein is not secreted into circulation)	☐ Not applicable to all doping proteins ☐ Depends on post-translational modifications that are specific to the cells expressing the recombinant protein ☐ Requires knowledge of the injection site and an invasive biopsy if the protein is to be detected at the site	Intermediate	[2,3,11]
Detection of delivery vector	Immunoassays to detect the viral proteins. PCR to detect the viral or the plasmid sequence	Blood, urine, or muscle.	☐ Individual may be infected with the virus via non-doping routes ☐ The vector may only be found at the injection site, e.g. muscle, which would call for an invasive biopsy	Intermediate	[3,6]
Detection of tissue-specific promoters/ligand inducible promoters	PCR with primers specific to the promoter sequence	Blood or muscle	☐ Random/multiplex testing, since several possible promoters may be used	Intermediate	

Table 4. Summary of gene-doping detection strategies

Detection strategy	Technique(s)	Possible sample(s)	Challenges	Chances of application	References
			☐ Possible false-positive results due to endogenous promoters, or sequence homologies ☐ New, undocumented promoters may be used for doping purposes		–
Detection of transgenic DNA sequence	spiPCR using specific primers that are able to differentiate between genes with and without intronic sequences	Blood or muscle	☐ Requires knowledge of the transgenic sequence ☐ Design of highly specific primers is fairly challenging	High	[3,16]
Expression profiling	DNA microarrays	PBMC	☐ Tedious DNA microarray result analysis ☐ Method standardization of DNA microarrays is needed. ☐ Need for race and gender-specific reference databases for expression profiles.	Low/intermediate	[2,4,11]
Proteomic profiling	Mass spectrometry	Blood or urine	☐ Need for identification of specific marker proteins and their expression patterns ☐ Need for reference databases for biomarker profiles that also incorporate factors like race and gender, as well as injury and exertion conditions	Low/intermediate	[3]
Assessment of the immune response to the delivery vector	Immunoassays (which employ recombinant antigens) that can detect host antibodies generated against the delivery vector	Plasma, serum	☐ Possible false-positive results if the athlete were infected with the virus via non-doping routes ☐ Potential use of tamed viral vectors eliciting insignificant or no immune response ☐ Need for extensive screening of athletes to determine normal infections and their serotypes	Low	[2,3,11]
DNA barcodes	PCR with primers specific to the barcodes within the transgene	Blood or muscle	☐ Requires extensive coordination between anti-doping authorities and other stakeholders ☐ Financial burden ☐ Need to establish and maintain extensive databases	Low	[2,4,11]

PBMC: peripheral blood mononuclear cells. PCR: polymerase chain reaction. spiPCR: single copy primer-internal intron-spanning PCR.
Chances of application – high: expected to come into practical application; intermediate: may come into practical application pending resolution of strong challenges; low: unlikely to come into practical application.

et al.[46] used matrix-assisted laser-desorption ionization (MALDI) MS to profile plasma proteins in pancreatic cancer patients. The method had a sensitivity of 88% and a specificity of 75%.

The main challenge facing the profiling approach would be the development of reference databases for different genetic and proteomic profiles that change following doping. These would have to be tailored per individual athlete. Expression profiling may also be misleading at times as it would not reflect the actual functionality of expressed protein, particularly when post-translational modifications play a determining role in the function. In such cases the mRNA levels would not be indicative of extent of function.[2–4,10]

DNA bar codes

Commercially available transgenes could be bar coded in a manner similar to that proposed to provide a way to track genetically modified agricultural products. A short stretch of DNA sequence unique to each transgene, to distinguish it from other transgenes and its endogenous counterpart, could be used to code transgenes and viral vectors. This bar code would identify the transgenic sequence and its source and can be identified by a simple PCR test.[2,3,47]

As simple and possibly effective as this strategy is, it may be the most challenging to apply. It would require construction of a database of bar codes and would require immense coordination among various organizational bodies. These include national and international sport federations and organizations, athletes, ethicists and the diagnostic and pharmaceutical corporations involved. The latter may be reluctant to cooperate due to extra cost and fear of jeopardizing commercial secrets and advantages. This is in addition to the possibility that illegal dopers may resort to using 'home-made' transgenes, not listed in the databases. Of course, this would require the back up of experienced scientists with independent resources.

Prospects for detection methods

The previous sections presented the strategies proposed for detection of gene doping but along with them come substantial challenges, which may make the practical applicability of a gene doping detection method, seriously questionable. Table 4 presents a summary of all possible detection methods and a rating of their expected applicability. Some strategies appear to have no real future as the logistical hurdles facing them are too difficult to overcome. This applies particularly to the use of DNA bar codes where the logistical challenges of coordination among all the stakeholders and reconciling their interests are too formidable. Assessment of host immune response also does not hold much promise of becoming a conclusive detection method as it lacks specificity and is likely to give a lot of false positive results. It could, however, be used as a preliminary screening method that would simply flag an athlete's sample as possibly doping. Even so, very careful analysis of the immune response pattern is needed as there are several possible non-doping causes for it.

Profiling approaches have a much better chance of coming into the practical application arena but some considerable time is required for standardization and the establishment of reference databases. Detection of the transgenic protein directly is not likely to become a general method for gene doping detection. However, it may be applied for targets like EPO, where there is a detectable post-translational difference between the endogenous and transgenic proteins.

As for detection of promoters and vectors, it would be feasible to focus on those currently used in gene therapy as they are the ones likely to be used in gene doping. Although the possibility of innovation and use of novel promoters and vectors does exist, it is considered a remote one, at least in the beginning, when and if gene doping does take place. The scientists and technological facilities that dopers would rely on are operating outside the acknowledged clinical system and thus they do not have the facilities and infrastructure for innovation. It is most likely that gene doping efforts would use the popular and established gene therapy tools. Thus it will be easier to focus the detection efforts on these targets (such as common promoters), thus minimizing the 'fishing' aspect of the detection strategies. At the moment, the current information regarding gene doping and its possible detection methods is obtained primarily from studies with therapeutic goals. In order to have an accurate assessment of the different techniques, several specialized gene doping studies need to be conducted.

One more initiative that would support detection strategies, namely the profiling approach, is an expansion of the World Anti-doping Agency's (WADA) initiative of the Athlete Passport. The aims of this project include gathering various biological data about athletes, including haematological ones.[1] This would provide a baseline reference for individual athletes. This could be expanded to include expression and/or proteomic and metabolic data related to the top gene-doping candidates. Such an initiative would not be easy and would take a long time to be of value but it does have a formal logistical framework in place and would be of immense value with time. At the moment, the occurrence of gene doping is undocumented and it remains an expected threat. The proposed detection strategies are an attempt to create a preparative measure against this threat. Perhaps what is to be done now is to carry on with the most promising strategies (both short- and long-term) and focus the efforts on the top candidates for gene doping. This way, detection methods for these specific targets can be optimized, for example spiPCR, and the sports arena would be ready for gene doping.

Future Perspectives

The detection strategies are based on the use of established analytical tools and methodologies. Nevertheless, promising technologies are emerging in the clinical analysis field, which are showing very strong potential. These tools and technologies such as nanoparticles, cantilevers, and lab-on-chip, would improve the performance of analytical tests in terms of sensitivity, turn-around-time, and cost-effectiveness, which would in turn affect doping detection. Enhanced assay sensitivity would be very important in gene doping detection. This is because the analytes, for example transgenes, vectors, or transgenic proteins, are expected to be present in limited concentrations in the athlete samples. The coming sections will introduce the technology concepts of nanoparticles, cantilevers, and lab-on-a-chip and their possible utilization in gene doping detection.

Nanoparticles

Nanoparticles are typically in the size range of 1–100 nm (1 nm = 10^{-9} m),[48] and can have different shapes and

compositions. Their very small size imparts physical and chemical properties that are very different from those of the same material in the bulk form. Two interesting and promising nanoparticles are quantum dots and gold nanoparticles,[49–51] whose main features and advantages are presented in Table 5. Both show significant potential utility in clinical diagnostics and are amenable to use in doping analysis.

Lab-on-a-chip/BioMEMS

Biological micro-electromechanical systems (BioMEMS) are the product of merging electronic and material engineering with biology and are also sometimes referred to as lab-on-a-chip or micro total analysis systems (micro-TAS).[52] They can act as actuators, sensing biological events such as molecular passage through pores of cell membranes or binding of proteins, and converting them into a measurable signal.[53,54]

The work of Liu *et al*.[55] illustrates the power of lab-on-a-chip technology. They developed a biochip for genetic testing that incorporated all the steps involved in the analysis from separation of blood cells and DNA isolation to target amplification by PCR and DNA microarray detection by an electrochemical method. The whole analysis was done on a plastic chip with dimensions of 60 × 100 × 2 mm. This biochip has been used successfully to detect pathogenic bacteria (*E. coli* K12) in whole rabbit blood (turnaround time was 3.5 hours). It was also used to detect the hereditary disease hematochromatosis by detecting single nucleotide polymorphism (SNP), associated with the disease condition. The entire analysis was completed in 2.7 hours from time of sample and reagent loading to result generation. Similar biochips adapted to different gene doping targets, for example transgenic protein, or a characteristic DNA sequence such as a viral promoter, would be of great benefit for on-site gene doping testing.

Another chip which is more related to the doping arena is exemplified by the recent work of Wellner and Kalish[56] who developed a chip-based capillary electrophoresis system for detection of hormones in human blood, urine and saliva. The chip can extract four hormones and detect them in a fast and accurate manner. Such a chip may be adapted for detection of transgenic hormones and used on-site in competition arenas.

Cantilevers

Cantilevers are small beams similar to those used in atomic force microscopy, whose use is based on the detection of nanomechanical deflections. The micro-machined silicon cantilevers can be used to monitor molecular events such as DNA hybridization or antigen-antibody binding. The capture molecule, for example DNA probe or specific antibody, is immobilized on the cantilever. The sample is then added and if the target is present it binds to the recognition moiety on the cantilever. This results in beam deflection due to mechanical stress. The nanodeflection is proportional to the amount of bound target and an optical or electrical signal is then generated.[57,58] One advantage of using cantilevers, in addition to sensitive detection, is the ability to detect unlabelled molecular targets in a homogenous format, thus reducing assay time and risk of error. Wee *et al*.[59] used piezoresistive cantilevers to detect markers of prostate cancer and cardiac disease. Cantilevers could also allow simultaneous detection of multiple targets and a mixture of DNA and protein molecules, and may be adapted to use in chip format. However, there are still problems of non-specific binding that require further optimization.[57,60] Nevertheless, if suitable antibodies and DNA probes can be designed for gene doping targets, this strategy would be extremely helpful; especially given that cantilever production is now a mature process.

Athletic Excellence: Good Genes, Talent, or Hard Work?

The case of the Finnish skier who had a natural mutation in his EPO receptors that gave him a significant medal-winning edge,[2,4] plus all the discussion of how gene manipulation can make super athletes may raise a question about what is the true essence of athletic excellence. Is it the talent, the genetic makeup, or relentless training? Are athletes born with natural edge-giving mutations to be regarded as talented or are they the same as gene dopers? But what is talent? Why do some people appear to be born for their sport not only in terms of physique but also in mindset? It would be safe to say that having the right genes, or mutations in them, is an important component

Table 5. Main features of quantum dots and gold nanoparticles

Nanoparticle	Description	Features and advantages	Application example
Quantum dots	Semiconductor nanocrystal fluorophores, with a typical diameter of 2–10 nm[49]	– Broad range excitation – Narrow strong emission bands – Multiplexing potential – Optical tunability – High photostability compared to organic fluorophores[49,73,74]	Quantum dots with two emission colours and different intensity levels were used in an assay for multiplex detection of 10 single nucleotide polymorphisms of cytochrome p450[75]
Gold nanoparticles	– Consist of either a thin gold shell surrounding a dielectric core, e.g. silica, or just gold nanoparticles (typically spherical in shape) – Their size ranges from 0.8 to 250 nm[49]	– Characterized by high absorption coefficients. – Strong and tunable optical signal[48,51,57]	Tanaka *et al*.[76] used secondary antibodies labelled with AuNPs in an immunochromatographic assay to detect human chorionic gonadotropin and total prostate specific antigen in serum with low detection limits of 1 pg/mL and 0.2 ng/mL, respectively. The colorimetric signal was detected within 15 minutes.

of talent. This is the physical predisposition to excellence in a particular activity. As a matter of fact, the knowledge obtained from the study of gene function and phenotype associations has inspired the emergence of the new field of 'athleticogenomics'. This discipline, which could come into practice fairly soon, aims at using expression profiling and DNA sequencing to determine the athletes better suited for certain sports based on their genetic makeup.[62] In other words, to identify those who would be more talented in different sports. It does not sound very different from scouts looking out for the talented kids at school.

One important point to note is that athleticogenomics would identify the 'trainable' athletes – the ones who would respond better to training. It is to be noted that athletic training can induce some favourable epigenetic changes, which may account in part for efficacy of certain training programmes with some athletes.[61] So, although genetic makeup is important and genetic mutations may give an extra edge, athletic excellence cannot be simply boiled down to good genes. A gene doper would not simply be transformed into a sports icon. This misconception may be held by young adolescents who may be lured by the appeal of enhanced physique, even outside the sports setting.[7] Athletic excellence comes from dedication, hard work, and discipline, and certainly good genes are needed.

Discussion

The enhancement potential and current undetectability are not the only special features of gene doping. It also differs from other conventional doping methods in the fact that its enhancement effect is long term or even permanent. The improved feature will continue to exist as long as the transgene is there and being expressed. At the same time, there are considerable safety concerns regarding the health of the doping athletes themselves. First, these dopers would not resort to legitimate gene therapy facilities and personnel, but would use underground ones, which are not properly monitored. The effects of overexpressing a transgene could be extremely dangerous – for example, increased blood viscosity in case of EPO and overload on tendons and bones in case of muscle growth increase. There is also the risk of insertional mutagenesis of the transgene (if it is incorporated in the wrong place in the genome), or unexpected regaining of the virulence of the viral delivery vector.[4,11] Clearly, classic sports ethics view regards gene doping as an infringement of the ethos of fairness of competition. But some may regard it as an equivalent to special training techniques that pump up the body's innate abilities. Moreover, the techniques of gene doping are fundamentally therapeutic. Would we deny athletes cutting edge-therapy due to fear of doping? Another view may be to allow genetic enhancement of athletes, yet to have them compete against each other in separate events from the unmodified athletes. This would raise other concerns about changing the very nature of competitive sports. Also, where would an athlete with a natural mutation stand? The keyword is 'natural' – athletes are not born equal, some are more talented than others, and some train harder, but ultimately they all invest in their innate abilities, which is generally regarded as fair competition. What is unacceptable and regarded as a violation of sports ethics is the use of a chemical modification method, be it gene doping or use of other conventional doping substances. The recent doping scandals of prominent athletes, and their disgraceful fall from the status of global role models to being the subject of public disdain and severe legal sanctions, are a testament to that notion.

Gene doping continues to be regarded as an imminent threat in the sports community. This feeling is strengthened by scientific advances in gene therapy and the increasingly strong association between athletic excellence and substantial financial gain. The lack of practical detection methods continues to raise alarm. Nevertheless, several potential strategies are being actively investigated. Also, advances in other areas such as nanotechnology promise to help. However, reaching reliable gene doping detection strategies would not be easy and would take some time. This is because of the multitude of challenges involved, not only analytical, but logistical and legal as well. But anti-doping efforts remain relentless in the hunt for gene dopers. If history is any indication, armed with good science and the support of the sports community to clean sport, they are bound to catch up with the dopers.

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