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Potential Use of Gene Transfer in Athletic Performance Enhancement

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After only a short history of three decades from concept to practice, gene therapy has recently been shown to have potential to treat serious human diseases. Despite this success, gene therapy remains in the realm of experimental medicine, and much additional preclinical and clinical study will be necessary for proving the efficacy and safety of this approach in the treatment of diseases in humans. However, a potential complicating factor is that advances in gene transfer technology could be misused to enhance athletic performance in sports, in a practice termed "gene doping". Moreover, gene doping could be a precursor to a broader controversial agenda of human "genetic enhancement" with the potential for a significant long-term impact on society. This review addresses the possible ways in which knowledge and experience gained in gene therapy in animals and humans may be abused for enhancing sporting prowess. We provide an overview of recent progress in gene therapy, with potential application to gene doping and with the major focus on candidate performance-enhancement genes. We also discuss the current status of preclinical studies and of clinical trials that use these genes for therapeutic purposes. Current knowledge about the association between the natural "genetic make-up" of humans and their physical characteristics and performance potential is also presented. We address issues associated with the safety of gene transfer technologies in humans, especially when used outside a strictly controlled clinical setting, and the obstacles to translating gene transfer strategies from animal studies to humans. We also address the need for development and implementation of measures to prevent abuse of gene transfer technologies, and to pursue research on strategies for its detection in order to discourage this malpractice among athletes.

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INTRODUCTION

Advances in gene technology and gene therapy may soon provide sportspersons with the means to enhance athletic performance unethically through manipulation of expression of performance-related genes. This practice is termed "gene doping". To such sportspersons, one major attraction of gene doping, as against "traditional" drug-based doping, lies in the apparent difficulty in detecting its use. This notion is based on the assumption that both the transgene and the expressed protein would be indistinguishable from their endogenous counterparts.

According to the World Anti-Doping Agency, gene or cell doping is defined as "the non-therapeutic use of genes, genetic elements and/or cells that have the capacity to enhance athletic performance" and, from 2003, gene or cell doping has been included in the World Anti-Doping Agency's list of banned substances and methods.[1] One very important justification for listing gene and cell transfer technology on the prohibited doping list is its actual and potential serious risk to the health, and possibly even the lives of athletes, as was learnt from experiences with legitimate gene therapy. Furthermore, many long-term effects of gene transfer to healthy people may not even be predicted from studies in experimental animal models or in a clinical setting in patients with debilitating diseases. Therefore, if such effects occur, they may remain unnoticed, uncontrolled, or untreated. In addition, gene doping, like doping in any form, violates the spirit of sport, threatens its integrity, and undermines the principles of fair play, and can involve potential harm to non-doping athletes and to society and the environment in general.[2,3] Moreover, it is speculated that gene doping could be a precursor to a broader controversial agenda of human "genetic enhancement"[2,4] that aims to improve "non-disease" human physical traits, such as physical appearance, strength, intelligence and social behavior, and to enhance the quality of life by manipulating existing genetic make-up. In this regard, as a preface to human genetic enhancement, gene doping has potential for a significant long-term impact on society and its relationship with the scientific community. It calls for a close analysis of the moral and ethical issues involved, and development and implementation of preventive measures.

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The growing possibility of misuse of gene technologies to enhance athletic performance, the ethical issues involved, and the danger to human health and harm to society in general, have attracted the attention of representatives from different fields of science and social science. Reviews, editorials, and news feature articles in a number of journals and on the internet aimed at a broad audience including medical professionals, scientists, policy makers and regulators, sports officials, athletes and the lay public. Several excellent reviews published in the last 2 years have analyzed, in varying depths, several aspects of this topic, including the background of genetics and gene therapy, targets for gene doping, health risks, associated ethical issues, regulatory and preventive measures, and strategies for detection of gene doping.[2–5] However, there is a need for an updated comprehensive review of the principles and technologies of gene transfer relevant to gene doping, of the genes with potential to influence physical performance, and of advancements in legitimate gene therapy that could be exploited in gene doping. This review provides an overview of recent developments in gene therapy that are closely related to gene doping with the major focus on candidate performance-enhancement genes identified mainly from research on animals and a few human studies. We also discuss the current status of relevant preclinical studies and clinical trials, and address issues associated with safety of gene transfer technologies in humans and obstacles in translating gene transfer strategies from animal studies to humans.

GENE TRANSFER FOR THERAPEUTIC PURPOSES

Most gene transfer strategies in gene therapy have, to date, been directed only at somatic cells and do not lead to heritable changes.[6] In essence, these are gene-addition therapies, in which a normal copy of a gene is transferred into the cells containing its mutant copy. More recently, the potential for gene therapy has extended to the use of small nucleic acids that silence endogenous gene expression at the transcriptional or post-transcriptional levels. Examples are short interfering RNA, which works through a process called RNA interference, and antisense oligonucleotides.[7,8] A novel approach with potentially unlimited gene therapy applications is the modulation of endogenous gene expression at the DNA level using genes encoding engineered zinc finger protein transcription factors.[9] In the past year, several strategies have been developed for correcting genetic mutations responsible for diseases in humans; one such strategy employs zinc-finger nucleases.[10] An approach that is attracting considerable interest relates to the treatment of the genetic disease Duchenne muscular dystrophy (MD), and involves the use of antisense oligonucleotides to allow production of a functional dystrophin protein from a mutant gene through manipulation of pre-messenger RNA splicing and exon skipping.[11]

Gene therapy vectors and their administration routes of potential relevance to gene doping

In gene therapy the required gene, together with appropriate regulatory genetic elements (referred to as transgene), is cloned into a gene expression construct and delivered to the appropriate tissue by a suitable vector. An ideal vector for human application should permit targeted gene transfer into specific tissues and provide efficient sustained production or regulated expression of the target gene, without causing pathogenic and toxic effects or host immune responses; it should be easily produced at high titer and not have a limited gene size capacity. Several excellent reviews on gene therapy vectors and their applications have been published in recent years including in this journal. **Table 1** summarizes the current use of different classes of gene therapy vectors in humans and, in particular, in clinical trials of relevance to gene doping.

For successful gene therapy, in addition to an efficient vector system, the choice of route and mode of its administration require careful consideration. The optimal mode of administration for a specific application will depend on target organ accessibility, the nature of the protein encoded by the transgene, the vector used and considerations of safety and simplicity of the procedure. For some genes, gene transfer must be directed to the natural site of expression and requires a cell-autonomous effect, while for other genes ectopic expression with a systemic effect may be optimal. The selection of target cell type has the potential to affect post-translational processing of the gene product, the level and duration of expression, the character and amplitude of the immune response, the feasibility of achieving the desired effect, and the choice of delivery technology.

Muscle has been considered an attractive target tissue for gene therapy applications because of its abundance, accessibility for vector administration, good vascularity, and post-mitotic state allowing for prolonged expression of episomal DNA. In gene-based doping, muscle would be the most convenient tissue for gene delivery for reasons similar to those pertaining to legitimate gene therapy. Moreover, many candidate genes for gene doping, reviewed below, encode either muscle-related or secreted proteins, and therefore their expression can be achieved by using muscle as the delivery target. It is therefore important to review recent developments in gene therapy that focus on muscle-directed gene delivery.

Gene transfer to muscle using plasmid DNA (pDNA) or viral vectors is being investigated in animal studies and clinical trials for treatment of muscle disorders such as MD, and for expression of secreted proteins. In animal models, intramuscular (IM) injection of pDNA (with or without electroporation) of lentiviral, adenoviral, or adeno-associated viral vectors (rLV, rAd and rAAV, respectively) has been used to achieve the expression of therapeutic levels of secreted proteins. Many of these proteins have relevance to physical performance, for example, growth hormone (GH), erythropoietin (EPO), and vascular endothelial growth factor (VEGF) (reviewed in refs. 12–15 for pDNA, in refs. 15–19 for viral vectors, and in text below in relation to particular genes). rAd efficiently transduces skeletal muscle fibers, but its *in vivo* administration often causes inflammation and only minimal gene expression, because of the induction of strong immunity (ref. 19 and references therein). A substantial increase in IM transduction with rAd has been recently achieved by conjugation of cell-binding ligand fibroblast growth factor (FGF) type 2 to rAd using bifunctional polyethylene glycol cross-linker.[20] rAAV-mediated gene transfer into skeletal muscle of mice, dogs, and nonhuman primates was well tolerated and was associated with long-term transgene expression even when the transgene encoded foreign proteins (ref. 21 and references

Table 1 Current use of gene therapy vectors in clinical trials

Vectors	% In total number of gene therapy clinical trials[a]	Current use in clinical gene therapy: Main applications[b]	Trials of relevance to "gene enhancement"	Reported adverse effects
Viral				
rRV	23.1	• Hematopoietic diseases (SCID-X1, adenosine deaminase-SCID, chronic granulomatous disease) • Alzheimer's disease, AIDS • Cancer	• EPO in anemia and end-stage renal disease • VEGF and angiopoietin-1 in PAD	Development of leukemia in 3 patients with SCID-X1 due to vector insertion in the vicinity of a proto-oncogene
rLV	0.6	• AIDS		
rAd	24.9	• Cystic fibrosis, ornithine transcarbamilase deficiency, hemophilia A, Parkinson's disease • Cancer	• VEGF, FGF4, HIF1A/VP16 in coronary and arterial disease (CAD and PAD) • EPO in anemia	One patient with ornithine transcarbamilase deficiency died due to immune response to a high dose of virus
rAAV	3.7	• Cystic fibrosis, hemophilia B, hereditary emphysema, arthritis, AIDS • Neurological disorders (Parkinson's, Alzheimer's, Canavan's, Batten's diseases) • Cancer		Transient asymptomatic transaminitis due to immune response to a high dose of virus after hepatic artery vector delivery in hemophilia B patients
rHSV	3.4	• Cancer	• Proenkephalin in bone pain from cancer metastasis	
Nonviral				
pDNA	18.1 (naked pDNA)	• Cystic fibrosis, multiple sclerosis, eye diseases, rheumatoid arthritis, hemophilia A, AIDS • Cancer	• VEGF, HGF, FGF, Del-1, ZFP-TF for VEGF in CAD, PAD • GHRH in cachexia associated with cancer	

Abbreviations: AIDS, acquired immunodeficiency syndrome; CAD, coronary artery disease; Del-1, developmentally regulated endothelial locus-1; EPO, erythropoietin; FGF, fibroblast growth factor; GHRH, growth hormone releasing hormone; HGF, hepatocyte growth factor; HIF1A, hypoxia-inducible factor-alpha; PAD, pheripheral arterial disease; pDNA, plasmid DNA; rAAV, adeno-associated viral vector; rAd, adenoviral vector; rHSV, herpes simplex viral vector; rLV, lentiviral vector; rRV, recombinant retroviral vector; SCID-X1, X-linked severe combined immunodeficiency; VEGF, vascular endothelial growth factor; ZFP-TF, zinc finger protein transcription factors.
[a]From ref. 75. [b]From ref. 75 and numerous review articles on specific vector applications in clinical gene therapy, reviewed by authors but not referenced here because of space constraints.

therein). Examples of successful gene transfer into muscle in animals using *ex vivo* genetically modified myoblasts and "bio-artificial muscles" for delivery of VEGF, EPO, GH and insulin-like growth factor 1 (IGF-1) have been reported (ref. 22 and references therein).

Several clinical trials have used plasmid and viral vectors to deliver transgenes into skeletal muscle. For example, rAAV—for coagulation factor IX (FIX) expression in patients with severe hemophilia,[23] plasmid—for VEGF, FGF, hepatocyte growth factor (HGF)[24] (reviewed in refs. 25, 26), and rAd—for VEGF[27] in peripheral arterial disease (PAD).

A number of approaches have been developed for systemic administration of vectors targeted to muscle. Hydrodynamic vascular injection has been used for nonviral gene delivery to limb muscles in rodents, dogs, and monkeys; brief blood flow occlusion or transient clamping of relevant blood vessels enhances gene expression after intravenous (IV) injection of naked DNA in animals (reviewed in ref. 28). However, hydrodynamic delivery is unlikely to be acceptable in a clinical setting because it calls for invasive surgery, large volumes of material to be delivered, and repeated injections. Hagstrom *et al.*[29] have recently developed a protocol for IV delivery of pDNA to skeletal muscle. This protocol does not require very large volumes or rapid injection, can be applied to short interfering RNA, does not require surgery, and is not associated with serious adverse effects. The procedure has been carried out in animals ranging in size from mice to monkeys; however, further optimization is required before it can be used safely in the clinic. Another method for muscle-targeted gene delivery in mice following IV administration was reported by Gregorevic *et al.*[30] This approach uses AAV6-pseudotyped vectors, which are very effective in transducing muscle, co-administered with VEGF, a potent effector of vascular permeability. The safety and applicability of this procedure in humans is yet to be established although, as indicated by High 2 years ago,[31] preliminary studies have not been encouraging. Regional intravascular delivery of rAAV by isolated limb perfusion has been developed to enhance delivery of transgenes to skeletal muscle of large animals.[32] However, the requirement for surgery and the use of a vascular-permeabilizing agent that has not been approved for human use impose limitations on the applicability of this method in humans.

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Regulation of transgene expression

In addition to selecting the most suitable route of administration for vector delivery, approaches have been explored to achieve cell-specific gene delivery and expression. These include the use of tissue-specific receptor-mediated uptake of the vector and/or the use of a tissue-specific expression cassette (extensively reviewed elsewhere). Tissue-specific promoters activate gene expression only in specific tissues. For example, of relevance to muscle-targeted gene doping, promoters derived from muscle creatine kinase, smooth muscle cell α-actin and myosin heavy- or light-chain have been used in cell culture and animal model studies to achieve muscle-specific transgene expression (reviewed in ref. 33).

In order to regulate timing and duration of gene expression after gene transfer, several regulated expression systems have been developed by incorporating sequence elements that respond to the local environment or particular stimuli in a specific tissue. As an example, and of potential relevance to gene doping, inclusion of a hypoxia-responsive element within a promoter allowed regulated expression of a transgene (*e.g.*, EPO or angiogenic genes) in response to hypoxia. Several ligand-inducible promoters have been developed which allow pharmacological control of transgene expression. Among these are the antibiotic tetracycline or its derivative doxycycline as used in the "Tet-on/off" systems, the synthetic steroid, mifepristone, as used in steroid response switches, and the antibiotic and immunosuppressive drug, rapamycin, as required in the dimerizer vectors (reviewed in refs. 33, 34). Other *cis*-acting regulatory elements of the expression cassette, as well as bacterial sequences in pDNA and immunostimulatory unmethylated CpG motifs, can control the kinetics and amplitude of gene expression.[33,34]

In summary, despite significant advances over recent years and ongoing research toward construction of an ideal "designer" vector (*e.g.*, the development and evaluation of nonhuman or pseudotyped rAd or rAAV to overcome pre-existing immunity to these viruses in humans), the technical challenges remain considerable with respect to vector design and administration techniques, efficiency of gene delivery, and transgene expression in targeted tissues. So far, most of the systems for optimal gene transfer have been developed and tested using *in vitro* or *in vivo* screening with reporter or therapeutic genes in cell culture or animal models. However, further evaluation of their safety, stability, and therapeutic effects in preclinical studies are required before they can be realistically considered for human use. Furthermore, some of the methods used for vector delivery in preclinical studies with animal models might not be applicable in humans for ethical reasons, and alternative approaches may be required.

Gene therapy in humans: successes and setbacks

The first gene therapy trials began in the early 1990s, and since then over a thousand trials have been approved worldwide. In 2000, the first clearly successful gene therapy was reported by Fischer and colleagues to treat children with X-linked severe combined immunodeficiency (reviewed in ref. 35). This was followed by a second major success in 2002 in children with another form of severe combined immunodeficiency caused by a deficiency in adenosine deaminase.[35] The final result in *ex vivo* gene therapy of these diseases has been genetic and phenotypic correction of the disease with normalization or significant improvement in immune parameters in most infants treated to date. These trials are considered as proof-of-concept for gene therapy. The latest advance in gene therapy (also *ex vivo*) is partial correction of the inborn genetic condition, chronic granulomatous disease, in two adult patients.[36]

Unfortunately, these trials have also demonstrated potential risks which may be inherently associated with the use of integrating retroviral vectors (rRV) in gene therapy. Due to the insertion of rRV in the vicinity of a proto-oncogene, three children with X-linked severe combined immunodeficiency developed T-cell leukemia, leading to the death of one of the children.[35] An earlier tragic setback from another gene therapy trial occurred in 1999 after an 18-year old volunteer died in a phase I trial that used rAd to deliver *in vivo* a gene encoding a deficient liver enzyme ornithine transcarbamilase. The patient died of an acute and uncontrollable reaction to the vector that was directly administered to the liver through the hepatic artery, and which caused intravascular coagulation and multi-organ failure.[37]

Most clinical trials conducted or currently under way are in Phase I or II with less than 3% of all trials in Phase II/III or III clinical development. In addition to the already mentioned trials involving immunodeficiencies, some other studies have shown limited clinical benefits, for example, in the treatment of patients with coronary artery disease and PAD with angiogenic gene transfer (reviewed in ref. 26) and cancer therapy (reviewed in ref. 38). In a recent clinical trial for severe hemophilia B, gene therapy with rAAV containing the transgene for FIX, infused through the hepatic artery, achieved the expression of therapeutic levels of FIX at the highest viral dose tested, but this was short-lived because of T-cell mediated immunity to rAAV capsid, which caused destruction of transduced hepatocytes. It also resulted in sub-clinical transient side effects which manifested in elevated levels of liver enzymes.[39]

As pointed out earlier, there are many obstacles that hinder the translation of strategies for design and delivery of gene therapy vectors from animal studies to humans, and even from studies in small to large animals. Moreover, results from animal models do not always anticipate the outcome of similar gene therapy protocols in humans. As reviewed by High,[31] the cure of genetic diseases by gene transfer approaches in mice is now commonplace, whereas successful treatment of genetic diseases in large animals is relatively uncommon, and in humans it is very rare. For example, in hemophilia B, therapeutic levels of circulating FIX could be obtained in mice, hemophilic dogs, and nonhuman primates, whereas in humans the levels of FIX or the duration of its increased expression were lower than required for therapeutic efficacy.[23,39] There are some possible explanations for the difficulties experienced in applying to large animals the same strategies that are successful in small animals, and the inability of animal models to translate accurately into human therapies. These include: the anatomical and size differences in terms of composition and activity of muscles and their microcirculation, the efficiency of promoter/enhancer, the nature of the vector, differences in immune response between species, as well as differences in general metabolic aspects and life duration. In gene therapy for hemophilia in the case of IM injection, because of the saturable

capacity of muscle to synthesize biologically active FIX, the vector dose escalation required more than 80 injections at multiple sites, making the procedure extremely cumbersome (reviewed in ref. 31). The example of hemophilia also demonstrates how differences in immune response between species make it difficult to translate success of gene therapy in large animals to success in humans. The short-term FIX expression and hepatic inflammation seen in humans were explained by the immune response to rAAV2 capsid due to pre-existing immunity to this AAV serotype in humans. Neither short-term transgene expression nor transaminitis were observed in dogs, which are not natural hosts for AAV2.[39] In order to overcome this disadvantage of rAAV for gene therapy in humans, antibody-evading rAAV mutants and new pseudotyped rAAV are being developed.[40–42] Nevertheless, years of intense research to evaluate the safety and therapeutic effects of these vectors are required before they can be successfully used in humans. Another demonstration of the difficulty of translating results of gene therapy from small to large animals was the development of autoimmune response after gene therapy with EPO in nonhuman primates, while no such response was observed in mice (refs. 43, 44 and references therein).

In summary, more intense research and many years of treatment and follow-up of patients are required before commercially approved gene therapy becomes available. Gene therapy, though a clinical reality, is still in its infancy. The available tools are still imperfect and its history has demonstrated that, despite its potential to correct serious, often fatal, human diseases, it may also expose the patient to many dangers, even under a highly controlled and regulated clinical environment. Therefore the misuse of this technology for performance enhancement may carry the risk of inducing serious health problems or even death when used in healthy people outside a clinical setting. Apart from the adverse effects associated with transfer of specific performance-related genes (discussed later), serious general health risks also exist. These often depend on the type of vector used and include induction of immune responses to gene delivery and/or expression, potentially oncogenic effects due to mutations of normal human genes or their regulatory sequence(s) as the result of transgene integration in the host genome, generation of replication competent virus, and side effects associated with the quality of the vector preparation and the procedure of vector administration itself, without close medical surveillance and in an uncontrolled setting. Importantly, gene doping imposes potential risks not only on the doping athletes themselves, but also on their relatives, non-doping athletes and society and the environment in general and these are reviewed in detail in refs. 2, 3.

POTENTIAL APPLICATION OF GENE TRANSFER IN THE FIELD OF SPORTS

It has been predicted by many that, in the future, gene transfer technology may be applied not only for the treatment of life threatening diseases, but also in the field of sports for enhancing the physical performance of athletes. Gene doping could potentially be used to increase muscle size and power, enhance endurance, promote more rapid healing of sports injuries, and reduce associated pain. The most relevant candidate genes for gene doping and their use in gene therapy are summarized in **Table 2**. Although the genes are categorized in accordance with their likely effect on aspects associated with physical performance, some may relate to more than one category, given their complex biological functions. Recombinant proteins for many of the gene products discussed are already used in clinical applications. Pharmacological agents are also available which may stimulate or inhibit expression of the genes or block function of an active protein. As new sophisticated methods for detecting drug doping become available, gene doping may become the preferred strategy for those competitors who are determined to seek an unfair advantage, because genetic manipulation may be more difficult to detect.

Endurance genes

Erythropoietin. Performance in endurance sports can be improved by enhancing oxygen delivery to tissues. One way of achieving this is by increasing the amount of red blood cells in the circulation. Regulation of the number of erythrocytes in peripheral blood is mediated by EPO that interacts with the EPO receptor (EPOR) on erythroid precursor cells in the bone marrow. EPO is a naturally occurring hormone secreted mostly by the kidney with a small contribution from the liver and other organs. Elevated levels of serum EPO stimulate erythropoiesis leading to higher hemoglobin and hematocrit levels and boosting the amount of oxygen delivered to tissues. It has also been suggested that the EPO/EPOR signaling pathway plays a role in the vascular system through the induction of angiogenesis and the maintenance of endothelial cells.[45,46] The therapeutic potential of EPO and erythropoietic agents capable of activating the EPO signaling pathway lies in the treatment of severe anemia (**Table 2**). The safe delivery of this hormone by gene transfer techniques rather than by repeated injection could have clinical and economic benefits. Some endurance athletes have misused recombinant EPO to improve performance, the most public incident being by cyclists in the 1998 Tour de France. It could be expected that once EPO gene therapy is developed for human application, it will attract interest from some people involved in elite sports.

The two approaches that have been developed to date for *EPO* gene transfer in animals and humans are *in vivo* and *ex vivo*. *In vivo* approaches in animal models include IM injection of pDNA with or without muscle electroporation[47–49] and IM or subcutaneous injection of rAAV-*EPO* (ref. 50 and references therein) or rLV-*EPO*.[16,51] In a murine model an increase in plasma EPO has been also reported after injection of rAAV-*EPO* into adipose tissue or salivary glands.[52,53] For *ex vivo EPO* delivery, polymer encapsulation of xenogenic or allogenic fibroblasts, myoblasts, bone marrow stromal cells, or vascular smooth muscle cells engineered to secrete EPO have been investigated.[54–58]

Results from the first clinical trial on EPO gene therapy for patients with anemia associated with chronic renal failure have demonstrated limited success of the "Biopump" approach, which utilized implantation of autologous *ex vivo* rAd-*EPO* transduced dermal cores.[55] Another trial in patients with end-stage renal disease is currently under way to test the safety of EPO delivery via rRV-transduced smooth muscle cells seeded into polytetrafluoroethylene vascular grafts.[59]

In order to prevent adverse effects associated with excessive levels of EPO production following gene therapy in animals, such as

Table 2 Genes with potential for performance enhancement

Gene	Performance enhancement potential	Function of expressed protein	Current gene therapy	Models/examples of enhanced physical characteristic(s) and/or performance	Clinical therapeutic use
EPO	Increased endurance	Stimulates erythropoiesis, increases blood oxygenation and oxygen delivery to tissues	*Preclinical:* *Ex vivo*: encapsulated transduced cells of different types *In vivo*: pDNA with or without electroporation, rAAV, rLV *Clinical trials:* *Ex vivo*: rAd- or rRV-transduced cells	EPO/EPOR signaling pathway: Heterozygous mutation in the gene for the EPOR in one human kindred resulted in stimulated erythropoiesis and increased endurance in an Olympic athlete from this family	Treatment of severe anemia associated with renal failure, AIDS, chemotherapy of cancer
PPARD	Increased endurance	Nuclear hormone receptor, transcriptional regulator of fatty acid catabolism in adipose tissue and in skeletal muscle		"Marathon" mice with transgenic overexpression of constitutively induced PPARD had increased endurance and lower accumulation of fat on a high-fat diet	Potential treatment of impaired muscle activity, inability to exercise, dyslipidemia, obesity, diabetes and coronary artery disease
PPARGC1A *PPARGC1B*		Nuclear transcriptional co-activators; enhance transcription of genes involved in mitochondrial biogenesis and uncoupling. PPARD and PPARGC1A promote conversion of muscle type II to type I. PPARGC1B promotes formation of muscle type IIx fibers		Transgenic PPARGC1A or PPARGC1B mice had greater resistance to fatigue Transgenic PPARGC1B mice remained leaner on a high-fat diet, despite consuming more food	
VEGF, FGF, HGF, HIF1A, gene for Del-1	Increased endurance	Increase production of new blood vessels, tissue perfusion and supply of oxygen and nutrients	*Preclinical:* *Ex vivo*: implantable "bioartificial muscle" containing rRV-*VEGF*-transduced myofibers *In vivo*: pDNA, rAd, rAAV (VEGF, FGF, gene for Del-1, HIF1A, ZFP-TF for VEGF) *Clinical trials:* *In vivo*: rAd (VEGF, FGF, HIF1A) pDNA (HGF, gene for Del-1, ZFP-TF for VEGF)		Vascular (coronary artery or peripheral arterial) diseases

Table 2 continued on next page

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Table 2 Genes with potential for performance enhancement (continued)

Gene	Performance enhancement potential	Function of expressed protein	Current gene therapy	Models/examples of enhanced physical characteristic(s) and/or performance	Clinical therapeutic use
IGF1 transcripts (for mIGF-1 and MGF)	Anabolic effects—increase in muscle mass and strength	Stimulate muscle regeneration and repair by triggering proliferation and differentiation of satellite cells, promote their incorporation into existing muscle fibers, increase nuclear content, and promote upregulation of protein synthesis	*Preclinical:* *In vivo*: pDNA, rAAV	• Mice transgenic for mIGF-1 show muscle hypertrophy with little body fat and increased force generation • In rodents, mIGF-1 or MGF expression resulted in muscle hypertrophy and improvement of strength • In rats, combination of resistance training and overexpression of mIGF-1 induced greater muscle hypertrophy than either treatment alone • In mice, mIGF-1 expression in muscles promoted muscle regeneration following injury	Injury-induced muscle hypotrophy, muscle-wasting diseases, such as muscular dystrophy, cancer cachexia, muscular weakness with aging
Genes for inhibitors of Myostatin (*e.g.*, its propeptide, GASP-1, FLRG, SKI) Suppression of myostatin expression by siRNA or antisense oligonucleotides	Stimulated muscle growth and regeneration	• By reducing myostatin level, enhance activation, proliferation and differentiation of muscle progenitor cells into mature myofibers, repopulating and regenerating injured muscle • May also switch muscle fiber type from slow to fast twitch • Following muscle injury, may accelerate inflammatory cell responses, myoblast migration to the injury site, reduction of fibrosis, improvement of healing		• Myostatin-null cattle (natural mutation) and mice (targeted deletion mutation) have a "double-muscled" phenotype and decrease in body fat • Point mutation in the 3′ untranslated terminal repeat of MSTN gene results in translational downregulation of myostatin and contributes to muscular hypertrophy in Texel sheep • Human baby with a loss-of-function mutation in myostatin gene has almost double muscle mass; the baby's mother, a heterozygous carrier of the mutation, was a professional athlete and several members of the same family have been unusually strong • Mice overexpressing myostatin propeptide show increased muscularity and do not deposit fat although on a high-fat diet	Muscle-wasting diseases, such as muscular dystrophy, inflammatory myopathies, cachexia, sarcopenia
Gene for follistatin		Positive regulator of muscle growth, at least partially via inhibitory effect on myostatin		Transgenic mice overexpressing follistatin in skeletal muscles had dramatic increase in muscle growth	

Table 2 continued on next page

Table 2 Genes with potential for performance enhancement (continued)

Gene	Performance enhancement potential	Function of expressed protein	Current gene therapy	Models/examples of enhanced physical characteristic(s) and/or performance	Clinical therapeutic use
GH, GHRH	Anabolic effects - increase muscle mass and strength	Essential for growth, homeostasis of carbohydrates, proteins and lipids; stimulate GH-IGF-1 axis leading to anabolic effects	*Preclinical:* *Ex vivo*: GH *In vivo*: rAd, rAAV (GH), pDNA (GHRH) *Clinical trials*: pDNA (GHRH)		Disorders associated with decreased growth, cancer- and chronic renal failure-associated cachexia and anemia
Genes for BMP, LMP, IL-1 receptor antagonist, IGF, FGF, TGF-β, TNF receptor-Fc Ig fusion gene, parathyroid hormone gene	Bone fracture and joint healing	Wide range of functions associated with bone formation	*Preclinical:* *Ex vivo*: mesenchymal stromal cells, myocytes, chondrocytes, fibroblasts (*e.g.*, BMP, IGF1, FGF-2) *In vivo*: rAd (BMP, LMP); rRV, rLV, rAAV (BMP), pDNA (BMP and the parathyroid hormone fragment) *Clinical trials* (rheumatoid arthritis): *Ex vivo*: rRV (IL-1 receptor antagonist gene) *In vivo*: rAAV (TNF receptor-Fc Ig fusion gene)		Bone fracture, cartilage, tendons and ligament healing, rheumatoid arthritis
Genes for GAD, POMC, Preproenkephalin, IL-4	Pain relief	Block transmission of pain signals from damaged nerves to brain by triggering production of powerful inhibitory neurotransmitter GABA (GAD) or of endogenous opioid peptides endorphin (POMC) or enkephalins (preproenkephalin)	*Preclinical:* *In vivo*: rHSV (GAD, POMC, preproenkephalin) *Clinical trials:* *In vivo*: rHSV (preproenkephalin)		Chronic pain (inflammatory or due to nerve damage)
Genes for DREAM inhibitors		DREAM suppresses prodynorphin gene transcription and expression. DREAM inhibition stimulates dynorphin expression, activates dynorphin/k-opiate receptor system and leads to reduction in pain		DREAM-deficient mice exhibited attenuated pain responses in several models of pain	

Abbreviations: AIDS, acquired immunodeficiency syndrome; BMP, bone morphogenetic protein; Del-1, developmentally regulated endothelial locus-1; DREAM, downstream regulatory element antagonistic modulator; EPO, erythropoietin; EPOR, EPO receptor; FGF, fibroblast growth factor; FLRG, follistatin related protein; GABA, gamma-aminobutyric acid; GAD, glutamic acid decarboxylase; GASP-1, growth and differentiation factor-associated protein-1; GDF8, growth differentiation factor 8; GH, growth hormone; GHRH, growth hormone releasing hormone; HGF, hepatocyte growth factor; HIF1A, hypoxia-inducible factor-alpha; Ig, immunoglobulin; IGF, insulin-like growth factor; IL-1, interleukin-1; LMP, LIM mineralisation proteins; MGF, mechano growth factor; mIGF, muscle IGF; MSTN, myostatin; pDNA, plasmid DNA; POMC, pro-opiomelanocortin; PPARD, peroxisome-proliferator-activated receptor delta; PPARGC1A, peroxisome-proliferator-activated receptor-gamma co-activator-alpha; rAAV, adeno-associated viral vector; rAd, adenoviral vector; rHSV, herpes seimplex viral vector; rLV, lentiviral vector; rRV, recombinant retroviral vector; siRNA, short interfering RNA; TGF-β, transforming growth factor-β; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor; ZFP-TF, zinc finger protein transcription factors.

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polycythemia and associated thrombosis, hypertension, hyperviscosity and heart failure, regulated expression systems have been developed. These are controlled by administration of low doses of a drug such as rapamycin or its analogs, doxycycline, mifepristone, and glucocorticoid.[49–51,53,60] A system has also been developed for physiologically controlling the regulation of EPO expression using a promoter containing a hypoxia-responsive element and tested in anemic mice using IM administration of rAAV.[61] Importantly, another serious adverse effect—autoimmune anemia—has been reported even in a regulated gene therapy expression system in nonhuman primates, caused by the appearance of neutralizing antibodies against the endogenous and transgene-derived EPO.[43] A similar outcome was observed in nonhuman primates that received the *EPO* gene in the absence of a regulated promoter.[44]

Stimulation of erythropoiesis and associated increased oxygen transport capacity are also observed when the negative feedback in the EPO-signaling pathway is interrupted due to very rare heterozygous naturally occurring mutations in the *EPOR* gene (ref. 62 and refs. 8, 14 therein). These cause hypersensitivity of erythroid progenitors to EPO in the presence of low serum EPO levels. One such mutation, leading to benign elevation of hematocrit and increased endurance, was identified in a Finnish skier who won three Olympic gold medals and also in members of his family.[63] Alteration of the *EPOR* gene in erythroid precursor cells in the bone marrow with gene transfer technology for performance enhancement does not seem feasible, although screening for this mutation may be considered by some in talent identification for endurance sports.

A novel phenotype of erythropoiesis, mediated by increased synthesis of hepatic EPO in response to inhibition of VEGF by different mechanisms including rAd-mediated expression of VEGF receptor ectodomain, has been recently described in animal models.[64] The relevance of this pathway in humans remains to be investigated.

PPARD, PPARGC1A, PPARGC1B and mitochondrial genes. Other genes that are found to significantly enhance physical performance, on the basis of results from animal studies, are the peroxisome-proliferator-activated receptor delta *(PPARD)* and closely related peroxisome-proliferator-activated receptor-gamma co-activator 1-alpha *(PPARGC1A)* and -beta *(PPARGC1B)* genes (reviewed in ref. 65).

Expression of either *PPARD* or *PPARGC1A* has been shown to promote a switch in muscle fiber type from "fast-twitch" type IIb to "slow-twitch" type I/IIa. Mitochondria-rich "slow-twitch" fibers use oxidative phosphorylation to produce energy in the form of adenosine triphosphate, and can contract for sustained periods. In contrast, "fast-twitch" muscle fibers rely on glycolytic metabolism as a major source of energy, generate rapid muscle contraction, and are susceptible to fatigue. Exercise promotes conversion of muscle fibers from type II to type I naturally,[66] but a similar effect could be achieved by expression of PPARD or PPARGC1A even in the absence of training.[66,67]

Evans *et al.* recently engineered "marathon" mice, in which transgenic overexpression of constitutively induced PPARD promoted a characteristic switch in the type of muscle fiber. This manifested functionally in a dramatic improvement in exercise profile and protection against accumulation of body fat even on a high-fat diet.[66] Strikingly, although there was no significant difference in muscle mass and daily activity between transgenic and control mice, mice overexpressing the active form of PPARD were characterized by a remarkable increase in endurance. In another study, targeted expression in mouse skeletal muscles of PPARGC1A resulted in greater resistance to fatigue.[67] Consistent with these findings, PPARGC1A knockout mice were characterized by impaired strength and reduced capacity to sustain running exercise.[68]

Recently it has been found that PPARGC1B also increases the capacity of the animal for aerobic exercise.[69] Mice that are transgenic for PPARGC1B had muscles rich in oxidative type IIx fibers and can run for longer durations and tackle higher work loads than their wild-type counterparts. When placed on a high-fat diet, the PPARGC1B-transgenic mice consumed more food but remained leaner than control mice due to their elevated energy expenditure.[70]

The discovery of these functions of *PPARD*, *PPARGC1A* and *PPARGC1B* may provide scientists and clinicians with new tools to treat a range of human health problems (**Table 2**). However, these genes may also become a target for abuse by sportspersons. Activation of these gene pathways can be achieved with some pharmacological agents, as observed in mice with PPARD-specific synthetic ligand, GW501516.[66] However, gene doping with activated *PPARD*, *PPARGC1A*, and *PPARGC1B* might be preferred for those seeking an unfair advantage in sports competitions, given the potential difficulty in detecting this approach.

In addition to these genes, other genes located in the mitochondrial or nuclear genomes that encode proteins involved in oxidative phosphorylation may also benefit athletes involved in endurance sports. Mutations in these genes may result in an age-related decline in mitochondrial function and a wide range of diseases including chronic muscle and cardiac dysfunction and degeneration (reviewed in ref. 71). The discovery of clinically relevant mutations in mitochondrial genes and demonstration of correction of mitochondrial function in mitochondrial disease by metabolic or genetic therapies[71] has improved the likelihood of treatment for these diseases. However, this has also opened possibilities for using gene therapy to enhance performance in sports. Although several gene therapy approaches to mitochondrial diseases could be designed,[71] research in this area is still in its infancy. The study that was most relevant to future clinical application was on the IM rAAV-assisted transfer of the nuclear gene for the heart-muscle isoform of the adenine nucleotide translocator for treating mitochondrial myopathy in a murine model.[71] With further progress in this area, it is conceivable that some athletes may want to change the energy-producing ability of muscle mitochondria by altering their mitochondrial genome.

Angiogenic genes. Members of the VEGF and FGF families and HGF are other potential targets for gene doping, because their expression increases angiogenesis (reviewed in ref. 25). Enhanced expression of these proteins increases tissue perfusion and delivery of oxygen to heart, muscles, liver and other organs, and may thereby increase the endurance of athletes. Stimulating new blood vessel growth in ischemic hearts or limbs has been

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extensively investigated as an approach for treating patients with advanced vascular disease. Although angiogenic growth factors may be delivered in the form of a recombinant protein, gene transfer is preferred in the clinical setting because of the potential for continuous expression, reduction in systemic exposure to growth factors, and ease of delivery to peripheral tissue.[25]

Clinical trials have demonstrated that *in vivo* IM, intramyocardial, or intracoronary viral and nonviral delivery of the angiogenic growth factors (VEGF, FGF, HGF) can be used in the treatment of PAD and coronary ischemia.[24,25,27,72,73] Following success of the approach in mice and rabbits,[9,15] a clinical trial has been approved for investigating an engineered zinc finger protein transcription factor delivered IM as a plasmid for the induction of VEGF-A and improvement of blood flow in patients with intermittent claudication of lower limb in cardiovascular disease.[74] An alternative strategy is gene therapy based on transcription factors such as hypoxia-inducible factor (HIF) 1α that regulate the expression of multiple angiogenic genes (reviewed in ref. 25). Clinical trials in patients with PAD or coronary artery disease have been carried out with IM or intramyocardial injection of rAd-delivered *HIF1A*, respectively (*e.g.*, refs. 74, 75). rAd that expresses a constitutively active hybrid form of the HIF1α subunit is being utilized in a recently-started Phase II clinical trial in patients with PAD.[74]

The developmentally regulated endothelial locus (Del)-1 gene encodes an angiomatrix integrin binding protein Del-1 which, by interacting with $\alpha v\beta 3$ integrins, promotes migration, adhesion, and proliferation of endothelial cells and vascular smooth muscle cells.[76] Gene therapy clinical trials in patients with PAD or coronary artery disease are currently underway using IM or IV nonviral delivery of the gene, respectively[77] (ref. 75, search the website for "Del-1" as "Gene transferred").

Several potential adverse effects are associated with gene therapy with angiogenic genes. The major concerns are an increased risk of neoplastic disease and malignancy, and worsening of atherosclerosis or retinopathy (reviewed in refs. 25, 26). Safety records from clinical trials performed to date, however, show no major problems, and the most common adverse effect of therapeutic angiogenesis has been local edema[27,73] (reviewed in refs. 25, 26). Nonetheless, it is noteworthy that the issue of safety of this type of gene therapy will require more data from more patients and longer follow-up-times, and the potential risks to health of angiogenic gene transfer should not be ignored.

Muscle growth and repair genes

Muscle growth and regeneration can be achieved by using positive effectors of muscle growth such as IGF-1, mechano growth factor (MGF), FGF and HGF, but also by repression of negative growth regulators such as myostatin.

IGF-1 isoforms mIGF-1 and MGF. Muscle-specific IGF-1 isoforms mIGF-1 (for muscle-IGF-1, under the control of strong muscle regulatory elements) and MGF (or IGF-1-Ec in human, IGF-1-Eb in rodents) play important roles in muscle regeneration (**Table 2**). Mice transgenic for mIGF-1 showed hypertrophy in trunk and limb musculature, with little or no body fat and increased force generation when compared against their age-matched wild-type siblings.[78] As demonstrated in rodent model studies, IM injection of rAAV encoding mIGF-1 resulted in muscle hypertrophy and improved strength,[79,80] and IM delivery of the gene for mIGF-1 by electroporation in mice promoted regeneration in a muscle injury model.[81] Similarly, after IM injection into normal mice of pDNA encoding MGF, which is normally expressed in response to mechanical stimulation and/or damage, there was a 25% increase in the mean cross-sectional area of muscle fiber within 3 weeks.[82]

Gene therapy using mIGF-1 or MGF is an attractive and potentially promising approach for patients with one of several muscle-wasting diseases (**Table 2**). However, these isoforms of the *IGF1* gene will undoubtedly be of great interest to athletes who are engaged in sports that require physical strength. Although injection of human recombinant IGF-1 to trigger muscle hypertrophy is relatively easy and safe, it is limited by the high concentrations required for producing a substantial effect, given its rapid clearance and short half-life. Also, because of its glucose-lowering properties and potential effects on other muscles and organs, and the likelihood that it can cause cardiac problems and increase the risk of cancer progression, systemic administration of recombinant IGF-1 in a clinical setting has only limited safety. Gene therapy, however, may prove to be an effective method for delivering stable high concentrations of IGF-1 to a muscle and could be relatively safe, given that the effects seem to be localized to the targeted muscle.[78,79,81] The attraction of this approach for athletes could also stem from the observation that a combination of resistance training and IGF-1 overproduction resulted in a more significant gain in muscle strength than was observed with either alone.[80] Interestingly, other growth factors such as HGF and FGF, discussed earlier as proteins with angiogenic properties, have also been shown to affect muscle regeneration positively by stimulating satellite cell activation and, in the case of HGF, downregulating myostatin expression (ref. 83 and references therein).

Myostatin and follistatin. Myostatin, or growth differentiation factor 8, is a secreted transforming growth factor-β family member that functions as a negative regulator of skeletal muscle growth and regeneration. It is synthesized almost exclusively in skeletal muscle as a precursor protein that undergoes proteolytic processing to generate a propeptide and a disulfide-linked C-terminal dimer, which is the biologically active ligand. The circulating form of myostatin consists of a non-covalently held complex with other proteins, including its propeptide, follistatin related protein, and growth and differentiation factor-associated protein-1 which maintain the C-terminal dimer in a latent, inactive state. Proteolytic cleavage of the complex by a metalloproteinase of the bone morphogenetic protein (BMP)-1/tolloid family at the site of signaling releases the activating C-terminal dimer of myostatin, which then binds to cell surface activin type II receptors (ACVR2B). This process then initiates a signal transduction cascade resulting in inhibition of transcription of muscle-specific genes (reviewed in refs. 84, 85). Myostatin is thought to be important in maintaining satellite cells (which play a major role in muscle growth and regeneration) in a quiescent state, and to inhibit differentiation of myoblasts. Following injury to myofibers, myostatin levels are reduced, leading to activation, proliferation, and differentiation of muscle progenitor cells into

mature myofibers, thus repopulating and regenerating injured muscle (reviewed in ref. 84). The absence of myostatin also causes a switch in muscle fiber type from slow to fast[86] and, following muscle injury, it results in accelerated inflammatory cell response and myoblast migration to the site of injury, reduction of fibrosis, and improvement of healing.[83]

The absence of a functional version of myostatin in cattle, mice, or a human child results in the "double-muscled" phenotype and decrease in body fat[87] (reviewed in refs. 84, 85) (**Table 2**). Translational microRNA-mediated downregulation of myostatin, resulting from a point mutation in the growth differentiation factor 8 3′-untranslated terminal repeat, contributes to muscular hypertrophy in Texel sheep.[88] Induction of maternal myostatin antibodies in mice enhanced the growth performance of offspring and influenced their body composition by increasing the crude protein and reducing crude fat.[89] Contrary to conditions associated with the absence of functional myostatin, muscle-specific overexpression of myostatin in mice causes muscle atrophy and muscle loss.[90,91]

In contrast to myostatin, the secreted glycoprotein follistatin is a positive regulator of muscle growth. Transgenic mice overexpressing follistatin in skeletal muscles showed a dramatic increase in muscle growth.[92] Chronic infusion of follistatin to neonatal rats stimulated protein synthesis[93] and myoblasts expressing exogenous follistatin displayed an increased number of nuclei, greater cell size, and enhanced fusion.[94] Conversely, follistatin-null mice died soon after being born with several defects including insufficient muscle development and skeletal abnormalities.[95] This effect of follistatin is at least partially attributed to its known inhibitory effect on myostatin. Follistatin binds myostatin with high affinity, inhibits its binding to the ACVR2B, and reduces myostatin activity in a transcription-based reporter assay.[91,92,96] The antagonistic actions of follistatin and myostatin are also observed by their opposing expression following skeletal muscle injury when follistatin gene expression is induced, whereas myostatin expression is reduced.[83,94]

It is noteworthy that another gene whose muscle-specific overexpression in transgenic mice leads to a distinct muscular phenotype is c-*SKI* which encodes a nuclear protein SKI.[97] As this protein is capable of blocking the activity of Smad proteins involved in myostatin signaling, it has been suggested that *in vivo* overexpressed SKI negates the action of myostatin by blocking myostatin-mediated activation of Smad proteins.[85]

The clinical interest in myostatin was triggered by its potential significance in the treatment of muscle regeneration in patients with muscle loss diseases (reviewed in refs. 84, 85) (**Table 2**). Myostatin function could be blocked with specific antibodies against the protein or with soluble or mutant forms of its receptor, ACVR2B, follistatin, overexpression of propeptide, inhibitors of the metalloproteinases, and a mutant form of myostatin propeptide resistant to BMP-1/tolloid metalloproteinases[87,98] (reviewed in ref. 84). Blocking the activation of myostatin resulted in increased muscle size, on account of both hyperplasia and hypertrophy. Interestingly, transgenic mice overexpressing myostatin propeptide showed minimal fat deposition when fed a high-fat diet, thereby suggesting that inhibition of myostatin shifts dietary fat utilisation from adipose tissue toward muscle tissue.[87]

The potential usefulness of the myostatin inhibition approach for therapy of muscle-wasting diseases has been confirmed in studies with some murine models of MD. In those studies functional improvement of dystrophic pathophysiology was achieved with either intraperitoneal injections of a stabilized version of the myostatin propeptide or myostatin blocking antibody, targeted deletion of the myostatin gene (all reviewed in ref. 84) or rAAV-mediated expression of mutated myostatin propeptide.[99] In order to test the therapeutic role of myostatin inhibition in human disease, Wyeth has developed an anti-myostatin antibody, MYO-029, which is being tested in a multicenter safety trial in adults with MD.[74] It could be envisaged that endogenous inhibitors of myostatin, including its propeptide, follistatin related protein, growth and differentiation factor-associated protein-1, and follistatin could potentially also be used in pharmacological therapy of diseases associated with muscle loss. In fact, it was recently reported that deacetylase inhibitors induce muscle-specific expression of follistatin and stimulate myoblast recruitment and fusion into myocytes in a follistatin-dependent manner; in animals with muscle injury they stimulated follistatin production and produced signs of *de novo* fiber formation.[94]

Potential gene therapy approaches to degenerative muscle conditions using the myostatin-related pathway could aim either at inactivating myostatin expression using antisense or short interfering RNA strategies (as reported in zebrafish[100]), or at boosting the expression of genes encoding myostatin inhibitors, as in transgenic myostatin propeptide mice, or in mice IM injected with rAAV encoding a mutated myostatin propeptide[87,92,99] (**Table 2**). The myostatin-targeted pathway appears more attractive for treating human diseases, because its inactivation has highly specific effects on muscle tissue. On the other hand, the follistatin-targeted pathway may lead to non-specific effects, given the ability of follistin to inhibit other members of the transforming growth factor-β superfamily.

Even while pharmacological and gene transfer approaches are of great potential benefit to people with MD and many other diseases, they may also appeal to sportspersons seeking to achieve rapid muscle growth and who are already aware of myostatin as a potential means of increasing muscle mass and strength. Several manufacturers of nutrient supplements claim that the myostatin-binding properties of the active ingredient, sulphated polysaccharide extract from brown sea algae, result in a significant gain in muscle mass and decrease in fat mass when combined with weight training. Contrary to these claims, it has been reported that 12 weeks' intake of a recommended dose of one such product combined with heavy resistance training was ineffective in inhibiting serum myostatin, increasing muscle mass and strength, or decreasing fat mass, when compared with training alone.[101] Moreover, resistance training, while inducing muscle strength and mass, was accompanied by an increase in myostatin expression regardless of whether the supplement was taken or not.[101–103] This finding suggests that numerous other factors may be contributing to training-mediated muscle growth, and that these factors may counter myostatin's contribution as a muscle growth inhibitor. Interestingly, other studies have reported a decrease in myostatin messenger RNA expression following heavy-resistance strength training.[104,105] Decreased expression of myostatin messenger RNA

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was also seen after a short-term resistance exercise that followed a period of strength-training.[103]

Importantly, on the basis of several experimental observations in animal models, it has been suggested that myostatin blockade is not always beneficial for muscle function (refs. 99, 106 and references therein). In one recent study it was shown in two murine models that the lack of myostatin, although promoting growth of skeletal muscles, resulted in impairment of muscle force in association with the loss of oxidative characteristics.[106] Similarly, inhibition of myostatin-signaling pathway through overexpression of SKI in transgenic mice resulted in type IIb glycolytic fiber hypertrophy accompanied by decrease in muscle force.[107] These findings further question the potential effect of myostatin inhibition on performance enhancement, using pharmacological or gene doping approaches.

GH and GH-releasing hormone. GH is synthesized and released as several different isoforms[108] from the anterior pituitary gland in a pulsatile pattern. Its production is stimulated by GH-releasing hormone (GHRH), a hypothalamic peptide hormone. The effects of GH (**Table 2**) are mediated by hepatic and peripheral synthesis of IGF-1, the downstream effector of GH in the GHRH-GH-IGF-1 axis. In humans, insufficient production of GH results in abnormally low growth rates in affected children, and daily subcutaneous injection of recombinant human GH is an effective treatment for this and other disorders associated with low growth rates.

As recently reviewed in refs. 109, 110, there is either insufficient or non-existent evidence regarding the "enhancing" effects of GH on body composition, muscle strength, and cardiac and pulmonary function in highly trained healthy subjects. On the contrary, side effects and health risks, including impaired glucose tolerance and insulin resistance, detrimental effects on the cardiovascular and pulmonary system, and possibly an increased risk of cancer, have become increasingly evident.[109] However, despite this, official and unofficial reports suggest that GH is widely abused by athletes as a performance enhancing agent (*e.g.*, references in ref. 110). The development and recent introduction into anti-doping control of a test to detect GH abuse may deter athletes from taking this approach.

There have been several published reports of *in vivo* or *ex vivo* transfer of the *GH* gene in animals. *In vivo* rapamycin-regulated GH expression was observed after IM injection of either rAd or rAAV delivering human *GH* in immunodeficient mice.[18] In immunocompetent mice, prolonged regulated expression of transgenic human GH was achieved using rAAV, whereas GH expression after gene transfer by rAd was not sustained because of the activated immune response. Glucocorticoid-regulated human GH expression, following transplantation of microcapsulated allogenic fibroblasts engineered to produce GH, has been demonstrated in a study with rats.[111] In animal models of growth deficiency, GH was produced after IM injection of GH-secreting *ex vivo* engineered myoblasts, after grafting of GH-producing keratinocytes, or after IV delivery of rAd-*GH*[112] (reviewed in ref. 113).

An alternative method to increase GH production is by administering GHRH. GHRH stimulates synthesis of GH in a pulsatile pattern and is capable of feedback regulation of pituitary release of GH. This avoids adverse effects associated with the recombinant GH therapies, in which feedback regulation is lost. Gene therapy with *GHRH* has been successfully applied in several animal models (mice, dogs, pigs) and has demonstrated that IM injection of plasmid containing *GHRH* under the control of a muscle-specific promoter stimulates the GHRH-GH-IGF-1 axis and results in anabolic responses and improvement of hematological parameters.[114] In addition, a mifepristone-regulated *GHRH*-plasmid expression system was developed and used in different murine models with IM vector delivery.[115] A clinical trial to test this regulated system in patients with cancer-associated cachexia has been proposed (ref. 75, search for "GHRH" as "Gene transferred").

Fracture repair genes

During competition and training athletes place considerable strain on elements of the skeletal system, including bone, ligament, cartilage, and tendon. Presumably athletic performance can be enhanced by engineering these tissues to resist injury and/or to repair more efficiently and, more generally, by reducing the pain and inflammation of repetitive trauma.

Bone tissue development involves activation of several genes leading to the differentiation of osteoblasts from mesenchymal precursor cells. A complex network of regulators of this process involves several bone-specific and non-specific proteins, transcription and growth factors, such as BMP and LIM mineralisation proteins, IGF, FGF, transforming growth factor-β, and VEGF (reviewed in refs. 116, 117). Locally delivered recombinant growth factors have been shown to improve the rate of fracture healing in animal models and in the clinic, but their use is limited because of their short half-lives and the requirement for sophisticated delivery methods (reviewed in refs. 117, 118). Gene therapy has several advantages over the use of recombinant proteins (reviewed in ref. 118) and legitimate gene transfer approaches have been suggested for treating several pathologies of the skeletal system (**Table 2**).

Bone is very responsive to gene transfer, and varied *in vivo* approaches have been successfully employed to deliver growth factor genes directly into the bone lesion or to skeletal muscle for fracture healing in animal models. As tissue repair in bone fracture and joint healing requires a short-term expression of osteoinductive genes, rAds have proven to be the most suitable vector and have been successfully used for delivering genes encoding BMP and LIM mineralisation proteins in some animal models[119] (reviewed in refs. 116–118). Interestingly, in a recent study on bone healing in sheep, retardation of bone healing and a strong immune response were observed after a single local administration of rAd-*BMP2*;[120] a similar outcome had been previously observed in immunocompetent rats (reviewed in ref. 117). rAAV and integrating rRV and rLV delivering genes for BMP have also been used for *in vivo* bone formation in animals, as well as electroporation- and chemical carrier-facilitated nonviral gene transfer (reviewed in refs. 116, 118). Evidence for the applicability of gene therapy in bone formation has been derived from *ex vivo* cell-mediated gene transfer animal studies using either mesenchymal stem cells or autologous differentiated cells (reviewed in ref. 117). These preclinical studies, mostly focusing

on the use of transgene expressing BMP, show promise, and clinical trials could begin this year.

Progress is less advanced in the gene-based approach to the treatment of ligaments, tendons and cartilage. Cartilage frequently incurs damage as a result of injury, but being an avascular, aneural and alymphatic tissue, it does not have an intrinsic ability to heal, and is difficult to repair by existing medical procedures (reviewed in refs. 121, 122). The use of gene therapy has been proposed as an alternative to surgical and pharmacological strategies for cartilage repair, and several approaches have been tested in cell culture, in animal models using genetically manipulated bone marrow, chondrocytes, mesenchymal stem cells, or synoviocytes to express various gene products to enhance chondrogenesis, such as BMP, IGF-1 or FGF-2 (reviewed in refs. 121, 122),[123] or by using direct rAd-*IGF1* or rAAV-*FGF2* intra-articular injection[124,125] (reviewed in ref. 122). Although some evidence of cartilage repair or elevation of transgene protein has been obtained, these approaches require further refinement before they can be used in the clinic. A limited number of *in vitro*, *ex vivo*, and *in vivo* gene therapy studies have been performed on menisci, ligaments, and tendons, which also have limited ability to heal spontaneously and are often damaged in sport[126,127] (reviewed in refs. 121, 128). A novel gene-based approach for healing of the anterior cruciate ligament using gene-activated matrices technology with rAd gene delivery has been recently developed in an *in vitro* system, and *in vivo* gene therapy protocols based on this approach are currently being investigated.[126] Research in this area, however, is at the developmental stage and clinical applications remain some way off.

The impressive efficacy of gene therapy involving the use of a number of anti-inflammatory genes and delivery protocols in several animal models of rheumatoid arthritis, an inflammatory disease of the joints, justified the first clinical application of gene therapy for this disease using *ex vivo* retroviral delivery of interleukin-1 receptor antagonist gene.[129] The results reported in the year 2005 demonstrated that the approach was effective in achieving intra-articular transgene expression and did not cause adverse events.[129] A phase I study in rheumatoid arthritis patients, using *in vivo* intra-articular administration of rAAV containing the tumor necrosis factor receptor-Fc immunoglobulin fusion gene, is currently underway (ref. 75, search for "Rheumatoid Arthritis" as "Indication"). A similar approach in an animal model was shown to be safe and effective in reduction of arthritis.[130]

Pain relief genes

Another strategy to enhance athletic performance that could be abused by sportspersons is pain relief. The most effective therapy for pain relief in many clinical conditions is the use of opioid drugs, such as morphine, that mimic endogenous opioid peptides to inhibit the pain signal. Although opiate drugs are highly effective, their continuous use for treatment of chronic pain is limited by side effects such as sedation, constipation, and respiratory suppression, and also by the development of tolerance over a period of time, which reduces the pain-relieving effect.

An alternative approach that has been proposed for treatment of chronic pain is gene transfer to induce expression of peptides which can block transmission of pain signals from damaged nerves to the brain. Among the genes that encode analgesic proteins are the genes for glutamic acid decarboxylase, pro-opiomelanocortin, and preproenkephalin (**Table 2**). Successful transfer of these genes using both nonviral and viral vectors has been demonstrated in many animal models of chronic pain.[131–134] The most promising has been the use of vectors based on herpes simplex virus (rHSV) which have a natural high affinity for peripheral sensory neurons. *In vivo* subcutaneous delivery of rHSV containing complementary DNA for glutamic acid decarboxylase, preproenkephalin, or anti-inflammatory cytokine interleukin-4 achieved targeted and localized transgene expression and reduced pain-related responses in several rodent models of chronic inflammatory pain, neuropathic pain, or pain resulting from cancer in bone.[132,133,135] Based on success in animals, the first phase I safety clinical trial of rHSV encoding proenkephalin is planned in human patients with pain from metastatic cancer.[132]

Recent interest in pain management has been directed towards an endogenous ligand for another family member of opiate receptors, dynorphin. A strain of mice was developed containing a knockout gene for downstream regulatory element antagonistic modulator, a transcriptional repressor for the prodynorphin gene. These mice exhibit attenuated pain responses in several models of acute, inflammatory, and neuropathic pain, and this is attributable to de-repression of prodynorphin gene transcription and activation of dynorphin/k-opiate receptor system.[136,137] This discovery of downstream regulatory element antagonistic modulator's function as a "transcriptional switch" for repressing and de-repressing endogenous modulators of pain processing may promote the development of novel approaches to the treatment of pain in the future, such as gene transfer technology involving inhibition of downstream regulatory element antagonistic modulator.

Having reviewed gene products that play a role in the improvement of physical performance and healing of sports injuries (which are therefore potential candidates for gene doping), it is important to stress that, as a map of performance-related genes in humans is drawn up, many new genes with the potential to enhance athletic performance will be identified, and consequently the number of candidate genes for use in doping will increase.

Athletic performance and inheritance

There is strong evidence that the natural "genetic make-up" of athletes influences their physical performance. Although environmental factors such as training and nutrition significantly contribute to human sporting performance, it has been shown in a multitude of studies that polymorphisms in many of the genes associated with various physical traits such as cardio-respiratory and skeletal muscle function could also impact on an athlete's performance (reviewed in ref. 138).

Several studies have demonstrated an association between athletic ability and the genotype of the angiotensin I converting enzyme gene.[138] The "I" allele (insertion of a 287-base pair Alu repeat element in intron 16), associated with a lower angiotensin I converting enzyme activity in serum and tissue, has been observed more often in athletes in endurance events, whereas the "D" allele (deletion of the Alu repeat element) has been associated with good performance in sprints and in sports that require physical power, presumably on account of the increased levels of angiotensin I

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converting enzyme and growth factor angiotensin II. Although other larger studies with a more heterogenous athlete cohort did not show such an association, possibly because of methodological differences, it is generally accepted that some association between the angiotensin I converting enzyme I/D polymorphism and athletic ability does exist in Caucasians. Interestingly, this polymorphism was not strongly associated with endurance ability in Kenyan athletes.[139]

Among other variants linked to athletic performance are a restriction fragment length polymorphism of the α_{2a}-adrenoceptor gene, an insertion/deletion polymorphism of the bradykinin β_2 receptor gene, and a common null polymorphism in the α-actinin-3 gene encoding for the protein which is specifically expressed in the fast-twitch myofibers responsible for rapid force generation (reviewed in ref. 138). As demonstrated in studies from Australia and Finland, the frequency of occurrence of the XX genotype (homozygous for a premature stop codon mutation R577X in the α-actinin-3 gene) with a loss of α-actinin-3 protein in fast-twitch muscle fibers was lower, and the frequency of the R allele higher, in sprint/power event athletes than in endurance event athletes, especially at the highest performance levels.[138,140] It has also been suggested that polymorphism in genes that encode proteins of mitochondrial energy metabolism is associated with good physical performance.[140] It was recently reported that the frequencies of occurrence of the mitochondrial genome haplogroups differed significantly between endurance event athletes and power event athletes, and none of the endurance event athletes in the study belonged to the mitochondrial genome haplogroup K or subhaplogroup J2.[140] The study by Walsh *et al.* indicates the gender-specific association between the haplotype structures of the *ACVR2B* and follistatin genes and skeletal muscle mass and strength.[141]

The discovery and characterisation of genetic variants that influence athletic performance may potentially make it feasible to carry out DNA testing in children to identify talent and suitability for a particular sport, or to optimize training programs. However, it is thought[138] that this may not provide additional benefit over existing talent identification programs that are based on physiological tests, at least until large well-controlled prospective cohort studies are performed.

CONCLUSION

To date, numerous genes have been identified whose products may affect physical performance and that are therefore potential candidates for gene doping. Many of these gene products have also been found to be linked to diseases in humans and intensive research has focused on developing gene therapy approaches for their treatment. Undoubtedly, the knowledge derived from this research will be of great interest to those sportspersons and their entourages who are out to seek an unfair advantage. It must be stressed nonetheless that research in the areas of vector design, vector delivery, and gene expression control is still at the developmental stage, and anyone who resorts to illicit gene manipulation would be taking major health risks without necessarily achieving the desired effects. Gene therapy in humans, despite immense effort in the last three decades, is a reality only for a very small number of disease phenotypes, and there are still numerous technical and biological challenges of effective gene transfer and expression, particularly in the short term. As such, effective performance enhancement through gene doping may not be achieved using current technologies and may therefore not be an immediate threat to sports. Nonetheless, scientists from different disciplines are working on developing innovative, specific, sensitive and non-invasive approaches to detect gene doping. Availability of methods for gene doping control together with the development and implementation of preventive measures, such as regulation, coordination, and education, that have been recently reviewed at length,[2,3] may dissuade athletes from gene doping and will assist in fighting this form of malpractice.

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