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A NOVEL MEANS OF DRUG DELIVERY: Myoblast-Mediated Gene Therapy and Regulatable Retroviral Vectors

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■ **Abstract** A potentially powerful approach to drug delivery in the treatment of disease involves the use of cells to introduce genes encoding therapeutic proteins into the body. Candidate genes for delivery include those encoding secreted factors that could have broad applications ranging from treatment of inherited single-gene deficiencies to acquired disorders of the vasculature or cancer. Myoblasts, the proliferative cell type of skeletal muscle tissues, are potent tools for stable delivery of a gene of interest into the body, as they become an integral part of the muscle into which they are injected, in close proximity to the circulation. The recent development of improved tetracycline-inducible retroviral vectors allows for fine control of recombinant gene expression levels. The combination of ex vivo gene transfer using myoblasts and regulatable retroviral vectors provides a powerful toolbox with which to develop gene therapies for a number of human diseases.

INTRODUCTION

Gene therapy is a natural outcome of landmark developments witnessed in the past few decades: the emergence of recombinant DNA technology and methods for identifying and characterizing an array of human genes implicated in disease. Since the first federally approved gene therapy protocol began in 1990 (1), the number of clinical trials underway worldwide has burgeoned into the hundreds. Initially, gene therapy was thought to be best applicable for replacing or correcting single defective genes, such as those implicated in various metabolic disorders. Although efforts aimed at treating single-gene defects are currently in progress, growing attention is being directed toward treatment of multi-gene disorders, including cancer and vascular diseases. In such disorders, expression of recombinant genes may complement genetic defects and alleviate or correct the disease

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pathophysiology. Whereas classical pharmacotherapy relies on delivering to the patient a chemical manufactured outside of the body, gene therapy strategies utilize the therapeutic transgene as the template of the drug and harness the patient's own cellular machinery to produce the drug.

A number of preclinical gene therapy approaches have employed skeletal muscle as a platform to treat both muscle- and non–muscle-related disorders. Skeletal muscle has a number of advantages for genetic manipulation over other tissue types, as is discussed below. The strategies for gene delivery into muscle fall into two categories: (*a*) in vivo approaches, in which a vector (of either viral or nonviral origin) harboring a therapeutic transgene is introduced directly into muscle tissue, and (*b*) ex vivo approaches, in which cells removed from the body are genetically engineered in culture and introduced into the patient, where they become integrated into preexisting tissue. Many of the features of skeletal muscle make it ideally suited for in vivo adenoviral, adeno-associated viral, lentiviral, and naked DNA delivery. However, the primary focus of this chapter is the use of muscle as a target tissue for ex vivo gene delivery, together with approaches for regulating gene expression to avoid toxic levels and achieve pulsatile drug delivery when desired. Ex vivo strategies for gene transfer into muscle surpass most other current methods, as they lead to long-term, stable delivery into the circulation of therapeutic proteins at physiological levels. Moreover, problematic immunological effects currently associated with most other methods can be avoided with ex vivo approaches by transplantation of autologous cells.

In this article we highlight the advances in cellular and molecular biology that have enabled gene transfer via myoblasts. We illustrate several potential applications of genetically engineered myoblasts to the treatment of human diseases. Because of the vast literature in this field, only a subset of references have been included.

EX VIVO GENE DELIVERY USING MYOBLASTS

Features and Advantages of Skeletal Muscle

Skeletal muscle has a number of properties that distinguish it from other tissue types. First, the in-depth knowledge accumulated over the years on this tissue provides insights for developing strategies of gene delivery and for assessing and controlling therapeutic protein expression. Skeletal muscle makes up approximately 10% of the total human body mass and is easily accessible for the delivery of recombinant genes. Myofibers—the typical striated differentiated cells found in skeletal muscle—are multinucleated, allowing delivery of multiple genes encoding products that can interact inside the cell. In addition, because myofibers are long-lived, they provide a stable environment for long-term expression of recombinant transgenes. Finally, although not widely recognized as a secretory tissue, genetically altered skeletal muscle tissue is surprisingly efficient at pro-

ducing and delivering recombinant gene products to the circulation. All these advantages make skeletal muscle an optimal target tissue for genetic manipulation in various gene therapy strategies.

Transplantation studies conducted by Partridge and coworkers (2–7) showed that donor myoblasts could coexpress exogenous genes together with host genes in myofibers. These experiments employed isoforms of the enzyme glucose-6-phosphate isomerase as markers for distinguishing between donor and host myoblast contributions to myofibers. Following allografts of minced muscle tissue (2) and pieces of intact muscle (3), or injection of muscle precursor cells (4–7), hybrid fibers expressing isoforms containing subunits derived from donor and host were observed. These observations indicated that muscle precursor cells of one genotype injected into muscle of another genotype fuse to form hybrid myofibers, which are capable of coexpressing donor and host genes.

Myofibers contain many nuclei because they are formed during development by the fusion of mononucleated precursor cells known as myoblasts. Myoblasts continue to fuse to neighboring mature myofibers throughout adult life, aiding in regeneration following injury to muscle tissue (8), and are retained in mature muscle as satellite cells that persist between the plasma membrane of the myofiber and the surrounding extracellular matrix (9). Myoblasts can fuse randomly with all fiber types encountered and adopt the pattern of myogenic gene expression characteristic of the host muscle fiber, as was demonstrated through several experiments. Studies of the generation of patterns of slow and fast fiber types within mammalian muscle have shown that although intrinsic properties and lineage clearly play a role (10, 11), extrinsic factors are important in the creation of fiber patterning. For example, myoblasts taken from different stages of early muscle development give rise to clones expressing slow myosin heavy chain upon differentiation in culture, even if only a small percentage of fibers in vivo express the protein at the developmental stage being examined (12).

In studies of cell fate during development in which vectors encoding the marker bacterial *lacZ* gene were injected directly into muscles of rats, labeled single satellite cells gave rise to clusters of labeled fibers (13). Migration of these satellite cells into multiple fibers did not appear to be hindered by the presence of the basal lamina, a connective sheath surrounding each myofiber. Additional experiments demonstrated that myoblast clones could give rise to both slow and fast muscle fiber types in their vicinity in vivo (14). Thus, in contrast with other cell types, myoblasts become an integral part of the muscle into which they are injected and adapt to local signals in their immediate environment. Moreover, the fused cells continued to express a transgene, the marker *lacZ* gene. These studies provided the rationale for utilizing myoblasts for gene transfer into mature muscle.

Primary Myoblasts as Gene Delivery Tools for Muscle

Primary myoblasts from a range of species, including human, can be readily isolated from muscle and expanded in tissue culture. The development of this process constituted a major advance, as in early studies only established myogenic

cell lines were available for use. Primary myoblasts can now be purified, genetically engineered, and extensively characterized in vitro; they then can be reimplanted back into muscle, where they fuse with preexisting muscle fibers of mice, rats, or humans. Primary mouse myoblasts stably express recombinant genes following transduction. Myoblasts that have been retrovirally transduced with the *lacZ* gene and injected into mouse skeletal muscle fuse with myofibers and express high levels of β-galactosidase for at least 6 months (15). Other studies have demonstrated stable levels of recombinant protein production via myoblast-mediated gene transfer for at least 10 months (16). Myoblasts genetically engineered to express a recombinant gene can thus be employed for long-term delivery of the gene-encoded protein within the body (Figure 1) (17).

Although not widely viewed as a secretory tissue, skeletal muscle is well-vascularized and recombinant proteins secreted from myoblasts readily gain access to the circulation. This was first demonstrated using a mouse myoblast cell line genetically engineered to express human growth hormone (hGH) (18, 19). hGH was chosen as the gene of interest because the protein has a very short half-life in mouse serum (4 min) (20), providing a stringent test for sustained production and secretion into the circulation over time. Following injection of genetically engineered myoblasts into mouse muscle, stable physiological levels of hGH

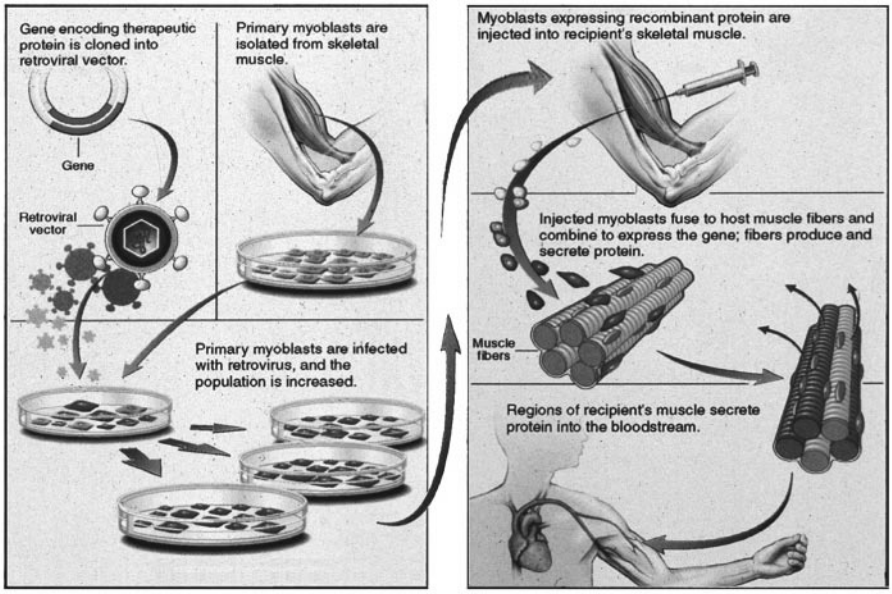

Figure 1 Muscle-mediated gene therapy. Genetically engineered myoblasts can be used for delivery of diverse therapeutic proteins either directly to muscle or, as shown, to the systemic circulation. From Reference 17 (copyright © 1995 Mass. Med. Soc. All rights reserved).

could be detected for at least 3 months (Figure 2). Moreover, muscle has also been demonstrated to be capable of carrying out posttranslational modifications normally performed by other tissue types (such as gamma carboxylation, essential for production of functional coagulation factors in the liver) (21–24). Thus, skeletal muscle may be enlisted as a ''factory'' for production of a range of secreted proteins for the treatment of nonmuscle disorders. These recombinant nonmuscle gene products are biologically active even when produced by muscle.

The ease of isolating myoblasts from both mouse and human muscle and purifying, growing, and transducing them in vitro is a major advantage of using myoblasts rather than other cell types for gene transfer. Primary myoblasts can be isolated from any mouse strain, including strains harboring genetic mutations or transgenic strains (25, 26), providing a broad array of genotypes either for study in tissue culture or for transplantation. Established myogenic cell lines, such as the C2 cell line, may also be implanted; however, these cells can proliferate and form tumors when implanted into mice (15). In contrast, although they exhibit an impressive capacity to proliferate in culture, primary myoblasts do not form tumors when injected into mouse muscle (15). In human myoblasts, senescence has been observed but transformation has not (27).

Myoblasts can be isolated from tissues of any age, but both the yield and number of doublings tend to be higher when obtained from younger donors. Primary cultures are derived from postnatal muscle using mechanical or enzymatic dissociation methods (15, 28) and are readily obtained from human biopsy

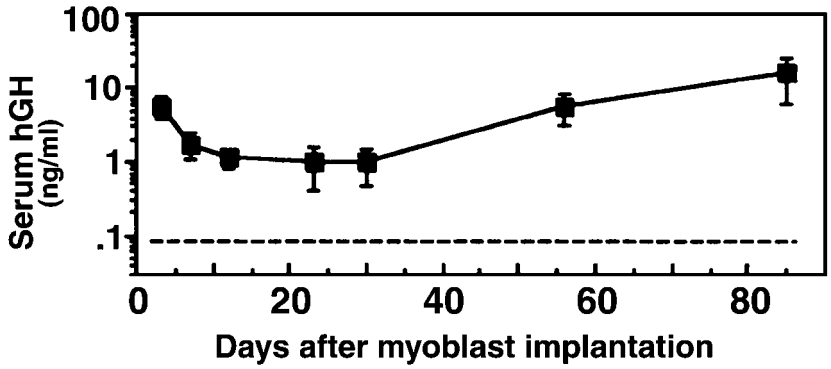

Figure 2 Persistent expression of human growth hormone (hGH) by virus-transduced myoblasts implanted into mouse muscle in vivo. C2C12 myoblasts were retrovirally transduced with hGH and injected into hind limbs of 24 C3H mice, and serum hGH levels were monitored for 85 days by radioimmunoassay of tail blood. More than 90% of the implanted cells expressed and secreted hGH, as determined by clonal analysis in culture. (*Points*) The mean ± standard deviation for 4–24 mice; (*dashed line*) the mean for serum samples from five uninjected control mice. Reprinted with permission from Science, 1991, 254 (5037):1509–12 (Reference 18) (copyright © 1991 Am. Assoc. Advance. Sci.).

or autopsy tissue (29, 30). Further purification to obtain myoblasts free from contaminating cell types can be accomplished using cell culture conditions that favor myoblast growth at the expense of other cell types, yielding a pure population of mouse myoblasts within 2 weeks of normal growth (15, 31). Alternatively, primary cells have been isolated by fluorescence-activated cell sorting using antibodies directed against α7 integrin for mouse (W Blanco-Bose, C Yoo, R Kramer, HM Blau, manuscript in preparation) and rat (32) cultures, or against the muscle surface antigen NCAM in human-derived cultures (28). Human cells isolated by fluorescence-activated cell sorting can undergo at least 40 cell doublings without differentiating (28), implying that a kilogram of cells for use in transplantation could theoretically be derived from a 5-mm^3 biopsy. Recombinant genes can be introduced into myoblasts by lipofection, by calcium-mediated transfection, or (more readily) by retroviral infection. Optimized conditions for retroviral infection of myoblasts at high efficiency enables >99% of primary myoblasts in culture to be transduced without a selectable marker (33). Thus, primary myoblasts can be genetically engineered with relative ease, and genetic alteration does not reduce the ability of myoblasts to mature and differentiate into myofibers.

CLINICAL APPLICATIONS

The many studies establishing myoblasts as potent vehicles for delivering donor genes into host muscle led to the concept of applying myoblast-mediated gene transfer to the treatment of disease. Implanted myoblasts are integrated into preexisting muscle tissue that is highly vascularized and, thus, can be used to deliver genes encoding both muscle-specific and circulatory proteins. This section briefly summarizes the clinical applications that have been tested using myoblast-mediated gene delivery.

Delivery of Genes Encoding Muscle Structural Proteins

The first studies applying myoblast transplantation to the treatment of disease used allografts of normal myoblasts to insert donor nuclei, containing a normal genome, into genetically abnormal muscle. Although technically not gene therapy because donor myoblasts were not genetically altered in any way, these ''cell therapy'' experiments were important in demonstrating the validity of myoblast-mediated gene delivery, and they are the only studies involving myoblast transplantation that have been translated into human clinical trials. Duchenne muscular dystrophy (DMD), the most common of heritable human muscular dystrophies, was the first disorder to which this therapeutic approach was applied. This disease causes progressive muscle weakness beginning in childhood and is caused by mutations in the *dystrophin* gene (34). Studies on mdx dystrophin-deficient mice, the mouse model of the disorder, showed that implantation of normal myoblasts

containing an intact *dystrophin* could counteract the cycle of muscle fiber degeneration and regeneration characteristic of mdx muscle (7, 35).

Clinical trials in which donor myoblasts taken from normal human muscle were introduced into DMD patients were initiated at multiple institutions (36–42). All these studies demonstrated that myoblast implantation into humans has no adverse effects. However, all but one group (36) reported the disappointing finding that only a very small percentage of host myofibers began to express *dystrophin.* Definitive evidence that donor *dystrophin* transcripts were being synthesized was first provided by polymerase chain reaction by Gussoni and colleagues (37). Recent studies employing fluorescent in situ hybridization together with immunohistochemistry to localize both the dystrophin protein and donor nuclei at the same time showed that for at least one of these studies, a large proportion of donor myoblasts did successfully integrate into host myofibers in almost every subject, because donor nuclei were interspersed and aligned with host nuclei (43, 44). Thus, viability and access were not problems. Indeed, in half of the patients tested, more than 10% of the injected nuclei were estimated to gain access to the human host fibers, a remarkable result. Furthermore, because the antibodies used in these studies were specific to the product for the deleted gene regions in the recipients, these experiments demonstrated that increased dystrophin protein production observed in recipient muscle was contributed by the donor nuclei. The dystrophin produced by a single nucleus spanned regions including 20–30 nuclei. The unresolved question at this point is why only a subset of transduced myofibers expressed *dystrophin*. Possible variables include factors specific to the DMD disease state itself, such as increased fibrosis with patient age, which could have created an environment in which the newly introduced nuclei remained transcriptionally inactive in fiber regions undergoing degeneration. An alternative hypothesis is that myoblasts are heterogeneous (45) and only a subset are capable of activating and expressing the *dystrophin* gene.

Treatment of DMD by cell therapy remains an ambitious undertaking, as it requires that a large proportion of muscle fibers be transduced in order to produce a beneficial outcome. In addition, all muscles must be implanted with myoblasts, including those that are difficult to access, such as the diaphragm or the heart. It is the failure of these two muscles that is often the cause of death in patients with DMD. For targeting a high percentage of muscle in humans, delivery of viral vectors or naked DNA encoding either full-length or truncated *dystrophin* genes (46–48), or up-regulation or delivery of the ubiquitous *utrophin* gene (49), may prove to be most effective.

Delivery of Genes Encoding Secretable Factors

Lysosomal storage diseases are a subset of disorders caused by a single-gene defect that may be amenable to gene therapy (50, 51). These recessive disorders, which are due to a single missing or defective lysosomal enzyme, result in the detrimental accumulation of lysosomal enzyme substrates within affected tissues.

Missing lysosomal enzymes manufactured by muscle and delivered to the serum can be internalized by distant tissues and appropriately targeted to lysosomes via mannose 6-phosphate receptors (52). Implantation of genetically engineered primary myoblasts encoding β-glucuronidase, a lysosomal enzyme, into muscle led to in vivo expression of the recombinant protein in adult β-glucuronidase–deficient mice (53). Production and secretion of the missing lysosomal enzyme by muscle in this case corrected phenotypic abnormalities in the liver and spleen of treated animals.

Hemophilia B, a bleeding disorder caused by a deficiency of clotting factor IX, is another single-gene defect well-suited for ex vivo gene therapy (54). Gene therapy may provide a safer and more convenient alternative to conventional protein replacement therapies that necessitate frequently repeated treatments and, for plasma-derived factors, run the risk of transmitting blood-borne pathogens (55). Implantation of C2C12 myoblasts transduced with a gene encoding human factor IX in mice led to a peak expression of recombinant protein (1 μg/ml) at day 12 and a subsequent decline back to basal levels thereafter (21). The drop in human factor IX expression was shown to be due to production of specific antibodies targeted against the protein in wild-type mice. Other experiments (22–24) have demonstrated that in immunodeficient mice, primary myoblasts engineered to constitutively express factor IX led to stable, low-level production of the protein for many months. Use of a β-actin promoter with muscle creatine kinase enhancers led to stable production of human factor IX at therapeutic levels in SCID mice for at least 8 months (16). Problems with immunogenicity are likely to affect only a percentage of hemophiliacs, because some are not null mutations but have reduced levels of factor IX (56). Of importance, recombinant factor IX manufactured in muscle undergoes the gamma carboxylation typical of liver that is required for functional activity of the protein (21–24). Future preclinical gene therapy studies should be facilitated by the recent creation of a mouse model for hemophilia B (57).

Erythropoietin (Epo)-responsive anemias, such as those associated with end-stage renal disease, are another class of disorders currently under study for muscle-mediated gene therapy. Delivery of recombinant Epo, a mammalian hormone controlling production of erythrocytes (58), has been employed as a successful treatment for anemias (59, 60). Treatments using recombinant Epo, however, require frequent hospital visits by patients and are costly. Muscle-mediated delivery of the protein could eliminate the need for multiple treatments. Implantation of primary or C2 myoblasts expressing Epo into mice led to an elevated hematocrit, a direct measure of Epo production, for 3 months (53, 61). Three months after implantation, myoblasts were shown to have fused and fully differentiated into myofibers (61). Additional experiments using implantation of human Epo-secreting C2 myoblasts and a mouse model of renal failure in which anemia is induced by nephrectomy led to reversal of the anemic phenotype (62). Levels of serum Epo, detected by ELISA, remained elevated for 2 months following myoblast implantation. Thus, myoblast-mediated expression of Epo

appears to be a potential treatment for certain types of anemias. As with other gene therapies, the ability to regulate expression of Epo may be desirable so that the amount of circulating hormone may be appropriately tailored to the individual patient.

An example that particularly illustrates this point is the delivery of genes encoding angiogenic factors. Therapeutic angiogenesis—the concept of using factors to stimulate new vessels to grow as treatments for ischemic diseases, including stroke, peripheral arterial disease, and myocardial infarction—has been a subject of much recent attention. Vascular endothelial growth factor (VEGF), a potent mitogen that stimulates growth of endothelial cells and increases permeability in vascular endothelium (hence its other designation, vascular permeability factor) (63–67), is the angiogenic factor that has received the most attention. VEGF plays an important role in the induction of angiogenesis by tumors (68), in the angiogenic response of normal tissue to decreased oxygen availability, and as a critical signal during vasculogenesis, the de novo growth of blood vessels from precursor cells during embryonic development (69, 70). Thus, VEGF is a crucial regulator of development of the vasculature pre- and postnatally.

Much effort has been devoted in recent years to investigating the clinical benefits of VEGF delivery to inadequately vascularized tissues as a means of stimulating new blood vessel growth. Injection of VEGF protein has resulted in angiogenic sprouting of vessels in ischemic muscle (71–73). However, presumably because of vascular permeabilizing and/or vasodilating properties, bolus injections of the protein have been reported to cause hypotension (74, 75). As a result, recent studies have examined the feasibility of localized delivery of VEGF using plasmid DNA or adenoviral vector-mediated gene transfer. Although these two delivery methods lead to only transient production of recombinant VEGF, angiogenic sprouting from preexisting vessels was observed in matrigel in vitro (76, 77) and in adipose tissues in vivo (78), as well as in ischemic skeletal or cardiac muscle (79–82).

Using myoblast-mediated gene transfer, the effects of long-term stable production of VEGF were recently investigated (83). Myoblasts transduced with murine cDNA encoding $VEGF_{164}$ were injected into the muscles of immunodeficient SCID mice, leading to an unexpected physiological response to VEGF. At day 11 postimplantation, although mice appeared outwardly normal, histological analysis of frozen muscle sections revealed that implanted VEGF-expressing myoblasts, but not control myoblasts, were invariably associated with regions of infiltrating mononuclear cells identified by fluorescent antibody staining as endothelial cells and macrophages. By days 44–47, large hemangiomas composed of vascular channels and pools of blood appeared in all legs injected with VEGF myoblasts, whereas control legs appeared normal (Figure 3). These studies highlight the potency of myoblast-mediated VEGF gene delivery, and the results suggest that this single growth factor can lead to a cascade of events resulting in the formation of complex tissues of multiple cell types. This study was also the first to show a physiological response to VEGF in nonischemic muscle and demon-

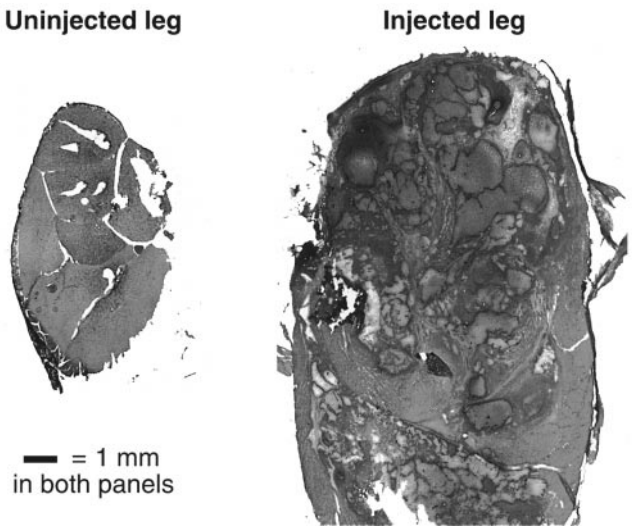

Figure 3 Large vascular structures developed in normal adult skeletal muscle implanted with vascular endothelial growth factor (VEGF)-expressing primary myoblasts. Histological analysis of hindlimb muscles was conducted at day 44–47 following implantation of myoblasts expressing the murine $VEGF_{164}$ gene, using hematoxylin/eosin staining of cryostat sections. Control legs that did not receive VEGF were normal in size and in morphology (*left*), whereas VEGF-injected legs were more than twice the diameter of control legs and consisted primarily of hemangioma and pools of blood (*right*). The two panels are shown at the same magnification. Adapted and reprinted with permission from Reference 83 (copyright is held by Cell Press).

strated that expression of VEGF at high levels or long duration can have deleterious effects. This latter point is of importance, as clinical trials of plasmid or adenoviral-mediated VEGF gene delivery are underway.

In contrast to studies aimed at promoting new blood vessel growth, other ongoing studies attempt to block blood vessel growth as a treatment for cancer. Both tumor growth and metastasis require persistent new blood vessel growth (84, 85); thus, therapies targeted at blocking vessel growth may arrest tumor development. The recent identification of anti-angiogenic factors such as angiostatin and endostatin may facilitate the development of such therapies. These proteolytic products of plasminogen and collagen XVIII, respectively, were isolated from fractions taken from serum and urine that were capable of inhibiting endothelial cell proliferation in vitro and metastatic tumor growth in vivo (86, 87). One recent study demonstrated that administration of plasmid DNA encoding endostatin into muscle could inhibit tumor growth and the development of metastatic lesions (88). An interesting therapy for cancer could be to engineer myoblasts to express these proteins, such that their secretion may inhibit metastases, or growth of tumors at distant sites. It will be critical to confirm that desired blood

vessel synthesis at sites of injury, for example, is not impaired. Because angiostatin and endostatin are difficult to produce in adequate amounts in bacteria, gene therapy protocols will be invaluable for discerning their biological function and possible application as anti-cancer agents in vivo.

GENE REGULATION USING TETRACYCLINE-REGULATABLE RETROVIRAL VECTORS

Most gene therapy studies have relied on constitutive expression of the introduced gene. However, as is made abundantly clear in the studies of myoblast-mediated transfer of the VEGF gene into normal adult muscle (83), regulation of gene expression is extremely important. Both the ability to increase expression levels if an insufficient amount of a recombinant protein is being produced and the option to intentionally reduce or cease expression are likely to be necessary for the health of the patient in many cases.

Regulation theoretically allows timing of gene expression and levels of the gene product to be optimized on a case-by-case basis. An advantage of using retrovirally transduced myoblasts for implantation is that they allow localized delivery of a recombinant gene at sustained levels; addition of inducible elements to retroviral vectors provides a mechanism for fine-tuning gene expression to the levels required for therapy. An ideal regulatable system should display five characteristics: specificity, efficiency, dose dependency, lack of immunogenicity, and lack of toxicity. The regulatable system should not require endogenous factors for activation, nor should it interfere with cellular regulatory pathways. The system should demonstrate inducibility from low basal levels to high levels of gene expression, and the potential for repression back to uninduced levels. The system also should respond to its inducer by modulating gene expression in a sensitive and homogeneous manner. Lastly, none of its components should elicit a host immune response or be toxic to the tissues or organism as a whole. To date, the four regulatable systems displaying some or most of these characteristics are the ecdysone, RU486, FK506/rapamycin, and tetracycline-inducible systems (for review, see 89, 90). This section focuses on the attributes and recent advances of the tetracycline system, which has been extensively studied by our laboratory and has already been incorporated into myoblast gene transfer strategies.

The tetracycline-inducible system originally developed by Bujard and colleagues (91, 92) has become one of the most widely used methods of regulating gene expression. Because most elements of the system are prokaryotic and are not endogenous to mammalian cells, pleiotropic effects and endogenous ligands are avoided. In addition, the inducer becomes an integral part of the transactivator directly responsible for turning on gene expression, allowing a direct correlation between the amount of transcription factor capable of binding DNA and the concentration of exogenous inducer (tetracycline or its synthetic analog doxycycline).

Tetracycline has been used for decades in humans and animals, and adverse effects from its use have only been seen at doses higher than those required for induction of transgenes.

The original tetracycline-inducible system incorporated a tetracycline transactivator (tTA), a hybrid factor composed of a bacterial tet repressor (tetR), and the viral transactivator domain VP16 (91). When bound to tetracycline or doxycycline, tTA cannot bind to tetracycline operator sequences juxtaposed to a minimal promoter, and gene expression is not turned on. When the inducer is absent, however, tTA is free to bind to the promoter and gene expression is induced. A variation of the system allows for induction of gene expression in the presence of inducer rather than its absence (92). In this second system, a chimeric protein containing a mutated version of tetR, designated reverse tTA (rtTA), binds to tetracycline operator sequences in the presence of tetracycline. Both tTA and rtTA efficiently regulate expression in tissue culture, fruit flies, and mice (93–96).

In the past year, two additional advancements of the tetracycline system have been made. The tetR transcriptional elements are modular; the VP16 transactivator domain of tTA, for instance, may be replaced with the KRAB transrepressor domain to create a tetracycline-regulated repressor of transcription (97). A problem with expressing two tetracycline modulators within the same cell, however, is that nonfunctional heterodimers may form because modulators have identical dimerization domains (Figure 4*a*) (98). Using sequence information and known crystal structures of tetR as well as mutational analysis (99–101), mutually distinct dimerization domains deriving from separate classes of gram-negative bacteria have been identified (102, 103). The use of tetracycline modulators harboring specific dimerization domains allows activators and repressors to be expressed within the same cell without formation of nonfunctional heterodimers. This system, designated RetroTet-ART (activators and repressors expressed together) (102), allows gene expression to be completely extinguished or induced in a fully dose-dependent manner, resulting in a greatly enhanced dynamic range of gene expression (up to five or six orders of magnitude) (Figure 4*b*). This improvement is of significant advantage in applications where basal expression from the inducible promoter must be negligible or totally absent. The RetroTet-ART system has been shown to be effective in reversibly silencing expression of p16, a growth arrest protein (102).

A second variation of the tetracycline system incorporates an altered DNA binding domain of tetR that interacts with a modified tetracycline operator sequence (103). tTA and rtTA proteins harboring distinct dimerization domains were engineered with the adapted and original DNA binding sequences. By placing two separate genes under control of old and new tetracycline operator sequences and expressing both of the modified tTA and rtTA proteins, Baron and colleagues (103) were able to either repress expression of both genes or express either gene alone simply by changing the doxycycline concentration (Figure 4*c*). The activity of two different genes could thus be reversibly controlled in a mutu-

ally exclusive manner. However, a means of turning both genes on at once has yet to be achieved.

The application of retroviruses was a major advance in broadening the utility of the tetracycline system, because retroviral gene delivery is much more rapid and efficient than transfection with plasmids. Genes can be introduced into tens of thousands of myoblasts at high efficiency, generating polyclonal populations within a week (33), an advantage over the few stable clones routinely obtained. In addition, retroviral vectors do not form concatemers and therefore should not form a repressive chromatin environment sometimes associated with plasmids (104). For these reasons, retroviruses are well suited for delivery of tetracycline-inducible systems to primary cells. The first studies with tetracycline-regulatable cassettes in retroviruses, however, met with problems. In one case, inclusion of an autoregulatory feedback loop required high background levels of expression in order to ''jumpstart'' the system (105). In other instances, overcomplexity of transcription and translation units led to low viral titers (106–110). These problems were first overcome using simplified retroviral vectors in which necessary elements were dispersed over more than one retroviral vector (111). In this setup, one retrovirus encoded rtTA, whereas the other contained an inducible Epo cassette. Primary myoblasts exhibited levels of induction approximating 200-fold following multiple rounds of infection. When the engineered myoblasts were transplanted into mice, Epo expression could be repetitively turned on and off over a 5-month period by controlling levels of doxycycline in drinking water. A further improvement of this approach was the inclusion of a selectable marker such as GFP, allowing purification of regulatable populations of cells by flow cytometry (96).

To summarize, tetracycline-regulatable retroviral vectors are powerful tools for regulating gene expression in a fully inducible manner both in vitro and in vivo. Moreover, when the rtTA protein was delivered to mice using myoblasts, an immune response to foreign elements was not observed (111). Retroviruses provide an efficient means of delivering tetracycline-regulatable vectors to large numbers of primary cells, such as myoblasts. Thus, tetracycline-regulatable retroviral systems, in conjunction with myoblast-mediated gene delivery, are powerful tools for fundamental in vivo studies of gene expression as well as for gene therapy.

FUTURE PROSPECTS

Circulating Muscle Precursor Cells (Stem Cells)

A problem in using myoblasts for gene delivery in the treatment of inherited myopathies, as noted earlier, is the difficulty of targeting a large enough proportion of muscle tissue in order for treatment to be effective. Histochemical staining and enzymatic activity assays of muscle transplanted with β-galactosidase–

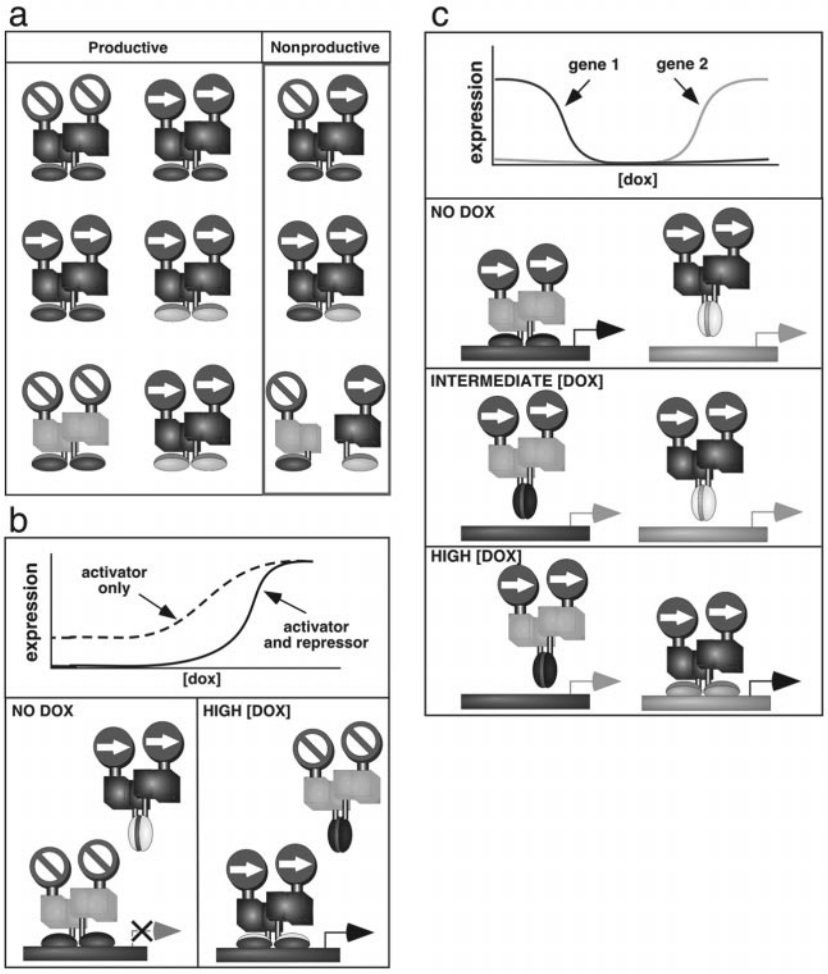

Figure 4 Improved tetracycline-regulatable systems. (*a*) The need for tetR dimerization specificity. Coexpression of tetR fusion proteins with different functional domains, such as repressor domains (represented by the ''do not enter'' sign) and activator domains (represented by the ''go'' sign), or DNA binding domains with distinct specificity (symbolized in the *middle row* by the light gray and dark gray ''feet''), leads to formation of both functional homodimers and nonfunctional heterodimers. Nonfunctional heterodimers can be eliminated by engineering distinct dimerization domains into the tetR portion of the tet modulators (symbolized by the dark gray and light gray midsections in the *bottom row*). (*b*) Increasing the dynamic range of the tetracycline system. A repressor and an activator that respond oppositely to doxycycline (dox) and that do not heterodimerize because of different dimerization domains can be coexpressed within the same cell. The result is a reduction in the basal expression level of genes under tetracycline control, without affecting the fully induced level. (*c*) Independent expression of two different genes.

expressing myoblasts have shown that fusion of myoblasts into myofibers is maximal in the region of the injection site; the number of fibers to which genes are delivered by intramuscular injection decreases with increasing distance from the site (112). For a sufficiently large percentage of fibers to be treated, many closely spaced injections would be necessary. Isolation of a myoblast population that can efficiently migrate to damaged or degenerated muscle appears to be the most promising solution for effectively treating a large percentage of muscle tissue.

One method of accomplishing this goal may be to introduce genetically engineered muscle precursor cells into the circulation, where they can reach muscles throughout the entire body. Recently, the observation that bone marrow–derived cells can become incorporated into areas of damaged muscle has suggested that these cells can travel through the circulation and from there enter into skeletal muscle tissue (113). Another study examined the feasibility of intraarterial delivery of genetically labeled, immortalized L6 myoblasts to skeletal muscle (114). Infusion of these cells into the arterial circulation led to a small number of labeled fibers in skeletal leg muscle, demonstrating that the circulation may be capable of delivering muscle precursor cells to differentiated myofibers, although some were also found in the lung. A powerful approach to targeting many muscle fibers may be to isolate muscle stem cells of the bone marrow, genetically engineer them ex vivo, and inject them back into the patient where they could serve as a continual pool of circulating cells harboring vectors for the treatment of myopathies.

Several lines of evidence have suggested the existence of a muscle stem cell. Populations of cells that are capable of self-renewal and remain undifferentiated when cultured in conditions designed to induce terminal differentiation have been identified both in the myogenic C2 cell line (115) and in clones of human myoblasts (116). A recent study has also shown that only a discrete minority of transplanted myoblasts participate in regeneration of host muscle, using two genetic markers with different modes of inheritance to examine the fate of myoblasts transplanted into skeletal muscle (45). The small population of cells identified in this study appeared to divide slowly in vitro, but they proliferated rapidly when transplantated into regenerating muscle (45). Methods for characterizing and isolating this muscle stem cell population would be a great boon to the development of ex vivo therapies for a number of inherited myopathies.

Figure 4 (Continued) By coexpressing two tetR-based activators that contain DNA binding domains with distinct specificity, that respond oppositely to doxycycline, and that do not heterodimerize, two independent genes can be regulated by the same inducer. In this system, the expression of each gene can be turned off at an intermediate concentration of doxycycline and activated at markedly different doxycycline concentrations. From Reference 98 (republished with permission of the Proc. Natl. Acad. Sci. USA, 2101 Constitution Ave., NW, Washington DC 20418 and by permission of the publisher via Copyright Clearance Center, Inc).

Encapsulation for Immunoprotection

Although myoblast-mediated gene delivery has proven effective for long-term secretion of recombinant proteins into the circulation, a major hurdle limiting its utility in the therapeutic realm is the requirement for using syngeneic (genetically identical) cells for transplantation in order to avoid immunological rejection (117). Myoblasts may be both isolated from and implanted back into the same individual; however, these procedures are both time intensive and costly. Alternatively, myoblasts can be encapsulated into an immuno-isolated environment prior to implantation to prevent immune cells from coming into contact with implanted cells. Encapsulation of cells within a matrix, for example one made of alginate (although other materials may be used), allows secreted proteins to leave the capsules while obviating the necessity of a genetically identical cell donor. Encapsulated myoblasts have been shown to be effective in delivering genes encoding mouse growth hormone (118) and human factor IX (119) intraperitoneally. The encapsulated cells were retrievable for as long as 213 days postimplantation and even at that point were found to be fully viable and capable of secreting recombinant proteins ex vivo at undiminished rates (119). More recently, encapsulated primary myoblasts were used to deliver VEGF to mice subcutaneously and intraperitoneally, leading to an angiogenic response (ML Springer, G Hortelano, D Bouley, J Wong, PE Kraft, HM Blau, manuscript submitted). This technology is a promising method for nonautologous gene therapy, in which universal donor cells can be created simply by encapsulation in a benign, immunoprotected environment. Myoblasts may be the cell type of choice for this mode of gene therapy, as cell types such as fibroblasts that are encapsulated overgrow and die, whereas myoblasts do not.

CONCLUSIONS

Myoblast-mediated gene delivery is a method that may be well suited for treatment of a number of disorders, not only for diseases caused by single-gene defects but also for complex multi-gene disorders such as cardiovascular diseases and cancer. Gene delivery using myoblasts offers a number of advantages. Genetically altered myoblasts may be fully characterized in vitro before in vivo injection to ensure secretion of recombinant products of correct size and function at pharmacologically useful levels. Moreover, isolated myoblasts are genetically engineered outside of the body, a process that (*a*) generally assures that only the proper cell type is transduced and (*b*) avoids inadvertent low-level transduction of cells other than those being targeted, for example cells of the germline. The recent development of improved tetracycline-inducible retroviral vectors provides a powerful means of controlling recombinant gene expression levels. Recent studies suggest that major limitations to ex vivo gene delivery, such as the difficulty in targeting a large proportion of muscle tissue and the requirement that syngeneic

myoblasts isolated from one patient be reinjected into that same patient to avoid rejection of cells by the immune system, may be overcome. Identification of a muscle stem cell could lead to methods of gene delivery to muscle throughout the body, allowing for treatment of myopathies using myoblasts. Encapsulation of myoblasts may obviate the requirement for a ''tailor made'' therapy, allowing allogeneic cells that are invisible to the immune system to be used, theoretically creating universal donor cells derived from muscles of a single patient that could be implanted at ectopic sites in different patients for delivery of diverse products. All these advantages make ex vivo gene delivery via myoblasts an attractive candidate for human gene therapy in the future.

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LITERATURE CITED

1. Blaese RM, Culver KW, Miller AD, Carter CS, Fleisher T, et al. 1995. T lymphocyte-directed gene therapy for ADA-SCID: initial trial results after 4 years. *Science* 270:475–80
2. Partridge TA, Grounds M, Sloper JC. 1978. Evidence of fusion between host and donor myoblasts in skeletal muscle grafts. *Nature* 273:306–8
3. Watt DJ, Morgan JE, Clifford MA, Partridge TA. 1987. The movement of muscle precursor cells between adjacent regenerating muscles in the mouse. *Anat. Embryol.* 175:527–36
4. Watt DJ, Lambert K, Morgan JE, Partridge TA, Sloper JC. 1982. Incorporation of donor muscle precursor cells into an area of muscle regeneration in the host mouse. *J. Neurol. Sci.* 57:319–31
5. Watt DJ, Morgan JE, Partridge TA. 1984. Use of mononuclear precursor cells to insert allogeneic genes into growing mouse muscles. *Muscle Nerve* 7:741–50
6. Morgan JE, Watt DJ, Sloper JC, Partridge TA. 1988. Partial correction of an inherited biochemical defect of skeletal muscle by grafts of normal muscle precursor cells. *J. Neurol. Sci.* 86:137–47
7. Partridge TA, Morgan JE, Coulton GR, Hoffman EP, Kunkel LM. 1989. Conversion of mdx myofibres from dystrophin-negative to -positive by injection of normal myoblasts. *Nature* 337:176–79
8. Campion DR. 1984. The muscle satellite cell: a review. *Int. Rev. Cytol.* 87:225–51
9. Mauro A. 1961. Satellite cell of skeletal muscle fibers. *J. Biophys. Biochem. Cytol.* 9:493–95
10. Hughes SM, Cho M, Karsch-Mizrachi I, Travis M, Silberstein L, et al. 1993. Three slow myosin heavy chains sequentially expressed in developing mammalian skeletal muscle. *Dev. Biol.* 158:183–99
11. Blau HM, Dhawan J, Pavlath GK. 1993. Myoblasts in pattern formation and gene therapy. *Trends Genet.* 9:269–74
12. Cho M, Webster SG, Blau HM. 1993. Evidence for myoblast-extrinsic regulation of slow myosin heavy chain expression during muscle fiber formation in embryonic development. *J. Cell Biol.* 121:795–810
13. Hughes SM, Blau HM. 1990. Migration of myoblasts across basal lamina during skeletal muscle development. *Nature* 345:350–53
14. Hughes SM, Blau HM. 1992. Muscle fiber pattern is independent of cell lineage in postnatal rodent development. *Cell* 68:659–71
15. Rando TA, Blau HM. 1994. Primary

mouse myoblast purification, characterization, and transplantation for cell-mediated gene therapy. *J. Cell Biol.* 125:1275–87
16. Wang JM, Zheng H, Blaivas M, Kurachi K. 1997. Persistent systemic production of human factor IX in mice by skeletal myoblast-mediated gene transfer: feasibility of repeat application to obtain therapeutic levels. *Blood* 90:1075–82
17. Blau HM, Springer ML. 1995. Muscle-mediated gene therapy. *N. Engl. J. Med.* 333:1554–56
18. Dhawan J, Pan LC, Pavlath GK, Travis MA, Lanctot AM, et al. 1991. Systemic delivery of human growth hormone by injection of genetically engineered myoblasts. *Science* 254:1509–12
19. Barr E, Leiden JM. 1991. Systemic delivery of recombinant proteins by genetically modified myoblasts. *Science* 254:1507–9
20. Peeters S, Friesen HG. 1977. A growth hormone binding factor in the serum of pregnant mice. *Endocrinology* 101: 1164–83
21. Yao SN, Kurachi K. 1992. Expression of human factor IX in mice after injection of genetically modified myoblasts. *Proc. Natl. Acad. Sci. USA* 89:3357–61
22. Yao SN, Smith KJ, Kurachi K. 1994. Primary myoblast-mediated gene transfer: persistent expression of human factor IX in mice. *Gene Ther.* 1:99–107
23. Dai Y, Roman M, Naviaux RK, Verma IM. 1992. Gene therapy via primary myoblasts: long-term expression of factor IX protein following transplantation in vivo. *Proc. Natl. Acad. Sci. USA* 89:10892–95
24. Roman M, Axelrod JH, Dai Y, Naviaux RK, Friedmann T, et al. 1992. Circulating human or canine factor IX from retrovirally transduced primary myoblasts and established myoblast cell lines grafted into murine skeletal muscle. *Somat. Cell. Mol. Genet.* 18:247–58
25. Charlton CA, Mohler WA, Radice GL, Hynes RO, Blau HM. 1997. Fusion competence of myoblasts rendered genetically null for N-cadherin in culture. *J. Cell Biol.* 138:331–36
26. Yang JT, Rando TA, Mohler WA, Rayburn H, Blau HM, et al. 1996. Genetic analysis of alpha 4 integrin functions in the development of mouse skeletal muscle. *J. Cell Biol.* 135:829–35
27. Webster C, Blau HM. 1990. Accelerated age-related decline in replicative lifespan of Duchenne muscular dystrophy myoblasts: implications for cell and gene therapy. *Somat. Cell. Mol. Genet.* 16: 557–65
28. Webster C, Pavlath GK, Parks DR, Walsh FS, Blau HM. 1988. Isolation of human myoblasts with the fluorescence-activated cell sorter. *Exp. Cell Res.* 174:252–65
29. Blau HM, Kaplan I, Tao TW, Kriss JP. 1983. Thyroglobulin-independent, cell-mediated cytotoxicity of human eye muscle cells in tissue culture by lymphocytes of a patient with Graves' ophthalmopathy. *Life Sci.* 32:45–53
30. Blau HM, Webster C. 1981. Isolation and characterization of human muscle cells. *Proc. Natl. Acad. Sci. USA* 78:5623–27
31. Springer ML, Rando TA, Blau HM. 1997. Gene delivery to muscle. In *Current Protocols in Human Genetics*, ed. AL Boyle, pp. 13.4.1–19. New York: Wiley
32. Kaufman SJ, Foster RF. 1988. Replicating myoblasts express a muscle-specific phenotype. *Proc. Natl. Acad. Sci. USA* 85:9606–10
33. Springer ML, Blau HM. 1997. High-efficiency retroviral infection of primary myoblasts. *Somat. Cell. Mol. Genet.* 23:203–9
34. Hoffman EP, Brown RH Jr, Kunkel LM. 1987. Dystrophin: the protein product of the Duchenne muscular dystrophy locus. *Cell* 51:919–28
35. Morgan JE, Hoffman EP, Partridge TA. 1990. Normal myogenic cells from new-

born mice restore normal histology to degenerating muscles of the mdx mouse. *J. Cell Biol.* 111:2437–49
36. Law PK, Bertorini TE, Goodwin TG, Chen M, Fang QW, et al. 1990. Dystrophin production induced by myoblast transfer therapy in Duchenne muscular dystrophy. *Lancet* 336:114–15
37. Gussoni E, Pavlath GK, Lanctot AM, Sharma KR, Miller RG, et al. 1992. Normal dystrophin transcripts detected in Duchenne muscular dystrophy patients after myoblast transplantation. *Nature* 356:435–38
38. Huard J, Bouchard JP, Roy R, Malouin F, Dansereau G, et al. 1992. Human myoblast transplantation: preliminary results of 4 cases. *Muscle Nerve* 15:550–60
39. Karpati G, Ajdukovic D, Arnold D, Gledhill RB, Guttmann R, et al. 1993. Myoblast transfer in Duchenne muscular dystrophy. *Ann. Neurol.* 34:8–17
40. Mendell JR, Kissel JT, Amato AA, King W, Signore L, et al. 1995. Myoblast transfer in the treatment of Duchenne's muscular dystrophy. *N. Engl. J. Med.* 333:832–38
41. Morandi L, Bernasconi P, Gebbia M, Mora M, Crosti F, et al. 1995. Lack of mRNA and dystrophin expression in DMD patients three months after myoblast transfer. *Neuromuscul. Disord.* 5:291–95
42. Miller RG, Sharma KR, Pavlath GK, Gussoni E, Mynhier M, et al. 1997. Myoblast implantation in Duchenne muscular dystrophy: the San Francisco study. *Muscle Nerve* 20:469–78
43. Gussoni E, Wang Y, Fraefel C, Miller RG, Blau HM, et al. 1996. A method to codetect introduced genes and their products in gene therapy protocols. *Nat. Biotechnol.* 14:1012–16
44. Gussoni E, Blau HM, Kunkel LM. 1997. The fate of individual myoblasts after transplantation into muscles of DMD patients. *Nat. Med.* 3:970–77
45. Beauchamp JR, Morgan JE, Pagel CN, Partridge TA. 1999. Dynamics of myoblast transplantation reveal a discrete minority of precursors with stem cell-like properties as the myogenic source. *J. Cell Biol.* 144:1113–22
46. Ragot T, Vincent N, Chafey P, Vigne E, Gilgenkrantz H, et al. 1993. Efficient adenovirus-mediated transfer of a human minidystrophin gene to skeletal muscle of mdx mice. *Nature* 361:647–50
47. Kochanek S, Clemens PR, Mitani K, Chen HH, Chan S, et al. 1996. A new adenoviral vector: replacement of all viral coding sequences with 28 kb of DNA independently expressing both full-length dystrophin and beta-galactosidase. *Proc. Natl. Acad. Sci. USA* 93:5731–36
48. Kumar-Singh R, Chamberlain JS. 1996. Encapsidated adenovirus minichromosomes allow delivery and expression of a 14 kb dystrophin cDNA to muscle cells. *Hum. Mol. Genet.* 5:913–21
49. Tinsley J, Deconinck N, Fisher R, Kahn D, Phelps S, et al. 1998. Expression of full-length utrophin prevents muscular dystrophy in mdx mice. *Nat. Med.* 4:1441–44
50. Akli S, Guidotti JE, Vigne E, Perricaudet M, Sandhoff K, et al. 1996. Restoration of hexosaminidase A activity in human Tay-Sachs fibroblasts via adenoviral vector-mediated gene transfer. *Gene Ther.* 3:769–74
51. Svensson EC, Tripathy SK, Leiden JM. 1996. Muscle-based gene therapy: realistic possibilities for the future. *Mol. Med. Today* 2:166–72
52. Pfeffer S. 1991. Targeting of proteins to the lysosome. *Curr. Top. Microbiol. Immunol.* 170:43–63
53. Naffakh N, Pinset C, Montarras D, Li Z, Paulin D, et al. 1996. Long-term secretion of therapeutic proteins from genetically modified skeletal muscles. *Hum. Gene Ther.* 7:11–21
54. Kay MA. 1995. Hepatic gene therapy for hemophilia B. *Adv. Exp. Med. Biol.*

386:229–34
55. Chuah MK, Collen D, VandenDriessche T. 1998. Gene therapy for hemophilia: hopes and hurdles. *Crit. Rev. Oncol. Hematol.* 28:153–71
56. Hedner U, Davie E. 1989. Introduction to homeostasis and the vitamin K-dependent coagulation factors. In *The Metabolic Basis of Inherited Disease,* ed. C Scriver, AL Beaudet, WS Sly, D Valle, pp. 2107–34. New York: McGraw-Hill. 6th ed.
57. Wang L, Zoppe M, Hackeng TM, Griffin JH, Lee KF, et al. 1997. A factor IX-deficient mouse model for hemophilia B gene therapy. *Proc. Natl. Acad. Sci. USA* 94:11563–66
58. Koury MJ, Bondurant MC. 1992. The molecular mechanism of erythropoietin action. *Eur. J. Biochem.* 210:649–63
59. Evans RW. 1991. Recombinant human erythropoietin and the quality of life of end-stage renal disease patients: a comparative analysis. *Am. J. Kidney Dis.* 18:62–70
60. Naffakh N, Danos O. 1996. Gene transfer for erythropoiesis enhancement. *Mol. Med. Today* 2:343–48
61. Hamamori Y, Samal B, Tian J, Kedes L. 1994. Persistent erythropoiesis by myoblast transfer of erythropoietin cDNA. *Hum. Gene Ther.* 5:1349–56
62. Hamamori Y, Samal B, Tian J, Kedes L. 1995. Myoblast transfer of human erythropoietin gene in a mouse model of renal failure. *J. Clin. Invest.* 95:1808–13
63. Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N. 1989. Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science* 246:1306–9
64. Plouet J, Schilling J, Gospodarowicz D. 1989. Isolation and characterization of a newly identified endothelial cell mitogen produced by AtT-20 cells. *EMBO J.* 8:3801–6
65. Conn G, Soderman DD, Schaeffer MT, Wile M, Hatcher VB, et al. 1990. Purification of a glycoprotein vascular endothelial cell mitogen from a rat glioma-derived cell line. *Proc. Natl. Acad. Sci. USA* 87:1323–27
66. Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, et al. 1983. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science* 219:983–85
67. Ferrara N, Henzel WJ. 1989. Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. *Biochem. Biophys. Res. Commun.* 161:851–58
68. Hanahan D, Folkman J. 1996. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 86:353–64
69. Pardanaud L, Yassine F, Dieterlen-Lievre F. 1989. Relationship between vasculogenesis, angiogenesis and haemopoiesis during avian ontogeny. *Development* 105:473–85
70. Risau W, Flamme I. 1995. Vasculogenesis. *Annu. Rev. Cell Dev. Biol.* 11:73–91
71. Takeshita S, Pu LQ, Stein LA, Sniderman AD, Bunting S, et al. 1994. Intramuscular administration of vascular endothelial growth factor induces dose-dependent collateral artery augmentation in a rabbit model of chronic limb ischemia. *Circulation* 90:II228–34
72. Takeshita S, Zheng LP, Brogi E, Kearney M, Pu LQ, et al. 1994. Therapeutic angiogenesis. A single intraarterial bolus of vascular endothelial growth factor augments revascularization in a rabbit ischemic hind limb model. *J. Clin. Invest.* 93:662–70
73. Bauters C, Asahara T, Zheng LP, Takeshita S, Bunting S, et al. 1995. Site-specific therapeutic angiogenesis after systemic administration of vascular endothelial growth factor. *J. Vasc. Surg.* 21:314–24
74. Hariawala MD, Horowitz JR, Esakof D, Sheriff DD, Walter DH, et al. 1996. VEGF improves myocardial blood flow

but produces EDRF-mediated hypotension in porcine hearts. *J. Surg. Res.* 63:77–82
75. Horowitz JR, Rivard A, van der Zee R, Hariawala M, Sheriff DD, et al. 1997. Vascular endothelial growth factor/vascular permeability factor produces nitric oxide-dependent hypotension. Evidence for a maintenance role in quiescent adult endothelium. *Arterioscler. Thromb. Vasc. Biol.* 17:2793–800
76. Mesri EA, Federoff HJ, Brownlee M. 1995. Expression of vascular endothelial growth factor from a defective herpes simplex virus type 1 amplicon vector induces angiogenesis in mice. *Circ. Res.* 76:161–67
77. Muhlhauser J, Merrill MJ, Pili R, Maeda H, Bacic M, et al. 1995. VEGF165 expressed by a replication-deficient recombinant adenovirus vector induces angiogenesis in vivo. *Circ. Res.* 77: 1077–86
78. Magovern CJ, Mack CA, Zhang J, Rosengart TK, Isom OW, et al. 1997. Regional angiogenesis induced in non-ischemic tissue by an adenoviral vector expressing vascular endothelial growth factor. *Hum. Gene Ther.* 8:215–27
79. Isner JM, Pieczek A, Schainfeld R, Blair R, Haley L, et al. 1996. Clinical evidence of angiogenesis after arterial gene transfer of phVEGF165 in patient with ischaemic limb. *Lancet* 348:370–74
80. Takeshita S, Weir L, Chen D, Zheng LP, Riessen R, et al. 1996. Therapeutic angiogenesis following arterial gene transfer of vascular endothelial growth factor in a rabbit model of hindlimb ischemia. *Biochem. Biophys. Res. Commun.* 227:628–35
81. Tsurumi Y, Takeshita S, Chen D, Kearney M, Rossow ST, et al. 1996. Direct intramuscular gene transfer of naked DNA encoding vascular endothelial growth factor augments collateral development and tissue perfusion. *Circulation* 94:3281–90
82. Mack CA, Patel SR, Schwarz EA, Zanzonico P, Hahn RT, et al. 1998. Biologic bypass with the use of adenovirus-mediated gene transfer of the complementary deoxyribonucleic acid for vascular endothelial growth factor 121 improves myocardial perfusion and function in the ischemic porcine heart. *J. Thorac. Cardiovasc. Surg.* 115:168–76
83. Springer ML, Chen AS, Kraft PE, Bednarski M, Blau HM. 1998. VEGF gene delivery to muscle: potential role for vasculogenesis in adults. *Mol. Cell* 2:549–58
84. Gimbrone MA Jr, Leapman SB, Cotran RS, Folkman J. 1972. Tumor dormancy in vivo by prevention of neovascularization. *J. Exp. Med.* 136:261–76
85. Brem S, Brem H, Folkman J, Finkelstein D, Patz A. 1976. Prolonged tumor dormancy by prevention of neovascularization in the vitreous. *Cancer Res.* 36:2807–12
86. O'Reilly MS, Holmgren L, Shing Y, Chen C, Rosenthal RA, et al. 1994. Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. *Cell* 79:315–28
87. O'Reilly MS, Boehm T, Shing Y, Fukai N, Vasios G, et al. 1997. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell* 88:277–85
88. Blezinger P, Wang J, Gondo M, Quezada A, Mehrens D, et al. 1999. Systemic inhibition of tumor growth and tumor metastases by intramuscular administration of the endostatin gene. *Nat. Biotechnol.* 17:343–48
89. Rossi FM, Blau HM. 1998. Recent advances in inducible gene expression systems. *Curr. Opin. Biotechnol.* 9:451–56
90. Harvey DM, Caskey CT. 1998. Inducible control of gene expression: prospects for gene therapy. *Curr. Opin. Chem. Biol.* 2:512–18
91. Gossen M, Bujard H. 1992. Tight control

of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc. Natl. Acad. Sci. USA* 89:5547–51
92. Gossen M, Freundlieb S, Bender G, Muller G, Hillen W, et al. 1995. Transcriptional activation by tetracyclines in mammalian cells. *Science* 268:1766–69
93. Mayford M, Bach ME, Huang YY, Wang L, Hawkins RD, et al. 1996. Control of memory formation through regulated expression of a CaMKII transgene. *Science* 274:1678–83
94. Kistner A, Gossen M, Zimmermann F, Jerecic J, Ullmer C, et al. 1996. Doxycycline-mediated quantitative and tissue-specific control of gene expression in transgenic mice. *Proc. Natl. Acad. Sci. USA* 93:10933–38
95. Bello B, Resendez-Perez D, Gehring WJ. 1998. Spatial and temporal targeting of gene expression in Drosophila by means of a tetracycline-dependent transactivator system. *Development* 125:2193–202
96. Kringstein AM, Rossi FM, Hofmann A, Blau HM. 1998. Graded transcriptional response to different concentrations of a single transactivator. *Proc. Natl. Acad. Sci. USA* 95:13670–75
97. Deuschle U, Meyer WK, Thiesen HJ. 1995. Tetracycline-reversible silencing of eukaryotic promoters. *Mol. Cell. Biol.* 15:1907–14
98. Blau HM, Rossi FM. 1999. Tet B or not tet B: advances in tetracycline-inducible gene expression. *Proc. Natl. Acad. Sci. USA* 96:797–99
99. Hillen W, Berens C. 1994. Mechanisms underlying expression of Tn10 encoded tetracycline resistance. *Annu. Rev. Microbiol.* 48:345–69
100. Kisker C, Hinrichs W, Tovar K, Hillen W, Saenger W. 1995. The complex formed between Tet repressor and tetracycline-Mg2 + reveals mechanism of antibiotic resistance. *J. Mol. Biol.* 247:260–80
101. Schnappinger D, Schubert P, Pfleiderer K, Hillen W. 1998. Determinants of protein-protein recognition by four helix bundles: changing the dimerization specificity of Tet repressor. *EMBO J.* 17:535–43
102. Rossi FM, Guicherit OM, Spicher A, Kringstein AM, Fatyol K, et al. 1998. Tetracycline-regulatable factors with distinct dimerization domains allow reversible growth inhibition by p16. *Nat. Genet.* 20:389–93
103. Baron U, Schnappinger D, Helbl V, Gossen M, Hillen W, et al. 1999. Generation of conditional mutants in higher eukaryotes by switching between the expression of two genes. *Proc. Natl. Acad. Sci. USA* 96:1013–18
104. Garrick D, Fiering S, Martin DI, Whitelaw E. 1998. Repeat-induced gene silencing in mammals. *Nat. Genet.* 18:56–59
105. Hofmann A, Nolan GP, Blau HM. 1996. Rapid retroviral delivery of tetracycline-inducible genes in a single autoregulatory cassette. *Proc. Natl. Acad. Sci. USA* 93:5185–90
106. Hwang JJ, Scuric Z, Anderson WF. 1996. Novel retroviral vector transferring a suicide gene and a selectable marker gene with enhanced gene expression by using a tetracycline-responsive expression system. *J. Virol.* 70:8138–41
107. Lindemann D, Patriquin E, Feng S, Mulligan RC. 1997. Versatile retrovirus vector systems for regulated gene expression in vitro and in vivo. *Mol. Med.* 3:466–76
108. Paulus W, Baur I, Boyce FM, Breakefield XO, Reeves SA. 1996. Self-contained, tetracycline-regulated retroviral vector system for gene delivery to mammalian cells. *J. Virol.* 70:62–67
109. Hoshimaru M, Ray J, Sah DW, Gage FH. 1996. Differentiation of the immortalized adult neuronal progenitor cell line HC2S2 into neurons by regulatable suppression of the v-myc oncogene. *Proc. Natl. Acad. Sci. USA* 93:1518–23
110. Sah DW, Ray J, Gage FH. 1997. Bipotent progenitor cell lines from the human CNS. *Nat. Biotechnol.* 15:574–80

111. Bohl D, Naffakh N, Heard JM. 1997. Long-term control of erythropoietin secretion by doxycycline in mice transplanted with engineered primary myoblasts. *Nat. Med.* 3:299–305
112. Rando TA, Pavlath GK, Blau HM. 1995. The fate of myoblasts following transplantation into mature muscle. *Exp. Cell Res.* 220:383–89
113. Ferrari G, Cusella-De Angelis G, Coletta M, Paolucci E, Stornaiuolo A, et al. 1998. Muscle regeneration by bone marrow-derived myogenic progenitors. *Science* 279:1528–30
114. Neumeyer AM, DiGregorio DM, Brown RH Jr. 1992. Arterial delivery of myoblasts to skeletal muscle. *Neurology* 42:2258–62
115. Yoshida N, Yoshida S, Koishi K, Masuda K, Nabeshima Y. 1998. Cell heterogeneity upon myogenic differentiation: down-regulation of MyoD and Myf-5 generates 'reserve cells.' *J. Cell Sci.* 111:769–79
116. Baroffio A, Hamann M, Bernheim L, Bochaton-Piallat ML, Gabbiani G, et al. 1996. Identification of self-renewing myoblasts in the progeny of single human muscle satellite cells. *Differentiation* 60:47–57
117. Chang PL. 1996. Microencapsulation—an alternative approach to gene therapy. *Transfus. Sci.* 17:35–43
118. al-Hendy A, Hortelano G, Tannenbaum GS, Chang PL. 1995. Correction of the growth defect in dwarf mice with non-autologous microencapsulated myoblasts—an alternate approach to somatic gene therapy. *Hum. Gene Ther.* 6:165–75
119. Hortelano G, al-Hendy A, Ofosu FA, Chang PL. 1996. Delivery of human factor IX in mice by encapsulated recombinant myoblasts: a novel approach towards allogeneic gene therapy of hemophilia B. *Blood* 87:5095–103