Studies of myoblasts have contributed to our understanding of pattern formation in muscle. In this review we describe such studies and how they suggested that genetically engineered myoblasts could be used as vehicles for the delivery of genes in vivo, or gene therapy.

Control of pattern formation
Extrinsic and intrinsic regulation

The development of pattern in tissues depends on an interplay of cell-autonomous behavior (lineage) and cell-cell interactions (environment). To establish that a behavior is autonomous, the intrinsic properties of individual cells can be studied after ablation of their neighbors in vivo or in isolated clones in culture. To understand the origin of cell-autonomous behavior, and the development of cell memory, it is necessary to establish cell lineage by marking cells in vivo. A key question in development is the extent to which intrinsic programs are modulated by extrinsic factors, such as cell-cell interactions or diffusible signals (reviewed in Ref. 1).

In invertebrates, there are examples in which differentiation is primarily controlled by cell-autonomous behavior and others in which cell-cell interactions predominate. In Caenorhabditis elegans, the differentiation of sex myoblasts provides an example of lineage control2. These cells specialize early before reaching their final position, and once relocated express their phenotype irrespective of their neighbors. Thus, pattern results from directed migration and selective adhesion of cells committed to a particular fate. By contrast, in Drosophila, a clear example of environmental control is provided by cells of the ommatidia3. These cells specialize late, remaining plastic until interaction with their immediate neighbors dictates their expression of a particular phenotype.

Until recently, it has been difficult to address questions regarding pattern development in mammals. However, genetic marking of cells in situ using replication-incompetent retroviral vectors now allows the fate of individual cells and their progeny to be monitored4. This method has revealed that in the central nervous system both cell-autonomous behavior and cell-cell interactions establish pattern. Lineage determines the fate of the precursors of neurons and glia5,6, whereas environmental cues control terminal differentiation of retinal neuroblasts7.

Pattern formation in muscle

Mammalian skeletal muscle tissue is composed of a complex pattern of fibers, multinucleated cells that grow by the fusion of mononucleated myoblasts. Fibers differ in their rate of contraction, a property that is in part determined by the complement of myosin heavy chain (MyHC) isoforms expressed; fast and slow MyHCs have high and low ATPase activities, respectively8. In general, slow fiber types are responsible for holding posture, whereas fast fiber types perform movements. Although both fast and slow fiber types occur in all muscles, the ratio of the two classes differs dramatically among muscles, and even among regions of a single muscle. Within any given region, fibers may be mixed in a salt-and-pepper fashion.

Myoblasts in pattern formation and gene therapy

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The tissues of a multicellular animal are composed of diverse cell types arranged in a precisely organized pattern. Features unique to muscle allow an analysis of pattern formation and maintenance in mammals. The progeny of single cells can be taken full cycle from the animal to the culture dish and back to the animal where they fuse into mature myofibers of the host. These features not only facilitate the use of genetically engineered myoblasts in studies of pattern formation, but also in cell-mediated gene therapy: a novel mode of drug delivery for the treatment of muscle and nonmuscle diseases such as hemophilia, cardiac disease and cancer.

Superimposed on this basic pattern are gradients of fast to slow muscle fibers, which can be entirely opposite in neighboring muscles9 (Fig. 1).

![Figure 1](image_url)
Muscle is particularly amenable to an analysis of patterning. Muscle precursor cells, or myoblasts, can be labeled in the animal with retroviral vectors, isolated from muscle tissue, grown to large numbers in tissue culture, characterized as clones or as bulk cultures, genetically engineered, and returned to the muscle of the animal. As a result, differentiation properties intrinsic to myoblasts can be monitored under controlled conditions in tissue culture. After transplantation into the animal, the differentiation of the same well-characterized cell population can be assessed in response to extrinsic signals encountered in vivo. These features make it possible to address questions regarding the generation and maintenance of pattern in muscle.

A mechanism based on lineage could lead to fiber-type diversity, if diversity derives from the distribution of distinct populations of myoblasts that differ intrinsically. Studies of myoblast clones in vitro\textsuperscript{10-12} and of myoblasts transplanted to sites of extensive damage in vivo\textsuperscript{13} have previously suggested that heterogeneity of muscle precursors is responsible for muscle fiber pattern. However, in both of these types of experiment the native environment was abolished and fibers formed de novo. In more recent experiments, avian myoblasts characterized in vitro and then transplanted into embryonic limb muscle expressed myosins typical of their muscle of origin, not their muscle of residence\textsuperscript{14}. These findings suggest that properties

\textbf{FIG1}

A single clone of myoblasts that contributes progeny to three fiber types. A clone generated by infection of a single myoblast in situ with MMuLV SVNLacZ at postnatal day 15 and analysed at day 37. Three fibers (indicated by arrows X, Y and Z) in the deep lateral gastrocnemius have received cells from the clone and contain β-gal-positive nuclei (A). Nearby unfixed frozen sections in the same region, shown schematically in (C), were histochemically stained for both β-gal activity and reactivity with antibodies 4A.840 (B), 4A.1519 (D), N3.36 (E) or 4A.74 (F). Each fiber has a distinct MyHC phenotype. Fiber X is a type A fast fiber (only N3.36\textsuperscript{+}), fiber Y is a type B fiber (N3.36, 4A.74\textsuperscript{+}, and 4A.1519\textsuperscript{+}) and fiber Z is a type C fiber (4A.840\textsuperscript{+}). The scale bar in (C) represents 25 μm. Reproduced, with permission, from Ref. 18.
intrinsic to muscle may play a role in the specification of the pattern of fiber types during development.

Other studies suggest that extrinsic factors are critical in determining the pattern of muscle fiber types in embryonic development. In these experiments the relative roles of intrinsic and extrinsic controls were examined during the period of early development of human limb muscle when multiple fiber types are first forming. The proportion of fibers that expressed slow myosin was found to change markedly. During the first trimester, a maximum of 75% of fibers expressed slow MyHC, dropping to 3% at mid-gestation, and increasing by week 30 of gestation to 50%, a proportion that persisted through adulthood. To determine whether these changes were attributable to inherent differences in muscle fiber precursors, myoblast clones derived from muscle at the different stages of human development were analysed in culture. The results were striking: virtually all myoblasts, irrespective of their stage of development, gave rise to clones that expressed slow MyHC upon differentiation in culture. This was true even for myoblasts from muscle at mid-gestation, a time when only 3% of fibers expressed slow MyHC in vivo. These results suggest that culture conditions are permissive for an inherent ability of myoblasts from all developmental stages to express slow MyHC. By contrast, expression in vivo appears to be suppressed by factors extrinsic to muscle.

Another set of experiments showed that extrinsic controls are predominant in determining the pattern of fiber types at postnatal stages of development. Retroviral marking of endogenous myoblasts allowed an analysis in intact rat tissues of whether different fiber types derive from the fusion of distinct myoblast lineages. Replication-defective vectors encoding either nuclear or cytoplasmic LacZ were mixed and directly injected into muscles of postnatal rats to monitor the fate of satellite cells, mononucleate muscle precursors. Satellite cells have long been thought of as being restricted in their movement and as contributing only to the fibers to which they are juxtaposed, because they are ensheathed together with a single muscle fiber by a basal lamina. Contrary to expectation, the majority of ß-galactosidase-positive clones gave rise to clusters of multiply labeled fibers. In most cases these clusters had either cytoplasmic or nuclear labeling but not both, indicating that they were clones derived from the progeny of single labeled myoblasts. These studies indicate that the basal lamina does not constitute a barrier to myoblast migration and that migration of myoblasts from one muscle fiber to another is a frequent event in the course of normal postnatal development in rodents.

Retroviral marking has also shown that satellite myoblasts do not migrate to a subpopulation of fiber types, but fuse randomly with all fiber types they encounter (Fig. 2). The fiber types to which ß-galactosidase-positive clones contributed were identified in situ using a panel of monoclonal antibodies. To rule out the possibility that satellite myoblasts could fuse only with fiber types found in their immediate environment, cloned primary myoblasts were labeled with retroviral vectors in tissue culture and then returned to various muscles of the animal by injection. Again, the cells showed no preference: they fused with the entire spectrum of fiber types. Furthermore, these myoblasts, which could be identified by a relatively high localized concentration of ß-galactosidase, adopted the pattern of myogenic gene expression characteristic of the host muscle fiber they entered. These findings suggest that extrinsic signals (the environment within the multinucleate heterokaryon fiber to which the myoblast fuses) override the intrinsic commitment of myoblast nuclei to particular patterns of gene expression. Taken together, these results indicate that at late stages of muscle development in the postnatal rodent, muscle fiber pattern is not dictated by myoblast lineage.

In addition to the environment within the multinucleate fiber, other extrinsic factors such as innervation have an impact on the expression of muscle fiber type. Innervation has long been known to play a role in fiber-type determination, but it has not always been clear whether this role is permissive or instructive. Neurons are unlikely to be involved in establishing the initial pattern, since this can form in their absence. There is strong evidence from experiments in rodent and avian muscle that early primary fiber-type determination is not neuronally controlled. However, innervation clearly has an impact on fiber types once formed. In early development, innervation is thought to be responsible for regulating slow MyHC expression in secondary fibers. Postnatally, fast and slow fiber types can be interconverted to some extent simply by surgical alteration of innervation, such as innervation of fast muscle fibers by slow motorneurons. Thus extrinsic influences, such as innervation, can modulate muscle fiber type, even in adulthood.

In summary, although some experiments suggest that lineage and myoblast-intrinsic properties may play a role in muscle fiber pattern, the environment is clearly a major controlling influence both in the generation and maintenance of that pattern.

**Myoblasts: vehicles for gene therapy**

A novel approach to drug delivery in the treatment of disease involves using cells to introduce into the body genes that express therapeutic proteins continuously. Myoblasts appear to be well suited to this purpose because of their unusual biological properties. As shown in the studies of patterning above, in contrast with other cell types, myoblasts become an integral part of the muscle into which they are injected. As a result, myoblasts are currently being tested as cellular vehicles for delivery of several different endogenous and recombinant gene products for the treatment of both muscle and nonmuscle disorders.

**Isolation, purification and growth of primary human myoblasts**

A decade of research has revealed several features of human muscle that make it attractive for use in cell-mediated gene therapy. Human primary myoblasts can be readily isolated from biopsy or autopsy material, enriched to 99% (Ref. 31; Fig. 3), grown to large numbers (10^12-10^18 cells per original isolated cell...
Clinical trial of myoblast therapy in DMD patients

Duchenne muscular dystrophy (DMD) is characterized by a lack of dystrophin: this leads to progressive muscle degeneration and death around the second decade of life. One potential therapy for this disease is the restoration of dystrophin expression by myoblast transplantation. Although it may not be possible to cure this particular disorder by myoblast transplantation, since skeletal, heart and diaphragm muscles are affected, it may be possible to improve the patients' quality of life. In a recent phase I clinical trial, normal myoblasts from a father or an unaffected sibling were transplanted into the muscle of eight boys who had DMD, and the cells assessed for production of dystrophin. No deleterious effects were observed, a finding that has been confirmed in three other independent phase I clinical trials (G. Karpati, J. Mendel and J. Tremblay, unpublished). In addition, three patients who had deletions in the dystrophin gene expressed normal dystrophin transcripts in muscle biopsy specimens taken from the transplant site one month after myoblast injection, and in one case six months after the transplant. The polymerase chain reaction (PCR) was used to determine that the dystrophin in these biopsies derived from donor myoblast DNA, not from genetic reversions in muscle cells of the host. Such reversions, which appear to result from second mutations within the very large dystrophin gene, could restore the translational reading frame and lead to production of an aberrant truncated protein that is indistinguishable from the normal protein by immunofluorescence using the antibodies that are currently available.

Although detectable, the expression of dystrophin in human muscle in our clinical trial was surprisingly low: this was in contrast to the levels of expression seen when myoblasts were introduced into the mdx mouse (Refs 36, 37; G.K. Pavlath and H.M. Blau, unpublished). In these studies mouse and human myoblasts fused extensively with the muscles of the mdx mouse (which, like individuals with DMD, carries a mutation in the dystrophin gene). Another clinical trial has reported a higher efficiency of myoblast transfer. The basis of the differences between the two trials and the low efficiency of myoblast transfer in our DMD patients is not yet known. The infrequent persistence of injected myoblasts could reflect the biology of the human disease, differences between mouse and human, or technical aspects of delivering cells into human muscles. To distinguish among these possibilities, it will be necessary to mark and track injected myogenic cells in humans to assess their viability and contribution to muscle fibers. Furthermore, myoblast transfer must be tested in human muscle that does not suffer from infiltration by cells from adipose and connective tissue, a characteristic typical of DMD. The extensive fibrosis in DMD muscles, which increases with patient age, is likely to inhibit access of myoblasts to myofibers. Indeed, the three patients in which dystrophin was detected were among the youngest in our trial. Finally, a better understanding of the biology of the mdx mouse is required, since although it has the same genetic defect as the DMD patient, it does not die from the disease but has only transient muscle degeneration and in adulthood is as strong as a wild-type mouse.

Cell-mediated gene therapy

The use of cells to introduce into the body genes encoding recombinant proteins that enter the circulation may provide a method of drug delivery for the treatment of a wide range of diseases. This approach involves genetically engineering cells in tissue culture to produce the therapeutic protein. Engineered cells are then extensively characterized in vitro before injection in vivo into animal models to ensure stable high-level expression of the introduced gene and secretion of the recombinant product.

A number of different cell types, including fibroblasts, hepatocytes, lymphocytes, endothelial cells...
and myoblasts, have been tested as vehicles for the delivery of recombinant proteins to the circulation. Long-term stable secretion has previously been reported with fibroblasts and keratinocytes. However, the cells used were transformed and gave rise to tumors. In most other studies, the recombinant gene product was either undetectable in serum, or levels of the product rapidly dropped. In some cases, the reason for the lack of circulating protein was determined: extinction of vector expression, antibody degradation of the protein or failure of the protein to gain access to the circulation. In other cases, the decrease in serum levels of the secreted gene product was unexplained and the fate of the transplanted cells unknown (reviewed in Ref. 28). Nonetheless, these studies were a milestone in the development of cell-mediated gene therapy, since both novel vectors for gene delivery and methods for engineering cells were developed.

**Myoblasts as vehicles for systemic delivery of recombinant proteins**

Recent studies in mice suggested that myoblasts may be a particularly advantageous cell type for the systemic delivery of recombinant proteins. This appears to be because of their unusual ability to fuse randomly with all fibers in their vicinity and become an integral part of muscle tissue that is innervated and has access to the circulation. In addition, in contrast with other cell types, myoblasts of the C2C12 line and primary myoblasts genetically engineered in vitro and injected in vivo express retroviral vectors for at least six weeks (Ref. 48; G.K. Pavlath, T.A. Rando and H.M. Blau, unpublished). A recombinant gene encoding human growth hormone (hGH) was stably introduced into cultured myoblasts of the C2C12 line either by transfection or infection with a retroviral vector. hGH was chosen for study because it has a very short half-life in mouse serum (4 min), providing a stringent test for continuous production and access to the circulation over time. In addition, sensitive assays distinguish the mouse and human hormones. After injection of genetically engineered myoblasts into mouse muscle, hGH was detected in serum for three months. Although after several months injected cells of the established C2C12 line give rise to tumors, we have not seen evidence that transplanted primary human cells are tumorigenic in mice (G.K. Pavlath and H.M. Blau, unpublished). The fate of injected myoblasts was determined by infecting the same cells with a retroviral vector encoding β-galactosidase, allowing an assessment of cell location and number. In addition to small fibers formed by fusion of implanted cells with one another, labeled cells were found in large diameter fibers typical of the surrounding tissue, suggesting that implanted myoblasts fused with the fibers of the host. Approximately $4 \times 10^6$ myoblasts could produce and secrete hGH at a steady-state serum level of 1 ng ml$^{-1}$, a level that is within the physiological range for mice (Fig. 4). Recent studies have confirmed and extended these findings using genetically engineered primary myoblasts isolated directly from mouse muscle tissue and another protein, Factor IX, which is missing in individuals with hemophilia B (Refs 49–51). In these experiments, stable production and secretion were observed over a six-month period.

**Conclusions and prospects**

Gene therapy has been hailed as the ultimate medicine. This mode of drug delivery bypasses the large-scale production of pure proteins for injection and enlists the body’s own cells for both production and delivery. Fundamental studies of muscle biology revealed that myoblasts might be useful for this purpose. The ability to isolate and characterize myoblasts in vitro, and reimplant them in vivo into the muscle of mice and humans has made a valuable contribution to our understanding of the complex processes underlying patterning, growth and repair in normal and dystrophic muscle. These properties now appear advantageous in myoblast-mediated gene delivery for the treatment of disease. Candidates for delivery in the near future include genes that encode hormones, coagulation factors, growth factors and antitumor agents, and that have broad applications ranging from the treatment of inherited hormone deficiencies to symptoms of ageing, hemophilia, Parkinson’s disease, heart disease and cancer. Current cell-mediated gene therapy involves constitutive expression of a recombinant gene product. The challenge of the next decade will be to test and further refine myoblast-mediated gene delivery by developing vectors that can be regulated and can be induced when needed, allowing the treatment of a wider range of inherited and acquired diseases.

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**Figure 1**

Persistent expression of hGH by virus-transduced myoblasts implanted in mouse muscle in vivo. A pool of transduced myoblasts was implanted in o 24 strain C3H mice and serum hGH levels were monitored for 85 days by radioimmunoassay of blood taken from the tail. More than 90% of the cells expressed and secreted hGH and 30% expressed β-gal, as determined by clonal analysis in vitro. Each point represents the mean ± SD for 4–24 mice. The dashed line is the mean ± SD (0.08 ± 0.08 ng ml$^{-1}$) for serum samples from five un.injected control mice. Reproduced, with permission, from Ref. 48.
The chromosome abnormality database (CAD) is a centralized repository of records of human chromosomal abnormalities and is available for researchers free of charge. Established in early 1991, the CAD now contains nearly 40,000 records of acquired and constitutional chromosomal abnormalities contributed by over 40 cytogenetics laboratories throughout the UK. Although there is a considerable bias towards recent cases, the data stretch back some 20 years and include information on the availability of cell lines and other stored material.

To date, the CAD has received well over 100 inquiries, but this represents only a fraction of the potential level of use. Searches of the database may be performed free of charge by contacting the database manager, Dr Mercer, or directly by the researcher through the computing facilities maintained by the Human Genome Mapping Program.

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