GENE THERAPY

Muscle transfection made easy

Terence A. Partridge

AT first sight, skeletal muscle is not an obvious tissue on which to attempt gene therapy — it is highly organized and stable, and the nuclei of mature muscle fibres do not undergo division, thus giving no opportunity for genomic integration of exogenous DNA. Last year, however, to general surprise, it was found that simple injection of circular plasmid DNA containing a reporter gene led to a long-term expression of that gene within a low proportion of fibres[1].

This same procedure has now been applied to insertion of the very large gene which is defective in Duchenne muscular dystrophy[2] into skeletal muscle of the mdx mouse, a genetic equivalent of Duchenne muscular dystrophy. On page 815 of this issue[3], Acsadi *et al.* report that injection of plasmids containing the 12-kilobase or 6.3-kilobase complementary DNAs coding for competent versions of the gene leads to expression of its protein product, dystrophin, in about 1 per cent of muscle fibre profiles. Yet more surprising is the longevity of this effect. Although the study reports on dystrophin expression for only a week after injection of the plasmid, the earlier work demonstrated stable expression of reporter genes for several months, apparently from plasmids which remain circular and episomal (independent of the chromosome) in the non-replicative environment of the muscle fibre. John Wolff, the initiator of this work, says that expression still remains stable after one year (J. Wolff, personal communication).

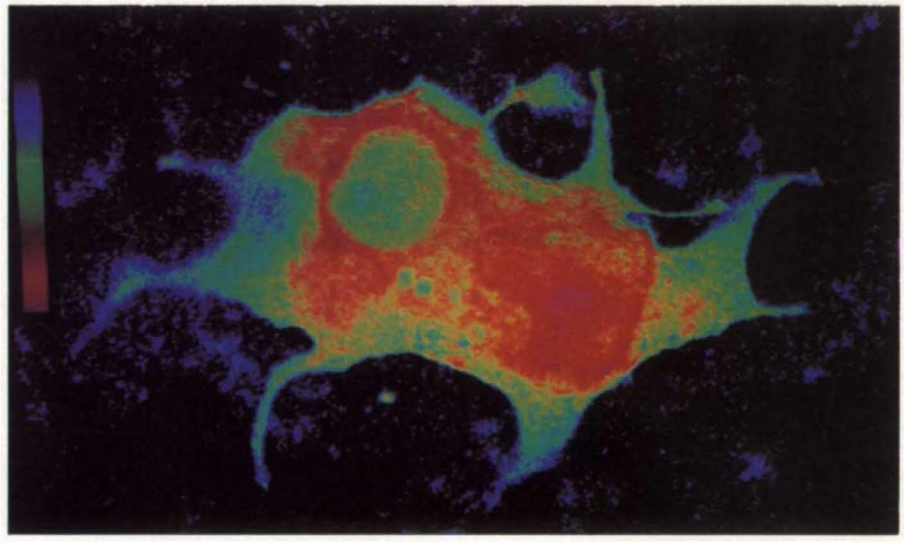
As it stands, the technique for inducing synthesis of dystrophin within muscle fibres is of great interest as a potential therapy for recessively inherited myopathies such as Duchenne muscular dystrophy. But such notions must be treated with reserve, pending a dramatic improvement in the proportion of muscle fibres which can be induced to synthesize dystrophin. The overall inefficiency is breathtaking — injection of 400 μg of dystrophin cDNA, equivalent to the total genomic dystrophin gene copies of several mice, leads to the synthesis of detectable dystrophin in only some 50 fibre profiles out of 5,000 in a single muscle. A therapeutic effect would require an increase of 10–50 fold in dystrophin-positive fibre profiles. This might be attained by increase in the efficiency of transfection or by 'gene-targeting'. Given such an improvement, the production of large amounts of plasmid DNA is a relatively trivial matter. The widely used replication-defective retroviral vectors are unable to accommodate the full length dystrophin gene (although they could carry the 6.3-kilobase 'minigene'[4]) but other viruses, such as adenoviruses, which are being tested for carrying the gene for α1-antitrypsin into cells lining the lung[5], may be of value. The possibility of encouraging uptake of genes into muscle fibres by way of specific cell-surface receptors is also worth examination. By whatever means the constructs are introduced into muscle fibres, it is to be hoped that the stability of expression of the directly transfected constructs would continue to apply.

It is encouraging that the same technique can be used to transfect the dystrophin gene into cardiomyocytes of the mdx heart, thus providing a potential treatment for the cardiomyopathy of Duchenne muscular dystrophy. In this respect, direct transfection is superior to the other main method being tested for introducing the dystrophin gene into dystrophic muscle, namely myoblast transplantation[6,7]. No means are currently known for inducing normal heart myocytes to proliferate. And even if we could inject large numbers of such cells into hearts, they would not be able to share their dystrophin with the existing heart cells because, unlike skeletal muscle precursors, heart myocytes do not fuse to form a syncytium.

In skeletal muscle, for the time being, injection of myoblasts obtained from normal mice into the muscles of mdx mice is far more effective than direct transfection at producing dystrophin-positive muscle fibres — injection of 100,000 cells gives up to 30–40 per cent dystrophin-positive fibres, spread over large areas[7]. These myoblasts also have the advantage of providing new sources of cells for formation of new normal muscle fibres and repair of dystrophic muscle fibre[8] within muscles where the endogenous repair processes are failing[9].

The question arises as to whether the dystrophin produced in muscle fibres as a result of direct gene transfection is functional. Much but not all of it is located in its normal position at the cell surface[10,11]. A high proportion of such surface-stained fibres appear to be normal, with the nucleus in a peripheral position, whereas most of the dystrophin-negative fibres carry their nuclei in a central position, indicating that they have probably degenerated and regenerated[8]. But until the yield of positive muscle fibres is increased, it will be difficult to determine whether this association is due to the protection of muscle fibres by dystrophin or to loss of dystrophin expression by fibres which had degenerated and regenerated during the seven days since transfection.

Irrespective of its therapeutic value, direct gene transfection into muscle fibres will surely become a preferred method for preliminary assessment of intramuscular expression of DNA constructs containing muscle-gene promoters; it is cheaper and faster than analysis in transgenic animals, and more relevant to normal physiology than expression in tissue culture. Transplantation of myo-



G. Acsadi

Vivid expression – a colour-enhanced confocal image of a transfected mouse 3T3 cell, showing the expression, in red, of human dystrophin.

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blasts transfected *in vitro* offers an intermediate option in terms of cost, time and yield of transfected tissue.

One of the more fascinating aspects of the phenomenon is the implication that the skeletal muscle fibre lacks a mechanism for eliminating these episomal DNA constructs. It seems strange that such a cosy ecological niche is not regularly exploited by DNA viruses as a lifelong home — or is it? □

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GLOBAL EXTINCTION

Acid rain at the K/T boundary

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THE events that ended the Cretaceous period and started the Tertiary, 66 million years ago, have been a source for much study and not a little wild speculation. Of particular interest has been how a large proportion of all species, most strikingly the dinosaurs, met their demise then. Russell[1] posed the question in a slightly whimsical manner when he envisaged the last dinosaur either crawling at its last gasp towards an ever-retreating sea or disappearing in a hurricane of iridium-laden dust and roasted dinosaur flesh. On a more serious note, models have been presented suggesting sudden global warming, something like a nuclear winter, and/or showers of toxic acid rain as agents of destruction. Either increased global volcanism or an extraterrestrial impact would be responsible for these extreme atmospheric conditions[2–4]. The emphasis here should be on the word models, as there has been a marked dearth of evidence in the geological record for any of these hypotheses. But Martin and Macdougall now suggest[5] that a large and sudden change in the strontium isotope ratio of seawater at the Cretaceous/Tertiary (K/T) boundary is evidence of rapid addition of radiogenic strontium to the oceans from the continents resulting from enhanced weathering by global, highly acidic rain.

The $^{87}Sr/^{86}Sr$ ratio of the oceans is largely controlled by the relative fluxes and isotope ratios from submarine hydrothermal activity ($^{87}Sr/^{86}Sr = 0.703$) and river inputs ($^{87}Sr/^{86}Sr = 0.712$). The ratio is recorded by microfossils that make up carbonate sediments, which reveal a gradual increase from 0.70755 80 million years (Myr) ago to 0.70785 just before the K/T boundary. At the boundary there is a sharp increase to 0.70793 followed by a slower fall to 0.7077 about 55 Myr ago. Fluctuations in the marine strontium isotope record as a result of variations in the magnitude and isotope ratios of hydrothermal and riverine fluxes are well documented in the Cenozoic (the past 66 Myr), but the rate of increase at the K/T boundary is exceptionally rapid and the shape of the spike suggests a rapid injection of radiogenic strontium to the oceans followed by a slower return to the background levels. What then are the possible causes of this spike?

Martin and Macdougall conclude that the most likely cause was a dramatic increase in weathering rates following generation of nitric acid rain by atmospheric reactions between water vapour and nitrous oxides. These reagents would have been produced by shock heating of the atmosphere by the impact of an extraterrestrial bolide. The highly acidic rain, as well as delivering radiogenic strontium to the oceans, released additional CO_2 during weathering, raising temperatures and prolonging the effects of the acid rain.

Direct addition of strontium to the oceans from the bolide or associated impact ejecta would not have provided sufficient radiogenic strontium to yield the observed spike. Martin and Macdougall also reject increased volcanic activity as the source of the additional strontium on the grounds that volcanism at the K/T boundary was dominated by formation of the Deccan basalts in southern India which have $^{87}Sr/^{86}Sr$ ratios similar to the hydrothermal input.

In contrast, Javoy and Courtillot[6] suggest that development of the Deccan traps resulted in extensive melting of the continental crust. This produced explosive acid volcanism that preceded eruption of the basalts and would have delivered radiogenic strontium to the oceans by their weathering and river transport or directly by dumping large volumes of ash in nearby seas. In addition, acid-forming gases released during intensive volcanic activity would also have accelerated the rate of continental weathering. Unfortunately, evidence of these proposed extensive acidic volcanics is thought either to be buried below the Deccan traps or to be weathered away. Of course this in itself does not disprove their hypothesis, but the lack of hard evidence is not particularly satisfying.

Part of the reason Javoy and Courtillot developed their hypothesis was concern in placing the strontium isotope spike right at the K/T boundary. Uncertainties in the age assignments of microfossils analysed for strontium isotopes arise because carbonate sediments are not conducive to direct radiometric dating. Instead age assignments are based on magnetostratigraphy, correlation of palaeontological zones and interpolated sedimentation rates. These techniques have an absolute accuracy of only 0.5–2 Myr for individual samples leading some to propose that the isotope anomaly actually occurred before the K/T boundary and was due to an increased riverine flux related to late-Cretaceous sea-level regression[7].

Nelson *et al.*[8] have also suggested that a rapid change in marine strontium isotope ratios occurred before the K/T boundary, although doubts have been raised about their stratigraphy and age assignments (J. A. McArthur, personal communication). The far more detailed study by Martin and Macdougall also reveals an increase in seawater $^{87}Sr/^{86}Sr$ ratios preceding the larger spike at the K/T boundary itself, but they ascribe this to normal fluctuations in the relative inputs from river and hydrothermal sources rather than to any special event. Even in this careful study, sceptics could argue, the combination of analytical errors in determining the strontium isotope ratio and imprecisions in the age assignments could mean that the sharp increase in $^{87}Sr/^{86}Sr$ ratios at the K/T boundary may actually be less intense and spread out over a longer period than suggested by the data, and simply represents part of the normal fluctuations established before the boundary.

Our present inability to obtain more accurate absolute ages for carbonate microfossils means that we will not be able to resolve fluctuations in the marine isotope record to a much finer degree than already achieved in these recent studies. Until this problem is solved it is unlikely that this record will provide conclusive evidence about events around the K/T boundary, but the attempt to address the problem in a novel manner and by collecting real data is a welcome alternative to the large number of arm-waving, computer-generated models that have characterized much of the debate so far. □

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