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Muscle Repair and Gene Therapy

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My research interest is in skeletal muscle. A classic picture of a body builder readily illustrates what normal skeletal muscle does: it is responsible for moving the various parts of the body, lifting weights, smiling, breathing, standing upright – a whole range of activities relating to the skeleton, posture and movement. The muscles lie just beneath the skin, they are organised into bundles and are attached at both ends to the bones or other parts of the body (Figure 1, Appendix 4, p. 129). Muscles come in many different shapes and sizes; some are long and thin, whereas others can be broad and flat. There are about 300 different kinds of skeletal muscles in the body and they make up about 50% of the entire weight. They are extremely long cells – they are much longer than the average cell in the body and that is because they are multi-nucleated, which I will tell you more about. Each muscle cell is filled up with well-organised contractile proteins and this is the machinery that does all the work – when they contract they shorten the length of the muscle (it gets fatter at the same time) and this moves the bones to make an arm lift up, etc. A splendid reference for detailed descriptions of all aspects of skeletal muscle biology are the books entitled Myology e.g. volume 1 in 1986 (see references at the end under Engel): this series was updated in 1994.

I will first outline research relating to the repair of skeletal muscle, and then discuss the situation relating to cell and gene therapy for muscle diseases.

Muscle repair

The appearance and structure of skeletal muscle cells

Figure 2, Appendix 4, p. 129 shows part of three muscle cells: they are very very long – sometimes up to 40 mm – and have many nuclei inside each cell. They also have a ‘striped’ appearance due to the highly organised arrangement of the contractile material inside them, so skeletal muscle is sometimes called striated muscle. These long muscle cells also have a nerve making a connection on to their surface as shown in Figure 2, Appendix 4, p. 129. It is only when you get an electrical signal coming down the nerve to stimulate this muscle that you actually get a contraction. Thus skeletal muscle is called voluntary muscle since you don’t just suddenly have your arm going up in the air without you telling it to, usually! You have to send a signal from the brain through the nerve, it sends the signal to the muscle, this releases calcium and causes the proteins to contract for striated voluntary muscle.

Most of the activities in our laboratory focus on factors controlling the regeneration of skeletal muscle (rather than the function). We are really interested in things that cause skeletal muscle to repair itself if it gets damaged. Our interest focuses on the way that muscle looks after itself in children and adults after birth, rather than the way it is initially formed in developing babies.
Figure 1
Function of skeletal muscle

Figure 2
Striated multinucleated skeletal muscle cells and nerves

Figure 3
How new muscle is formed
The scheme in Figure 3, Appendix 4, p. 129 shows the mono-nucleated muscle precursor cells which are call *myoblasts* (*myo* means muscle or flesh). The myoblasts look like many other mono-nucleated cells in the body: in the middle is a nucleus (which issues instructions) and this is surrounded by the cytoplasm (where all the business of the cell happens). The myoblasts can multiply to build up their numbers and then, in muscle – this is what makes skeletal muscle very unusual – they fuse together (like melting into each other) to form long thin multi-nucleated muscle cells called *myotubes*. A myotube with all the nuclei lying in the middle of it is also shown in Figure 3, Appendix 4, p. 129. The black striped lines represent the contractile material which gives rise to the movement of these muscle cells. As the myotube matures, it fills up with lots and lots of these contractile proteins and the nuclei get squashed out to the very edge of the cell – so that they are out of the way during contraction. The mature myotube is called a muscle fibre or *myofibre*.

These nuclei within the myofibre never contribute to new muscle formation – they have done their dash. The cells which are positioned just on the outside of the muscle fibre (bottom of Figure 3, Appendix 4, p. 129) are called *satellite cells* because of their geography. These satellite cells are normally ‘asleep’ but, if a muscle is damaged, they ‘wake up’, become active and multiply, and help form new muscle. I will dwell on these satellite cells in a moment.

When discussing skeletal muscle it is helpful to realise that a muscle fibre will look quite different when it is in cut in a longitudinal direction, compared with a cross-section. If you think of a bundle of muscle cells like a big bunch of spaghetti, then looking down at it along the length is called a longitudinal view (e.g. Figures 2–5, Appendix 4, pp. 129–130, Figures 7–9, Appendix 4, p. 131 and Figure 17, Appendix 4, p. 134). Such longitudinal sections readily show the contractile arrangements, the muscle nuclei within the muscle fibre, and the satellite cells lying outside the muscle fibre but beneath what is called a basal (or external) lamina (Figure 5, Appendix 4, p. 130). If you took this same long bundle (of spaghetti) and you actually pointed it towards you – imagine it coming directly out of the page at you – it would look like a bundle of many small tubes – this is called a cross or transverse view (e.g. Figures 11, 12, Appendix 4, p. 132).

If you cut a very thin slice (called a section) of muscle, stain it and then look down a microscope you can see the cells are greatly magnified – Figure 4, Appendix 4, p. 130 shows part of several very long muscle fibres cut in longitudinal section, stained with a routine stain called Haematoxylin & Eosin (H&E) and viewed with a light microscope (the asterisks are in the middle of 2 myofibres). You can see how enormous the multi-nucleated muscle cells are: all the contractile protein is in the middle and the (small dark) nuclei of these cells are squashed up right against the edge out of the way. It is very difficult to see whether these nuclei (e.g. arrow) belong to the muscle fibre itself or perhaps to other cells lying in between the muscle fibres, in what we call the *extracellular* (literally = outside the cell) space: I will be talking about this material between the cells later on. It used be considered that this extracellular material (or matrix) was rather boring and it was just a
Figure 4
View of real muscle cells (myofibres)

Figure 5
Satellite cell viewed with transmission electron microscope

Figure 6
Activated satellite cell (arrow) on myofibre (stained with desmin)
glue or cement in which cells sat, and only the cells were thought to be of real interest; however, recently the role of this material in between the cells has been attracting a lot of interest and it is actually very important (see later).

A similar section of muscle fibre (see part marked by an arrow in Figure 4, Appendix 4, p. 130) can be viewed under enormously high power with an electron microscope (Fig. 5, Appendix 4, p. 130) and shows a satellite cell on a muscle fibre. This very small part of the muscle fibre shows some of the highly organised contractile machinery (bottom half of the picture). It also shows several mitochondria (dark objects on the right-hand side); these are like power houses, and there are many of them in skeletal muscle to provide the huge amount of energy required for contraction. The satellite cell is what we are particularly interested in: one is clearly visible (in the middle) located outside the muscle fibre (small arrows indicate the boundary membranes of the muscle fibre and of the satellite cell) but lying beneath an outer layer (external lamina) made of extracellular matrix (long arrows).

Figure 6, Appendix 4, p. 130 is an image from our laboratory showing a muscle fibre stained with an antibody which detects a protein called desmin that is made only in muscle: under the light microscope this stains as a distinctive brown colour (shown as dark gray here). You can see how nicely the desmin stain shows up the striped banding pattern in the striated muscle fibre; it also shows a satellite cell that has become activated and is producing the desmin protein in the cytoplasm (arrow).

Figure 7, Appendix 4, p. 131 is a favourite picture of mine and it is included to illustrate different ways of viewing the tissue. This is viewed with a scanning electron microscope and is looking down on the (longitudinal) surface of the muscle fibre. You can see at very high magnification the lovely striations and a satellite cell lying in a groove on the surface of the muscle fibre. This shows how the satellite cell is really outside the muscle fibre but tucked in very nicely onto the surface of it.

**The repair of muscle in response to damage**

Figure 8, Appendix 4, p. 131 outlines some of the main events in muscle repair. It shows the basic diagram of a muscle fibre in longitudinal section, with the muscle nuclei inside and the satellite cells lying outside as we have discussed. What happens if you injure this, what happens if you get a cut with a knife or get a good tick on the footy ground or have a car accident, what happens if you rupture a muscle cell?

**Sealing off the damaged area:** The first thing the muscle does is to try and rapidly seal off the area of damage. It actually makes new cell membrane in both ends of the damaged or ruptured muscle fibre. This happens very quickly — within to 8 hours — and by 12 hours it is completely sealed so that the injured part is the only area that has to be fixed and the rest of the muscle fibre is ‘safe’.

**Invasion of inflammatory cells:** During this time large numbers of inflammatory cells start to come into this area and there are many of them by 24
Figure 7
Satellite cell(s) on surface of myofibre (m) viewed by scanning electron microscope

Figure 8
Diagram of muscle regeneration

Figure 9
Appearance of regenerating skeletal muscles
hours after the injury. You may have heard about some inflammatory cells as professional cells that eat up tissue debris and therefore, are called macrophages, which literally means 'big eaters': when you have a wound where you see pus, this is a result of many macrophages removing the dead or damaged tissue.

**New muscle formation:** At the same time as getting rid of this dead tissue, the satellite cells wake up, they divide like crazy and then fuse to form myotubes, these little myotubes grow and then fuse to the ends of the re-sealed muscle fibres to bridge the gap and repair the damaged zone.

We are interested in all aspects of this process: how quickly the damaged muscle can re-seal, the role of inflammatory cells, the factors that affect the proliferation of the satellite cells/myoblasts and the factors that affect the fusion of these cells (reviewed in Grounds 1991; Cullen 1998). We are also interested in trying to find out if you can limit the amount of damage, or make muscle fibres more resistant to injury.

Another histological picture (Figure 9, Appendix 4, p. 131) shows the appearance of regenerating muscle tissue under the light microscope, again in longitudinal section and stained with H&E. Healthy looking muscle is present (compare with Figure 4, Appendix 4, p. 130) along with an area that has been damaged and is starting to repair itself, with several long myotubes. You can see many nuclei lined up in the middle of the myotubes – these cells will grow and mature to become as 'fat' as the original muscle fibres. There is a mixture of myotubes and mononuclear cells all together at this stage. It is actually very difficult to identify some of the different mononuclear cells in such regenerating tissue.

**Whole muscle graft model of regeneration:** A model that we have found very useful in our regeneration studies is the transplantation of very small whole muscles (Figure 10, Appendix 4, p. 132). When you take out a muscle, in this case the extensor digitorum longus muscle from the leg of a mouse, you completely cut the blood and the nerve supply to that muscle. What happens is that the original muscle will die and it will be re-formed completely by a similar process to that described above: blood vessels must grow in from the underlying host muscle, then inflammatory cells come in to eat up all the dead tissue, myoblasts proliferate and fuse to form new muscle (Roberts and McGeachie 1990; reviewed in Roberts and McGeachie 1995; Grounds and McGeachie 1998).

If we take out one of these muscle grafts several days after transplantation, cut a thin slice transversely through the middle of the regenerating graft, stain it with H&E and observe under the light microscope (Figure 11, Appendix 4, p. 132), you can clearly see the graft and the underlying host muscle (which is essentially unaffected by the transplantation operation). It shows the central area of original muscle in the graft that is dying (asterisk), and the new zone of muscle regeneration (that is replacing the dying muscle) as it moves in from the edge of the graft towards the centre, fed by the blood vessels that are growing in from the host.

**Identification of myogenic cells:** It can be very difficult to identify the many different kinds of cells in regenerating grafts unless we use special techniques
Figure 10

Whole muscle graft

Figure 11

Transverse view through regenerating whole muscle graft

Figure 12

Myogenic cells, identified by desmin staining, in regenerating muscle
(Lawson Smith and McGeachie 1998). The use of specific antibodies to show desmin in muscle cells was mentioned above, but the principle behind the use of antibodies to identify cells will now be discussed in a little more detail as it is a very useful tool for studying cell biology. You are probably aware that different tissues, e.g. a nerve, a kidney or a skin cell, all have very different genes switched on and these make quite distinctive proteins – that is what makes them different. What we need to do is find out what particular gene is switched on, only in skeletal muscle and then develop some way of identifying that. In the last few years several new proteins have been identified that appear to only be made by skeletal muscle cells. If we purify one of these proteins (not an easy task!) e.g. purify desmin from mouse muscle, and then inject it into another animal e.g. a rabbit, the immune system of the rabbit will detect the mouse desmin protein as ‘foreign’ and will make specific antibodies to it (the same system is used by you to get rid of unwanted invaders/infections of your body). These specific antibodies can be collected and then used to detect the original desmin on sections of experimental mouse muscle tissues (e.g. Figures 6, 12, Appendix 4, pp. 130 and 132).

If (transverse) sections of the same muscle graft are stained with desmin antibody, all of the muscle cells – early activated satellite cells, myoblasts, myotubes – stain a dense brown in the cytoplasm where the desmin protein is located. This allows us to readily distinguish muscle cells from other cells such as macrophages and fibroblasts. Using markers like this we can estimate how good the regeneration is and exactly what is happening with these various cellular events. A major aim of our research is to find out what factors help to improve muscle regeneration.

**Growth factors:** Many studies have been done in tissue culture using a wide range of molecules called *growth factors* that affect many aspects of cell behaviour. Some of these growth factors have been shown to make myoblasts start to proliferate, whereas combinations of other factors will stop the myoblasts proliferating and make them fuse. These observations derived from tissue culture studies are useful but what we really want to know is ‘which factors are of critical importance *in vivo*’ because these are the factors we need to manipulate if we want to enhance muscle repair in real live animals and humans.

**Importance of the extracellular matrix:** The extracellular matrix, which is the material lying outside the muscle fibres, is also very interesting. We are studying the molecules that make up this material and the effect that they have on the behaviour of muscle fibres and myoblasts. Returning to the picture of the satellite cell viewed by the electron microscope in Figure 5, Appendix 4, p. 130 I will concentrate on the fuzzy layer (long arrows) that covers the outside of muscle fibre and lies over the top of the satellite cell. This is the external or basal lamina (also known as the basement membrane). An even higher power view (Figure 13, Appendix 4, p. 133) shows in greater detail the close proximity of the external lamina (long arrows) and the underlying muscle cell membrane (short arrows). Part of the ‘striped’ contractile apparatus at very high magnification is also visible within the myofibre (bottom one third). The external lamina (long arrows) looks very insignificant. However, it has recently been recognised that the composition of the external lamina (basement membrane) is extremely important: changes here
Figure 13
High powered view of muscle cell membrane and external lamina

Figure 14
Diagram of important molecules associated with the muscle cell membrane

Figure 15
Muscle biopsy from a boy with DMD
and in the closely associated area of the cell membrane can give rise to severe muscle diseases such as the muscular dystrophies (Campbell 1995). It appears that if there are deficiencies in this area then, as the muscle contracts and works, you get weaknesses in the cell membrane. Since muscle exerts a great deal of force when it contracts and shortens, the structure of the cell membrane has to be very strong. The connection between the cell membrane and the external lamina is absolutely critical and if some of these molecules are missing then the muscle fibre is very susceptible to damage.

**Defects in cell membrane associated molecules lead to muscle diseases**

Some of the crucial molecules in the vicinity of the muscle cell membrane are shown diagrammatically in Figure 14, Appendix 4, p. 133. Genetic defects in many of these molecules that lead to muscle diseases have been identified in the last 12 years.

In 1987, the protein that is responsible for Duchenne Muscular Dystrophy (DMD) was identified and named dystrophin. It is now known that if dystrophin is absent or defective, you get this lethal childhood disease, DMD, that I will be talking about later. The disease has been recognised for a very long time but the actual molecular basis of DMD is only now being identified. Dystrophin is located in the cytoplasm (also called sarcoplasm) of the muscle fibre immediately beneath the cell membrane, and binds to proteins in the cell membrane (as shown in Figure 14, Appendix 4, p. 133). If you get defects in any of these proteins here (e.g. a sarcoglycan) you can manifest a disease that can be quite indistinguishable from DMD (reviewed in Campbell 1995). The proteins in the cell membrane also form connections with proteins lying outside the muscle fibre, such as laminin-2 in the external lamina (basement membrane). In 1993, reduced laminin-2 (also called merosin) was associated with Fukuyama-type congenital ('apparent at birth') dystrophies, and a lack of laminin-2 was shown to account for many of the very severe congenital muscular dystrophies (Hebling-Leclerc et al. 1995). Defects in collagen VI (associated with the basement membrane) were shown in 1996 to result in Bethlem myopathy – a relatively mild disease (see Lamande et al. 1998). In 1998, another protein in the cell membrane, called caveolin-3, was identified as a gene responsible for some of the milder limb girdle muscular dystrophies (McNally et al. 1998). Once the specific molecule and gene responsible for a disease is identified, this allows for much more accurate diagnosis and counselling of the various families, and makes possible the development of targeted therapies. The identification of responsible genes is a major international effort.

It is amazing the specificity that is now being obtained to describe precisely what is wrong in these different muscle diseases. When you can identify the actual defect, this means that you then have the possibility of going about correcting the defect. If you have absolutely no idea why someone’s muscles are falling apart it is extremely difficult to come up with an intelligent approach to try and fix the problem. The kinds of talks that you have been hearing about in this series from people like Nigel Laing, illustrate the international co-operation that results in the identification of defects in many specific genes that are responsible for various diseases.
Cell and gene therapy

What I am going to talk about now is the disease DMD that results from dystrophin deficiency, and ways in which people are trying to replace dystrophin in the diseased muscle fibres to correct for this gene defect.

Duchenne Muscular Dystrophy (DMD): This is a disease that affects boys because the gene is carried on the X-chromosome. You have probably heard quite a lot about this, but boys only have one X-chromosome and a Y-chromosome. So if a boy has a ‘dud’ X-chromosome with the defective dystrophin gene, he has DMD. In contrast to males, women have two X-chromosomes (and no Y-chromosome). So even if a woman inherits the ‘dud’ X-chromosome, the other X-chromosome is usually normal and will compensate for the defective one: thus she will be carrying the gene that can cause the disease (hence she is called a carrier) but she will not manifest DMD. Sons can only inherit their X-chromosome from (one of the two X-chromosomes of) their mother. If a mother is a ‘carrier’ for DMD and her son inherits her normal ‘good’ X-chromosome then the boy is normal: but if the son inherits the ‘dud’ X-chromosome he will have DMD. Sometimes even a tiny change in the DNA of the gene coding for dystrophin will result in this terrible disease. The dystrophin gene is huge and is one of the biggest genes described: it has been calculated that it takes 24 hours to read off one molecule of the messenger RNA for this gene. Because it is so large there is unfortunately plenty of opportunity for things to go wrong – and they do: there is a high rate of spontaneous mutation.

Different families with DMD have different parts of the dystrophin gene that have gone wrong, but the end result is that the dystrophin protein is either missing or defective. DMD is usually diagnosed by about 4 years of age, by 12 years of age the boys are wheelchair bound and they usually die in their twenties. The lack of dystrophin causes the muscles to break down and over time they completely fail to regenerate themselves: as a result, the muscles waste away and are replaced by fat and connective tissue leading to death. Figure 15, Appendix 4, p. 133 shows a biopsy from a boy with DMD: most of the tissue consists of fat cells and only a few muscle fibres remain (dark-stained at the top and bottom): compare with normal muscle in Figures 4, 9, Appendix 4, pp. 130, 131.

There are several strategies you can think of to try and correct a disease like DMD; one of them is to replace the missing gene dystrophin and I will be talking about that in detail soon. Other strategies could focus on trying to stop or slow down the progress of the disease, e.g. alter the threshold of muscle damage and trying to stop the muscle breaking down. Another approach (even if the muscle does fall apart) is to try and enhance the repair process. These different approaches should all be pursued simultaneously.

Replacement of the missing dystrophin gene

There are two approaches to replacing the missing dystrophin gene: one is cell therapy (which is a major research interest of our laboratory) and the other is gene therapy. Cell therapy is where healthy normal myoblasts are used to replace the dystrophin in DMD muscle fibres. This approach takes advantage of the unique
biology of muscle regeneration and the fusion that occurs to produce multi-nucleated cells. If a healthy normal myoblast fuses with a dystrophic muscle cell, you will end up with a ‘mosaic’ muscle fibre that has a mixture of ‘good’ and ‘dud’ nuclei inside it. Most tissues in the body can’t be treated like this because the defective and ‘good’ nuclei would remain in separate mono-nucleated cells lying next to each other, rather than both kinds of nuclei being inside a single (multi-nucleated) cell. The multi-nucleated skeletal muscle is very unusual and lends itself to this kind of cell therapy. Before discussing some of the latest results relating to cell therapy, I will just comment on gene therapy as this has attracted considerable attention in the press.

**Gene therapy**

Gene therapy is based on the same idea of putting the missing dystrophin back into muscles of boys with DMD but, in this case, you are not putting in a whole healthy nucleus with all the normal DNA (using a normal myoblast as a vehicle) instead you are putting in only the piece of DNA for the dystrophin gene (reviewed in Turner et al. 1998). So, the question is ‘how do you get the DNA into these defective muscle fibres?’ One answer is to take advantage of viruses and hijack their biology. Viruses are specialised little organisms which are designed for sneaking their way into cells; when they get in they use the machinery of the cell to produce all of their own proteins. Now if you put the bit of DNA for dystrophin into a virus, and then the virus gets into a muscle, it will express all of its genes including the dystrophin gene. This is great in theory and people are very keen on the idea. One of the problems is that the viruses that are really good at getting into various tissues are particularly nasty things such as HIV and Herpes: therefore, these viruses cannot be used unless they are ‘engineered’ to remove the ‘nasty’ genes whilst still keeping the genes that make them useful as vectors. Meanwhile, most studies use existing viruses that are much milder, such as one called an Adenovirus which basically just gives you a mild cold. While this virus is quite good at getting in, the problem is that it does not stick around and disappears within about two weeks. Thus the lack of an ‘ideal’ virus is a major problem for gene therapy at the moment. Another complication is that the body recognises such viruses as foreign, which is what the body is meant to do, and it mounts an immune response (such as producing antibodies as mentioned above) and eliminates the virus. So, a lot of attention in gene therapy is devoted to engineering these various viruses that can carry the genes into tissues. Gene therapy is a very attractive idea and gets a lot of publicity, but it is now recognised that it faces major problems such as those outlined. Consequently, this has cast attention back onto the cell therapy approach which takes advantage of the normal biological situation that occurs during skeletal muscle formation.

**Myoblast transfer therapy**

*Human trials:* The cell therapy approach as outlined above is widely referred to as Myoblast Transfer Therapy (MTT). Clinical trials using MTT have already been carried out on boys with DMD. A small piece of normal skeletal muscle was taken from a relative of the boy (usually the father), myoblasts were extracted from this donor muscle and large numbers of normal myoblasts were grown up in tissue
Figure 16

Injection of normal myoblasts with the dystrophin gene into dystrophic muscle (nuclei lack dystrophin).

Figure 17

Lone male normal donor muscle nucleus in dystrophic (female) muscle.

Figure 18

Survival of male (normal) donor cell in muscle graft (at the bottom) implanted into dystrophic (female) muscle (at the top).
culture. These normal myoblasts were then injected into dystrophic muscles of the boys (often at many sites) to try and form mosaic muscle fibres. (Figure 16, Appendix 4, p. 134 illustrates the injection of normal myoblasts into dystrophic muscle: Note this is not drawn to scale.) It is hoped that if enough of the normal nuclei are present they will produce sufficient dystrophin to compensate for this deficiency. This is the principle of MTT. The clinical trials resulted from a meeting held in 1989 in New York where preliminary evidence for success of MTT in mice was presented (Karpati and Griggs 1990). Most scientists felt that human trials were highly premature but the clinicians were very keen to proceed. One consequence was that these trials diverted an enormous amount of money, energy and effort, and the experimental studies in animal models received little funding during this time. The trials, which were mainly carried out in America, found that effectively none of the normal donor muscle nuclei survived after injection in the boys. The human trials of MTT created a huge controversy which I will briefly touch upon, because it has received a lot of coverage in the media. Only one group in America claimed success with this treatment and they continue to claim success, despite the fact that this contrasts markedly with results from all the other groups (Partridge et al. 1997). This group has resisted requests for firm data to be presented to substantiate their claims and they continue to advertise and charge parents of these boys about $200,000 for this treatment. It is very difficult to regulate such things, but it illustrates some of the dangers of leaping in too early with trials of potential treatments. The rest of the scientific and medical community has now acknowledged that these trials of MTT have not worked and I will outline briefly the reasons that seem to account for the failure. As a result of this ongoing controversy with the human trials, the parents of boys who were treated by this person have formed a very strong and cohesive international group called the Parent Project (http://www.parentdmd.org) to help educate other parents and people around the world, and to focus attention back onto funding research into DMD. Attempts are still continuing to have the claims for success independently reviewed and accurately assessed. Everyone wants an accurate picture of what is going on. Meanwhile, the controversy and the failed trials made the concept of cell transplantation therapy unpopular.

Animal experiments: However, we continued with experiments with animals in our laboratory using a strain of mice called mdx which is a good model for Duchenne Muscular Dystrophy; these mice similarly lack dystrophin and their muscles also undergo necrosis followed by regeneration. The mdx mice actually look remarkably healthy (unlike the boys) and that in itself is of interest: possible reasons may be that mice are very small and therefore do not have such loading on their muscles as humans do, and mice also live for a relatively very short time (reviewed in Grounds and Yablonka-Reuveni 1993; Partridge 1998). However, the mdx mouse is a very useful model to study MTT. We really wanted to find out what happened to the myoblasts after injection. So, we mimic the clinical situation of growing normal myoblasts in culture and then injecting them into dystrophic muscles (Grounds 1996). First, we want to demonstrate that these myoblasts will survive after injection - this is a really important aspect. Ideally we want the myoblasts to increase in number, we would like them to move around in the
dystrophic muscle, and it is essential that they fuse with the host muscle and that they make dystrophin. So these are the kinds of conditions that we are looking for.

**Monitoring the injected myoblasts:** The mice can be selectively inbred so that all of the X-chromosomes carry the defective gene (the normal X-chromosome is eliminated from the colony): this means that all female (who now have two dud X-chromosomes) as well as all male mdx mice have the same disease. In our experiments we use a special piece of DNA that only recognises a matching piece of DNA on the Y-chromosome, to specifically identify the presence of male nuclei (this probe will not bind to female DNA since it lacks a Y-chromosome). We have got a probe that will only identify male cells and we use this to monitor exactly what is happening to these donor male myoblasts after injection into dystrophic female muscles: such close monitoring is something you cannot do easily in the clinical situation. Figure 17, Appendix 4, p. 134 shows an example of injected muscle at very high power; the striations of the muscle fibre in longitudinal section are visible and a lone male nucleus (asterisk) is present (identified using the specific Y-probe) several months after injection into a female dystrophic mdx mouse. A rare normal muscle nucleus like this is going to be completely ineffective at rescuing the dystrophic muscle. What we (and others) have found is that once the myoblasts have been extracted, cultured and injected, most of the donor myoblasts die very rapidly, within a few days (Fan et al. 1996b). In the clinical trials they didn’t even look until after three or six months! Why do the injected myoblasts die?

**Survival of injected myoblasts:** This question has attracted a lot of interest recently. I do not have time to discuss the reasons at this point, but the really big question is, 'can we enhance the survival of injected myoblasts?' While it became very unfashionable to study MTT for a few years, people seem to have recovered from the whole experience and are now very constructively addressing this particular question. They have identified the problem and have some understanding of why MTT didn’t work (reviewed in Tremblay and Guerette 1997). What is needed now are strategies to enhance the survival of the injected myoblasts. There is a large range of strategies that can be explored and many laboratories around the world are starting to participate. There are two main approaches: one is to play with the culture conditions for growing up the myoblasts, and the other is to influence the response of the host to the injected myoblasts.

With respect to the tissue culture conditions, it appears that something is happening to the myoblasts (possibly as a result of extraction from the muscle and growing them in culture) that makes them unhappy after injection, and it has been proposed that this treatment makes them change so that they become ‘foreign’. We know from studies that I will mention in a minute that if you take exactly the same kind of muscle and just put it in as a chunk it survives quite happily — so it must be something to do with the culturing/injection of these same muscle cells that makes them ‘unhappy’. Therefore, attention is focused on modifying the culture conditions to see if the problem can be overcome. The second approach (which we are doing in parallel) is to modify the other end of the equation — the host immune response — since there seems to be a very rapid inflammatory response by host cells which kills the donor myoblasts.
These kinds of studies have a lot of relevance to other diseases such as diabetes where it is a dream of people to be able to take the (islet) cells that make insulin in the pancreas, and implant them into diabetic people. If you could do that it would mean that those people never had to inject themselves with insulin again, because they would have their own little cell factory inside themselves producing the insulin as it was needed. Common problems occur across many of these kinds of cell transplantation procedures. At the moment, over half the activity in our laboratory is dedicated to such cell transplantation research.

**Influence of the host immune response:** What I will touch upon in the last part of the talk is ways of affecting the host immune response. There are various treatments to stop the host recognising something that is foreign and causing damage to it (and these are clearly of interest to survival of the injected myoblasts) but, 'what is the best and most appropriate method to use?' The work I will discuss now uses a slightly different model of sliced muscle grafts (rather than injected myoblasts) as the donor muscle for cell transplantation (Fan et al. 1996a). As emphasised above, the problem with the injected cells is that they die. In order to determine whether it is the donor muscle itself or alternatively the culturing/injection process that is the problem, we transplanted a small slice of the same kind of donor muscle directly into a female host mdx mouse. This was exactly the same combination of mice, the difference being that the donor muscle was intact and the muscle cells had not been extracted, cultured and injected. In this situation, we find fantastic survival of the donor muscle cells! This is terrific – we now have a situation where the donor myoblasts will survive up to a year perfectly happily. If we look with the Y-chromosome specific probe, we can clearly see the surviving male donor muscle graft (dark nuclei in bottom half of Figure 18, Appendix 4, p. 134) but, in addition, these male nuclei have moved out into the adjacent female muscle often quite a distance from the graft. This model is very useful to us because (unlike the injected cells) we don’t have a problem with the donor myoblasts surviving and, furthermore, we show that they actually move away into the dystrophic muscle.

**Movement and fusion of donor myoblasts:** We have used this model to address another important question ‘how can we enhance the movement and the fusion of these donor myoblasts?’ All of these studies are carried out in parallel: with one group of researchers trying to get injected myoblasts to survive, and another investigating what conditions would enhance their movement and fusion. Potential strategies are: to try and reduce host barriers to donor myoblast movement, e.g. by modifying the extracellular matrix (the material between muscle cells) to make it less resistant to movement; or maybe stimulate the migration of donor myoblasts by using chemotactic signals; or modify the host immune response. I will just touch upon the host immune response and describe a procedure that we have used (also in the cell injection studies). The approach tries to actually re-educate the immune system of the host. This differs from the traditional approach of using immunosuppressants which can also make you feel sick: after transplantation of an organ like the heart or the liver, the patient is given strong drugs that suppress the whole immune response of the recipient and, of course, this means that they also can’t respond to any kind of infection by bacteria or viruses so it makes them very
Figure 19
Manipulation of the host immune response: depletion of host T-lymphocytes

Figure 20
Result of T-cell depletion on donor myoblast survival in dystrophic muscle

Figure 21
Some members of the Muscle Research group in Perth
Left to Right
- Gail Smythe
- Peter Hamer
- John McGeachie
- Jason White
- Stuart Hodgetts
- Miranda Grounds
- Marilyn Davies
vulnerable. The advantages of trying to re-educate the immune system of the host are that it is a one-off operation and highly specific – after treatment the host should never recognise the cells of interest (in this case the donor myoblasts) as foreign, although it should still respond to everything else.

The procedure used in our work (Fan et al. 1997) is indicated in Figure 19, Appendix 4, p. 134. In brief, specific antibodies are injected into the host (Figure 19, Appendix 4, p. 135) in order to temporarily get rid of (by killing) all the circulating lymphocytes (that express molecules called CD4 and CD8) – lymphocytes are the main cells that the body uses to respond to something that is foreign. Another clever little manipulation that we do in some mice, is to also inject into the thymus (not shown) some of the actual muscle cells that will be ultimately implanted as a sliced graft. The idea is that as the lymphocytes build up their numbers again, if these new lymphocytes encounter the foreign muscle when they are still very immature (especially in the thymus where new lymphocytes are educated) they will actually think it should be there and is ‘self’ (rather than ‘foreign’), and the whole new population of lymphocytes will become ‘tolerant’ to these molecules on the donor muscle cells. So this strategy works to selectively re-educate the immune system of the host.

This procedure has produced some very promising results (see one set of data, Figure 20, Appendix 4, p. 135). We did the kinds of manipulations to the host described above, sampled the muscles at first, third, or twelfth weeks after transplantation of the sliced muscle grafts, and used the Y-probe to look at tissue sections to see the number of male nuclei (i.e. normal donor muscle cells) that have moved out of the graft into the (dystrophic female) host muscle. We know the graft is there. We are only interested in the number of donor cells that get out of the graft and move away into the host muscle and survive there. In the untreated control animals (right hand column at each time), male cells do move out as shown in Figure 20, Appendix 4, p. 135 – there are a lot at one week but the numbers drop off by three weeks and then they seem to be fairly stable. This is interesting as it shows that while the donor myoblasts do move out, they appear to be ‘unhappy’ in the host environment and quite a lot of them perish.

The treated groups differ from the controls (above) although both treatment groups seem to give similar results (i.e. the thymus injection does not seem to have any additional benefit). At first week, clearly more cells have got out and survived in these treated groups as compared with the untreated animals (Figure 20, Appendix 4, p. 135). This is much more striking at three weeks, as we don’t see a decrease (as occurred in the controls at this time) and the beneficial effect is still sustained at twelve weeks. The results show that these treatments greatly increase the movement and survival (fusion) of the donor myoblasts in the dystrophic host muscle, and that the host immune response plays a role in these events (Fan et al. 1997).

It is very promising that these kinds of manipulations will actually enhance the behaviour of the myoblasts in this way. We are carrying out long-term experiments (up to a year) and doing the same kind of treatment with respect to the survival of the cultured/injected cells. These experiments are producing some very interesting
information. This just gives you an example of the kinds of approaches that we use. We are always very conscious of applying these findings to the clinical situation. Before this might be undertaken, we would want to be confident that this re-education really lasted over an extended period of time. There is a lot of interest in this area and I am fairly confident within the next two years there will be a much clearer understanding of exactly what happens to these myoblasts and how you can enhance their behaviour in MTT.

**Members of the research team:** Some members of my group sitting by the river near the Department are shown in Figure 21, Appendix 4, p. 135. Marilyn Davies is my terrific technician who has been with me for many years and Professor John McGeachie is a colleague who has collaborated extensively on the muscle regeneration studies. Dr Gail Smythe works on the sliced muscle grafts and is interested in the movement of myoblasts (funded by the Association Francaise contre les Myopathies) and Dr Stuart Hodgetts studies the survival of the injected myoblast (funded by the International Parent Project). The rest of us are working on other various aspects of muscle regeneration and we are funded by the National Health and Medical Research Council of Australia.

For further information on our research see:

http://www.anhb.uwa.edu.au/mg.html

**References**


