

Characterisation of Stem Cells in Adult Mouse Muscle

Katerina Blaveri and Jennifer E. Morgan

Muscle Cell Biology, MRC Clinical Sciences Centre, Royal Postgraduate Medical School Hammersmith Hospital, London, England

Abstract

There is evidence that some of the muscle precursor cells (mpc) implanted into adult mouse muscle remain as quiescent stem cells, able to give rise to more quiescent muscle precursor cells and to mature muscle. Rare myogenic cells of donor origin were extracted from irradiated mdx muscle implanted with normal *H-2K^b-tsA58* mpc; these extracted cells gave rise to new muscle and to cultureable myogenic cells when injected into a series of irradiated mdx nu/nu mouse muscles.

New muscle of donor origin was also formed even after a series of intramuscular injections of notexin into the legs of mdx nu/nu mice which had previously been injected with normal *H-2K^b-tsA58* mpc. This again indicates the presence of long-lived mpc, as notexin is a myotoxin and as such, destroys mature muscle, sparing only single cells. It is not yet known if these muscle precursor cells are derived from cells in the satellite cell position. Further studies on these persistent mpc will be of interest for improving the efficacy of myoblast transfer therapy.

A major problem in the investigation of putative muscle stem cells is the effect of the tissue culture environment upon the cells. We have noticed, in particular, a loss of myogenicity on prolonged culture of *H-2K^b-tsA58* mpc.

Key words: muscle stem cells, satellite cells, mdx mouse, muscle precursor cells, myogenicity.

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What is a Stem Cell?

A stem cell is defined as a cell which is present for the life of the individual and which can give rise to further stem cells and to differentiated cells. Stem cells may be pluripotent, giving rise to different cell lineages e.g. haematopoietic stem cells, or multipotent, giving rise to only one differentiated cell type e.g. keratinocyte or muscle.

Practical Uses of Muscle Stem Cells

Implantation of normal or genetically-modified muscle precursor cells (mpc) has been suggested as a means of treating myopathies or to systemically disseminate recombinant proteins. If normal muscle stem cells were injected into chronically degenerating myopathic muscle, they would give rise to muscle and to more stem cells, which could be recruited into myogenesis during further episodes of necrosis. Muscle stem cells would also be invaluable for muscle-based gene therapy for other diseases: they could be extracted, transfected with the gene of interest and then implanted back into skeletal muscle, where they would give rise to all the compartments of mature muscle (muscle fibres, satellite cells and/or muscle stem cells).

Are There Stem Cells Present in Adult Skeletal Muscle?

During muscle development, multinucleated skeletal muscle fibres form by the fusion of mononucleated muscle precursor cells (mpc) in a system controlled by muscle regulatory factors (MRFs) [11]. Satellite cells, quiescent cells which are situated under the basal lamina of the muscle fibre, are formed at around 17 days of development in the rat and are involved in postnatal muscle growth and in regeneration [4].

Often referred to as muscle stem cells, satellite cells are usually assumed to be the sole source of myogenic cells during muscle regeneration. Moreover, in tissue culture studies, the mpc derived from adult muscle are also presumed to be derived from satellite cells. However, this can only be certain when no other cell types have been included in the cell preparation. One way to ensure this is to culture single viable muscle fibres; the only cells associated with these fibres are satellite cells [23]. Adult muscle cells are a heterogeneous population in terms of characteristics such as colony size [29, 30], cell cycle times [28] and fibre types of resultant myotubes [24], so it is unlikely that they are all stem cells. In injured rat muscles, there is evidence for two distinct populations of myogenic cells - one which

differentiates without proliferation after injury, whilst the second divides slightly later after injury [22]. Baroffio et al. have shown that small, desmin-positive and alpha sarcomeric actin-negative human muscle cells behave like stem cells *in vitro* [2, 3] although the decrease in their self-renewal capacity with increasing time in culture indicates that their lifespan may not be sufficient for them to conform to the picture of true stem cells as depicted by Hall and Watt [10].

It is possible that muscle stem cells originate from a non-muscle source, e.g. from a mesenchyme stem cell [32]. Whilst there is evidence that dermal fibroblasts can be myogenic [8, 27, 34], muscle fibroblasts do not seem to be so. There is little evidence, therefore, that cells other than, or in addition to, mpc or satellite cells are a source of persistent myogenic cells.

Experiments in which skeletal muscle has been repeatedly damaged to force its degeneration have provided evidence that muscle contains persistent precursor, or stem cells: regeneration of rodent muscle occurred following several injections of bupivacaine, which destroys mature muscle, but spares other cell types [5, 12, 26].

Do Mpc Implanted into Adult Muscle Give Rise to Stem Cells?

Two groups have attempted to investigate whether mpc implanted into adult mouse muscle give rise to muscle stem cells as well as mature muscle of donor origin. Yao and Kurachi [35] pooled 10 colonies of mpc derived from adult nude mice, infected them with retrovirus containing LacZ and then implanted the infected cells into nude or SCID mouse muscles. These implanted cells gave rise to muscle (but not to tumours) and to extractable, donor-type myogenic cells, which again formed normal muscle on implantation into a second series of mouse muscles. Mouse muscle cells readily become spontaneously immortalised, although several [20, 33], but not all (Morgan et al., unpublished observations) mouse muscle cell lines form tumours upon their injection into regenerating mouse muscle. The possibility that the mpc implanted by Yao and Kurachi had become transformed cannot be discounted.

As it is difficult to distinguish transformed cells from stem cells, we resorted to the *H-2K^b-tsA58* mouse [13] as a source of cloneable mpc. When grown under conditions permissive for T antigen expression (33°C in the presence of gamma interferon), the cells proliferate and when placed in non-permissive conditions *in vitro* (39°C without gamma interferon) or *in vivo*, they differentiate to form normal muscle. A clone of *H-2K^b-tsA58* mpc was passaged through a series of three irradiated mdx nude mouse muscles, giving rise to muscle of normal appearance and extractable donor-type myogenic cells from each injected muscle. The extracted cells ceased DNA synthesis *in vitro* under conditions non-permissive for T antigen production with the same kinetics as non-injected *H-2K^b-tsA58* clones, indicating that they had not become transformed, but were behaving as stem cells [19].

In these experiments, only one out of 8 of the *H-2K^b-tsA58* myogenic clones was serially-passageable in this way. Adult mouse muscle yields very few myogenic cells when prepared either by explant culture or by enzymatic disaggregation; it is therefore quite likely that many more stem cells of donor origin were present than were actually cultureable. Preparation of single fibre cultures from adult mouse muscle gives a considerably greater yield of myogenic cells [23] but there still remains the problem of sampling only a proportion of the injected muscle.

Single fibre cultures from adult mouse muscles give rise to very pure cultures of myogenic cells. We therefore used this technique to prepare clones of mpc from *H-2K^b-tsA58* mouse muscles, to be used in our cell injection experiments.

Effects of Tissue Culture on the Myogenicity of Cells

In order to assess the myogenic capacity of our cultures, (prior to injecting them into myopathic animals or exposing to differentiating conditions) a reliable marker of myogenicity was necessary. To this end, we chose desmin as a marker, as it is known to be expressed at low levels in replicating myoblasts and to be up-regulated upon terminal differentiation [14, 15]. Desmin is considered to be one of the earlier myogenic markers [6] and has been widely used in this role [1, 16].

However, upon culturing a clone of myoblasts derived from a single fibre culture [23] of the *H-2K^b-tsA58* transgenic mouse EDL muscle, the number of desmin-positive cells decreased with increasing passage, when grown under permissive conditions (33°C in the presence of gamma interferon). With each subculture, the percentage of myotubes formed with transfer to non-permissive conditions (37°C without gamma interferon) declined, indicating that cells with less myogenic potential were being selected. Ultimately, the entire cell population became desmin-negative and were unable to fuse. However, on culture of a different aliquot of the same clone of myoblasts, a less severe loss of the desmin phenotype or myogenicity was observed.

It was therefore decided to assess the effectiveness of desmin as a myogenic marker for *H-2K^b-tsA58* myogenic cells, under the influence of a number of tissue culture parameters.

Materials and Methods

Muscle precursor cells were prepared from the TA and EDL muscles of a 36 day old, male *H-2K^b-tsA58* homozygous mouse by enzymatic disaggregation [19] (left leg) or by single fibre culture [23] (right leg). Cells prepared by enzymatic disaggregation were plated either on Matrigel (1 mg/ml) or on Primaria plastic; single fibres were plated on Matrigel (1 mg/ml). Cells were grown under permissive conditions in either Dulbecco's modified Eagles medium (DMEM) containing 20% fetal calf serum (FCS), 10% horse serum (HS), 0.5% chicken embryo extract (CEE) or DMEM containing 20% FCS. Clones of myogenic cells

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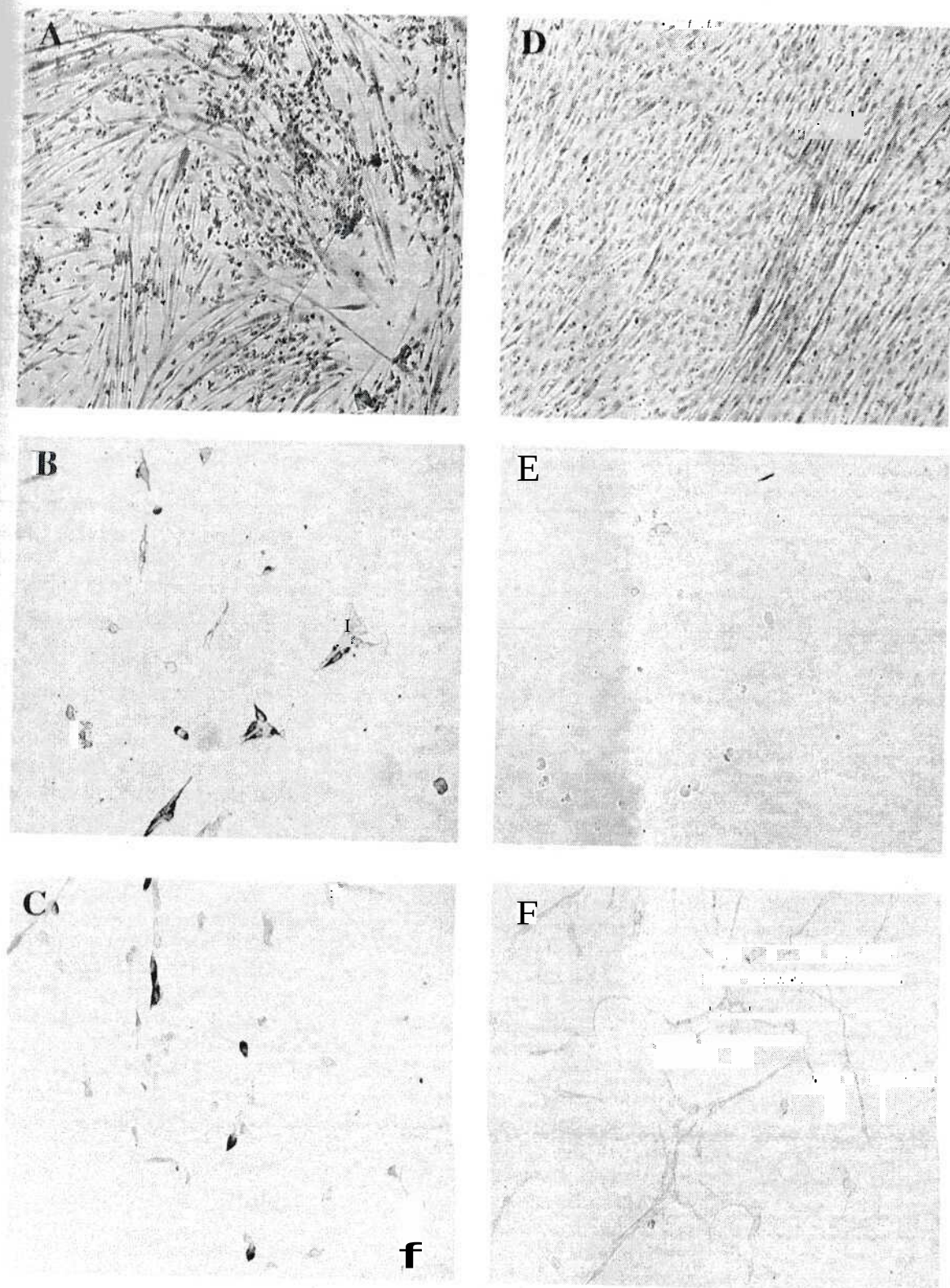


Figure 1. The behaviour of H-2K^b-tsA58 clone 25 (table 1) mpc at different passage numbers in vitro. A. Haematoxylin stain after 4 days in permissive conditions at high density (passage 4). B. Desmin immunostain after 4 days in permissive conditions at low density (passage 4). C. Desmin immunostain after 4 days in non-permissive conditions at low density (passage 4). D. Haematoxylin stain after 4 days in permissive conditions at high density (passage 11). E. Desmin immunostain after 4 days in permissive conditions at low density (passage 11). F. Desmin immunostain after 4 days in non-permissive conditions at low density (passage 11).

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were isolated by ring cloning [19] and expanded in plastic tissue culture bottles.

To assess the number of myogenic cells in the cell preparation, cell cultures at each passage were immunostained for desmin after four days at low cell densities in permissive conditions. To assess the fusion index, cells were plated at 5×10^4 cells/well in 8-well chamber slides in DMEM, 5% HS for four days at 39°C. The cultures were then fixed and stained with haematoxylin and the percentage of nuclei in myotubes was counted in three random fields.

Two clones derived from an EDL single fibre culture were sub-cloned and the myogenicity of the subclones assessed as described above.

Results

All of the clones showed a decrease in both desmin expression under permissive conditions and fusion index under non-permissive conditions with increased passage (figure 1; table 1). Neither the method of cell extraction (SF vs enzymatic disaggregation), nor the muscle of origin (TA vs EDL), nor the nature of the first substrate (Matrigel vs Primaria tissue culture plastic) had any effect on this change in phenotype.

The medium composition has an effect only on the myogenicity in the clones derived from the SF. The clones which were grown in 20% FCS failed both to express desmin and to fuse (not shown). However, it is unclear whether this was due to selection of solely negative clones or whether it indicates an effect of the composition of the

Table 1. Myogenicity of clones obtained from the tibialis anterior (TA) or extensor digitorum longus (EDL) muscles of a 36 day old male homozygous H2K^b tx A58 mouse.

Clone	Muscle	Cell Preparation	First substrate	Medium	Passage number	Percentage of desmin-positive cells	Fusion index
22	TA	enzymatic disaggregation	Matrigel	20% FCS	4	55	22
					7	4	10
23	TA	enzymatic disaggregation	Matrigel	20% FCS, 10% HS, 0.5% CEE	3	53	35
					9	20	12
28	EDL	enzymatic disaggregation	Matrigel	20% FCS	4	95	nd
					7	59	33
19	TA	enzymatic disaggregation	Primaria	20% FCS, 10% HS, 0.5% CEE	5	89	17
					11	10	11
25	EDL	enzymatic disaggregation	Primaria	20% FCS, 10% HS, 0.5% CEE	3	83	46
					9	0	1
31	TA	enzymatic disaggregation	Primaria	20% FCS	6	66	22
					10	0	12
36	EDL	enzymatic disaggregation	Primaria	20% FCS	5	35	29
					9	30	58
E1C7	EDL	single fibre culture	Matrigel	20% FCS, 10% HS, 0.5% CEE	5	54	56
					8	17	6
E1C7.6					12	89	90
					13	90	90
E1C9	EDL	single fibre culture	Matrigel	20% FCS, 10% HS, 0.5% CEE	5	29	20
					7	11	6
E1C9.2					13	90	88
					17	65	80
E1C9.3					13	72	85
					17	60	76
E1C10EDL		single fibre culture	Matrigel	20% FCS, 10% HS, 0.5% CEE	5	14	5
					7	5	7
T1C1	TA	single fibre culture	Matrigel	20% FCS, 10% HS, 0.5% CEE	7	15	0
					9	0	<1
T1C14TA		single fibre culture	Matrigel	20% FCS, 10% HS, 0.5% CEE	6	17	5
					8	0	<1

medium (for example whether the HS and CEE that were omitted are significant for demonstrating a myogenic phenotype).

The method of cloning used was ring cloning. In order to investigate any potential errors introduced by this technique, some of the clones were subcloned. The subclones (EC 7.6, 9.1 and 9.2: table 1) that were isolated were of a more desmin positive and myogenic phenotype than the clones from which they originated. As only a limited number of subclones were studied, a quantitative conclusion could not be drawn.

Overall, the following phenotypes of $H-2K^b$ -tsA58 myoblasts were observed in culture depending on their desmin expression under permissive conditions and their fusion capability under non-permissive conditions:

(i) cells that expressed desmin and were able fuse (d+,f+).

This is in agreement with the current suggestion that fusion might take place only between desmin-positive cells [14]. It was observed that clones in which the percentage of desmin-positive cells was high gave rise to long, branched myotubes. By contrast, in clones where the number of desmin-positive cells was low, the myotubes were thin in appearance and contained few myonuclei (figure 1).

(ii) cells that did not express desmin and were able to fuse (d-,f+).

One possible explanation might be that desmin expression occurred in these cells at levels too low to be detectable by immunocytochemistry. This would be in agreement with the above. This phenomenon may however, represent a stage where cells have begun losing their myogenic phenotype, a suggestion supported by the fact that the fusion index in cultures which contained no desmin-positive cells was always less than 50% (table 1).

It has been proposed that the entire muscle-specific phenotype of the early stages of development in proliferating myoblasts, including basal levels of desmin expression, is not essential to terminal differentiation *in vitro* but instead is related to events that are crucial to early development *in vivo*, such as cell migration and responsiveness to extracellular matrix proteins [9]. This may explain this 'paradoxical' phenotype as a normal occurrence *in vitro*, where cells can proceed to terminal differentiation under appropriate conditions, bypassing developmental stages which normally occur *in vivo* but are not essential under the current conditions.

(iii) cells that did not express desmin and were not able to fuse (d-, f-)

This may represent a phenotype that is born of extensive tissue culturing of the cells. It has been proposed before that myoblasts maintained in cell culture have undergone several changes, one of which is the reduction of the number of cells with a myogenic potential [7]. It is possible that such a change is accompanied by the loss of muscle specific proteins, which would be in agreement with the loss in desmin expression, although this has not yet been verified. A logical progression would be to inject these

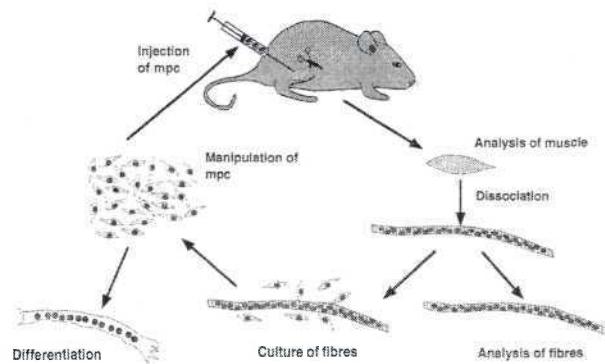


Figure 2. Study of the behavior of myogenic cells *in vivo* and *in vitro*. MPC are prepared from normal mouse muscle and injected into appropriate sites in the mouse leg (e.g. irradiated muscles of *mdx nu/nu* mice). Injected muscles may be analysed in a variety of ways, e.g. by sectioning and immunostaining. Injected muscles may also be separated into single fibres and analysed, or cultured. The composition of the fibres and of the satellite cells coming off the fibres may be determined. Any donor type myogenic cells emanating from single fibre cultures of injected muscles may be muscle stem cells; this may be verified by injecting the cells into a second mouse muscle, allowing muscle to form and then repeating the tissue culturing/injection steps.

cells into host animals and assess formation of muscle fibres *in vivo*.

A possible explanation for the loss of myogenicity with increasing passage is that the clones were impure and the cultures have been overrun by fibroblasts. This would have been unlikely to have occurred in every clone.

Finally, although it is not thought that SV40 large T antigen affects desmin expression or myoblast differentiation [21], it may have interfered in some way with their myogenic phenotype. It is clear that in addition to desmin, the expression of a number of other myogenic markers should be investigated in order to evaluate the myogenic capacity of muscle cells, including myf-5, MyoD and myogenin [17, 18, 25, 31].

Baroffio et al., [2, 3] consider α -sarcomeric actin to be a "predifferentiation" marker, expressing while human satellite cells are still proliferating. Satellite cells expressing both desmin and α -sarcomeric actin *in vitro* represent a population of cells committed to fusion. We may be able to assess the myogenic commitment of the $H-2K^b$ -tsA58 myoblasts using both α -sarcomeric actin and desmin. Creating a "battery" of myogenic markers is suggested as an appropriate way to assess the myogenic potential of myoblasts in culture, since *in vitro* phenotype may be subject to fluctuation.

Furthermore, it would be useful to investigate whether a clone which has become less myogenic with time *in vitro*, makes as much (if any) muscle *in vivo* when compared to the same clone at an earlier passage.

Searching for Muscle Stem Cells without Tissue Culture

In order to examine whether a population of mpc had self renewal capacity, without subjecting them to a tissue culture environment, we implanted enzymatically disaggregated mpc derived from newborn, *H-2K^b*-tsA58 mouse muscle into irradiated mdx nu/nu mouse muscles and then induced a series of degeneration events in the injected muscle by injecting the snake venom, notexin (Gross and Morgan, submitted for publication). Notexin destroys mature muscle fibres, but spares mpc and other tissues. Sufficient time was allowed for muscle regeneration between each notexin injection. The muscles were sampled one week after being given 1-4 notexin injections; the muscles and sections were immunostained for dystrophin and neonatal myosin. Fibres which contained both neonatal myosin and dystrophin were newly-formed fibres of donor origin. The finding of such fibres in all of the experimental muscles indicates that at least some of the implanted mpc had remained in the injected muscle as undifferentiated precursor cells, which were able to undergo myogenesis when the muscle was forced to degenerate. It is not known whether these fibres were derived from one cell, whether different mpc were being activated at each injury, or whether the persistent myogenic cells were in the satellite cell position. This will be resolved by marking single myogenic cells with retrovirus prior to their implantation into irradiated mdx mouse muscle which will subsequently be forced to degenerate as above. The unique insertion of the retrovirus will make it possible to identify the progeny of single clones.

Conclusions and Future Work

In much of our previous work, we implanted freshly-disaggregated mpc derived from newborn mice into myopathic mouse muscle. In order to be able to characterise mpc with possible stem cell characteristics, it is necessary to culture them. Conditionally-immortal *H-2K^b*-tsA58 mpc seemed ideal for the investigation of muscle stem cells, as they can be grown to large numbers *in vitro* and differentiate normally *in vivo*; it can also be easily demonstrated whether or not they have become transformed. Although there was no loss in the myogenicity of donor-type *H-2K^b*-tsA58 mpc extracted from a series of irradiated mouse muscles [19], in subsequent experiments where we have prepared single fibre cultures from irradiated and injected mouse muscles, the extracted donor cells became non-myogenic with increasing time *in vitro*. We have found no obvious reason why this occurred.

We are now analysing single fibre cultures prepared from irradiated mdx nu/nu mouse muscles into which we had implanted clones used in the original experiments [19]. Single fibre cultures were prepared as before [23], but the cells were grown thereafter on the same substrate and in the same medium as before [19]. The extracted, donor type mpc will be regularly checked for myogenicity, ploidy and transformation.

The combination of *in vivo* and *in vitro* techniques (figure 2) provides a powerful tool for examining the behaviour of myogenic cells, with the aim of isolating and characterising the behaviour of potential muscle stem cells for myoblast transfer therapy. The use of the single fibre tissue culture technique will enable us to ascertain whether cells which behave as stem cells also become sequestered in the satellite cell position. It must always be borne in mind, however, that the *in vitro* environment can modulate the behaviour of myogenic cells.

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Address correspondence to:

Dr. Jennifer Morgan, Muscle Cell Biology, MRC Clinical Sciences Centre, Royal Postgraduate Medical School, Hammersmith Hospital, Du Cane Road, London W12 ONN, tel. 0181 383 8265, fax 0181 383 8264, Email jmorgan@rpms.ac.uk.

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