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Conversion of Dermal Fibroblasts to a Myogenic Lineage Is Induced by a Soluble Factor Derived From Myoblasts

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Abstract The limb and axial skeletal muscles of mammals originate from somitic dermomyotome, which during early development separates to form two discrete structures, the dermatome and the myotome. The latter cell mass gives rise to the muscle-forming lineage while cells of the dermatome will form the skin dermal fibroblast population of the dorsal regions of the body. It has been generally accepted for some time that myotome-derived myoblasts were the sole source of muscle fibre nuclei, but evidence has recently been presented from several laboratories that fibroblasts can fuse with myoblasts to contribute active nuclei to the resulting myotubes.

We report here an investigation into the myogenic capacity of fibroblasts. Confluent monocultures of mouse dermal fibroblasts, muscle fibroblasts, and C2C12 myoblasts each retain their individual phenotype when maintained for periods up to 7 days in culture. We also grew isolated colonies of fibroblasts and myoblasts in an arrangement which allowed free exchange of tissue culture medium between the 2 cell types. We found evidence of the conversion of dermal fibroblasts to a myogenic lineage as measured by the appearance of MyoD-positive cells expressing the muscle-specific intermediate filament desmin. In addition, dermal fibroblast cultures contained multinucleate syncytia positive for MyoD and containing sarcomeric myosin heavy chain. In contrast, muscle-derived fibroblasts showed no evidence of myogenic conversion when maintained in identical culture conditions. We prepared conditioned medium from confluent cultures of C2C12 myoblasts and added this material to confluent monocultures of either dermal or muscle fibroblasts. While muscle fibroblasts showed no phenotypic alterations, cultures of dermal fibroblasts responded to myoblast conditioned medium by converting to a myogenic lineage as judged by expression of MyoD and desmin. We conclude that a proportion of dermal fibroblasts retain a myogenic capacity into stages well beyond their early association with myoblasts in the dermomyotome.

Key words: MyoD, myosin heavy chain, muscle, desmin, mouse, myogenesis

Skeletal muscles of the trunk and much of the head are thought to develop exclusively from somites [Christ et al., 1977], condensates of several thousand cells that bud from the segmental plate mesoderm in a pairwise progression. Somites are also the source of vertebral and rib cartilage, and the dermis. Cells within the medial region of a somite are the source of the myotome which gives rise to the axial and intercostal skeletal muscles [Ordahl and LeDouarin, 1992]. Lateral somitic cells migrate from the somite to give rise to the ventral abdominal muscles and the limb muscles [Bober et al., 1994]. Dorsal somite cells form the dermatome which disperses to give rise to cells of the dermis. The ventral somite gives rise to the sclerotome cell lineage that migrate ventrally to form the vertebral and rib cartilage [reviewed in Emerson, 1993]. This spatial description of the somite fate map disguises an early intimate association of myogenic and dermogenic cells in the dorsal dermomyotome epithelial sheet. Cells at the dorsal medial and basolateral lips of the sheet migrate away to differentiate into muscle, leaving behind the dermatome, which will form dermal cells [Buckingham, 1992].

The mononuclear precursors of skeletal muscle fibres are myoblasts, whose differentiation is under the control of a group of myogenic

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regulatory genes known as the bHLH or MyoD family [reviewed in Arnold and Braun, 1993]. In contrast, dermal cells derived from the dermatome are poorly characterised, usually classified as skin fibroblasts which only express standard mesodermal marker genes such as those for vimentin, fibronectin, and collagen as well as Thy 1.1 [Linge et al., 1989; Jones et al., 1990; Murphy et al., 1992; Caplan, 1991]. Mouse fibroblasts are easy to immortalise and amongst the many lines available, cells of the 10T½ line can be induced to differentiate into adipose, cartilage, or skeletal muscle tissue by exposure to 5′-aza-cytidine [Taylor and Jones, 1979]. This *phenomenon* was instrumental in the elucidation of the role of bHLH genes in muscle differentiation as it was found that while spontaneous conversion did not occur, over-expression of any one of this gene family was sufficient to convert 10T½ fibroblasts to myoblasts [Weintraub et al., 1991].

The first indication of a role for fibroblasts in myogenesis came from in vitro co-culture experiments. Normal fibroblasts [Courbin et al., 1989], particularly those derived from the dermis [Chaudhari et al., 1989] were found to be capable of fusing with myoblasts derived from the muscular dysgenic (mdg) mouse. This murine disorder presents as a lack of excitation-contraction coupling in affected animals [Pincon-Raymond et al., 1985; Powell, 1990], but the fusion of normal fibroblasts with mdg myoblasts resulted in the correction of the defect. More recently, Breton et al. [1995] have shown that a mixture of mouse fibroblasts and Schwann cells derived from the sciatic nerve are capable of fusing with both mdg and normal (wild-type) myoblasts in culture. The resulting myotubes express their own myogenic genes within the multinucleate cells so formed.

In the more complex in vivo milieu, we have recently shown [Gibson et al., 1995] that dermal fibroblasts derived from a normal mouse are not only capable of fusing with muscle cells of the muscular dystrophic (mdx) mouse, but that they can also convert to a myogenic lineage. Mdx muscle fibres do not express the muscle protein dystrophin, save for a few revertant fibres [reviewed in Watt and Jones, 1996] and can be depleted of endogenous muscle presursor cells (muscle satellite cells) by X-irradiation [Wakeford et al., 1991]. When such treated mdx mice were implanted with normal cloned or uncloned mouse dermal fibroblasts, new muscle fibres arose which were dystrophin-positive. The donor origin of the new fibres was confirmed by analysis of glucose-6-phosphate isomerase isoenzyme patterns. In mdx muscle where endogenous muscle precursor cells were present, injected donor dermal fibroblasts fused with host muscle to form dystrophin-positive fibres in which both host and donor gene products were simultaneously expressed. In the same report [Gibson et al., 1995] we also demonstrated that fibroblasts derived from muscle connective tissue failed to exhibit the capacity for myogenic conversion.

Our animal work and the in vitro studies of Breton et al. [1995] are both strongly suggestive of a myogenic conversion of dermal fibroblasts when subject to a muscle cell environment. In the present paper we ask the question whether such a myogenic conversion is still possible even when dermal fibroblasts are kept physically separated from muscle cells in culture. In addition, we examine whether mouse muscle fibroblasts, which we have shown from our in vivo work not to possess a myogenic potential, will convert to myoblasts under these circumstances.

MATERIALS AND METHODS

Animals and Routine Cell Culture

The Randall Institute maintains a colony of ROSAβ-geo26 transgenic mice bred onto a CBA 129 background. These mice express β-gal staining in all cells in vivo [Friedrich and Soriano, 1991] and retain this capacity when explanted cells are cultured in vitro (personal observation; Robson and Hughes, personal communication). Dermal fibroblasts were obtained by dissection of the dorsal surface at the position of the tail root or the tail tip of E17 to E18 mice. Fragments of tissue were treated by one of two methods in order to obtain primary cultures. Enzymic disaggregation of tissue followed the methods given in Jones et al. [1990] for the separation of myogenic and fibroblastic cells with modifications to the enzyme cocktail. Briefly, disaggregation was performed in the presence of 1.4 U/ml Dispase (Boehringer Mannheim, Indianapolis, IN), 1 mg/ml Collagenase (Boehringer Mannheim), and 5 mM $CaCl_2$ in phosphate-buffered saline (PBS) for 90 min at 37°C. The tissue was dissociated with gentle trituration using a wide-bore pasteur pipette. The cell suspension was filtered through an 80 μm mesh nylon filter (Millipore, Bedford, MA) and cells were pelleted by centrifugation at 300*g* for 5

min at 4°C. Cells were resuspended from the pellet in medium consisting of DMEM (ICN-Flow Laboratories, Costa Mesa, CA), supplemented with 10% heat-inactivated fetal calf serum (Globepharm, Surrey, UK), 1% L-glutamine, 1% penicillin/streptomycin mixture (ICN-Flow). Primary cultures were obtained by seeding cells into 35 mm tissue culture dishes (Nunc, Naperville, IL) at a concentration of 2×10^4 per ml. In some instances, populations of primary fibroblasts were obtained from explant cultures of tissue fragments following the method given in Jones [1990], which selects for migratory dermal fibroblasts. In both cases cells were cultured for 4 days, by which time substantial cell numbers were available for subculture. Secondary cultures of fibroblasts were then maintained in 25 cm^2 tissue culture flasks (Nunc). Propagation of cultures followed routine practice, using trypsin-EDTA dissociation (0.25 and 0.02% mixture, respectively, in PBS; ICN-Flow) and plating densities of 1×10^4 per ml of standard medium. Aliquots of passaged cells were regularly stained for the muscle-specific intermediate filament desmin [Jones et al., 1990; Murphy et al., 1992] to detect possible contamination from myoblasts. While occasional desmin positive cells were observed from primary cultures and more rarely at passages 1 and 2, later cultures were deemed myoblast-free by this criterion. Cultures were maintained until passage 5 upon which they were discarded. All cells were positive for β-gal activity when stained by the X-Gal reaction. Dermal fibroblasts used for experimental purposes were harvested at passage 3, 4, or 5.

Purified muscle fibroblasts were prepared as previously described [Jones et al., 1990; Watt and Jones, 1993] from ROSAβ-geo26 mice or normal C57 mice. In the latter case, fibroblast cultures were infected with the mouse Moloney retroviral vector carrying the lac Z gene [Watt et al., 1993] to provide lineage-marked muscle fibroblasts.

C2C12 mouse muscle myoblasts [Yaffe and Saxel, 1977] were obtained from ATCC (Rockville, MD). Cultures were routinely maintained on gelatin-coated tissue culture plastic dishes in DMEM containing 10% fetal calf serum, 1% L-glutamine, 1% penicillin/streptomycin mixture [Pizzey et al., 1988]. Subculturing and harvesting for experiments was as described for fibroblasts. Some cultures were grown to confluence and subsequently maintained for up to 5 days in standard media until straw-yellow in appearance. The medium was collected and centrifuged at 300*g*, 4°C for 10 min. The supernatant was sterilised by passing it through a 0.22 μm low protein-binding filter (Millex GV, Millipore) to remove remaining flocculates while retaining potential active solutes, and diluted 4:1 with fresh medium. This preparation was used within 4 h as myoblast-conditioned medium (MCM) as described below.

Cell Culture

For experiments we employed Lab-Tek tissue culture chamber slides (Nunc). These slides provide multiple growth chambers on a slide base and are a convenient and reliable device for establishing isolated cell populations on a single optically satisfactory surface. We used Lab-Tek model 178599, a 16-well glass slide base format. The glass chamber culture surface was precoated with sterile 0.01% gelatin in PBS prior to use to enhance the rapid attachment of both myoblasts and fibroblasts.

The cellular composition of the 16 wells was varied in order to test for a number of combinatorial associations of fibroblasts and myoblasts. Most commonly, dermal fibroblasts were seeded into the 8 wells at one end of the chamber slide, while myoblasts were seeded into the remaining 8 wells (paired cultures). This format was modified by the introduction of a central pair of empty wells, thus separating the fibroblast and myoblast sectors by a space of 1 cm. We also doubled the separation in some trials by leaving 2 central pairs of wells free of cells (also called paired cultures). Other spatial variants used in this study included the seeding of all 16 wells of a chamber slide with either fibroblasts or myoblasts (monocultures), and the seeding of a 1:1 mixture of myoblast and fibroblast suspension into each well (co-cultures).

Proliferating myoblasts and fibroblasts were harvested from routine culture. Cell density and cell viability (trypan blue dye-exclusion assay) were estimated via haemocytometry and appropriate dilutions made into standard myoblast proliferation medium as described above. C2C12 myoblasts were adjusted to 5×10^3 cells per ml and fibroblasts were adjusted to 1×10^4 cells per ml. Cells were plated out at 200 μl per well into the chamber wells and incubated overnight at 37°C in a humid 5% CO_2 atmosphere to allow for cell adhesion and spreading onto the gelatinised glass surface.

At this stage, chamber slides were treated in one of three ways. In the majority of cases, the upper chamber unit was removed from the slide so that the individual wells were now separated from each other by a plastic gasket. The gasket has a low profile (approximately 1 mm) and is also non-adhesive for cells. The slide was then placed into a sterile container (Quadriperm, Heraeus, Surrey, UK), flooded with fresh culture medium, and left undisturbed in a tissue culture cabinet for periods up to 7 days. Under these conditions, the cell population of each well remained isolated from the populations in surrounding wells but shared the same culture medium. In other instances the upper chamber units were left *in place* at the time of medium exchange, thus blocking any possibility that wells might share a common medium. A third set of experiments was performed on dermal and muscle fibroblasts. In this case, cells were allowed to grow for 2 days so that confluent monolayers were established in each well. At this stage medium exchange was accomplished using MCM rather than fresh medium and the upper chamber units were retained. The slides were left undisturbed in a tissue culture cabinet for 7 further days.

Cytochemistry

The following antibodies were used in this study.

1. An anti-MyoD (C-20) antisera from Santa Cruz Biotechnology (cat. no. sc-304; Santa Cruz, CA), diluted in 0.1% Triton-X-100/0.5% bovine serum albumin (BSA) in PBS.
2. An anti-sarcomeric myosin heavy chain (MyHC) monoclonal antibody (A4.1025), a gift from Dr. Simon Hughes, diluted in 1% BSA in MEM containing HEPES (BSA/MEM).
3. An anti-Desmin antibody, Sigma (cat. no. D1033; St. Louis, MO), diluted in BSA/MEM.
4. Biotinylated anti Rabbit IgG was part of the Vectastain Elite ABC kit from Vector Laboratories (cat. no. PK-6101; Burlingame, CA), diluted in 0.1% Triton-X-100/0.5% BSA in PBS.
5. A TRITC-conjugated Goat anti Mouse IgG antibody, Sigma (cat. no. T5393), diluted in BSA/MEM.

5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was from Boehringer Mannheim (cat. no. 651745), diluted to a stock of 40 mg/ml in dimethyl formamide.

Cultures grown as described earlier were fixed and stained for the identification of cell markers using the following protocols.

MyoD staining. Cells were fixed in freshly made cold 4% paraformaldehyde in PBS for 5 min on ice and then permeabilised 2 × 5 min in 0.5% Triton-X-100/0.5% BSA in PBS. Exogenous hydrogen peroxidase activity was quenched by a 10 min incubation in cold 0.5% hydrogen peroxide in methanol at −20°C, which was followed by 2 × 5 min washes in 0.5% Triton-X-100/0.5% BSA in PBS. Anti-MyoD antibody was diluted 1:50 and 50 μl added to each experimental well. To control wells, 50 μl 0.1% Triton-X-100/0.5% BSA in PBS was added and slides were incubated in a humidified box for 1 h. Slides were washed 2 × 5 min in 0.5% BSA in PBS and 50 μl biotinylated anti rabbit IgG, diluted 1:200, was added to all wells and incubated for 30 min in a humidified box. Cells were washed 2 × 5 min in 0.5% BSA in PBS, incubated for 30 min in Vectastain solution (Vector Laboratories) and then washed for 10 min in PBS. Diaminobenzidine (DAB) was dissolved to 1 mg/ml in 0.1M Tris pH 7.2 and mixed 1:1 with hydrogen peroxide (4 μl 30% solution in 5 ml PBS) to give a reaction mix, in which slides were incubated for 10 min at 50 μl/well. Slides were rinsed in distilled water and the bottom gasket of the chamberslide was carefully peeled away. Slides were mounted in Entellan mounting medium (BDH) or Gelvaltol mounting medium containing DABCO antifading agent if cells were to be stained for other markers.

Sarcomeric myosin heavy chain staining. Cells were pre-fixed by adding 37°C 5% acetic acid in ethanol directly to the culture medium for 10 min. This fixative was removed and 200 μl/well cold 5% acetic acid in ethanol was added and the cells placed at −20°C for 20 min. Slides were washed 3 × 10 min in 1% BSA in MEM containing HEPES (BSA/MEM), and then 50 μl per well anti-MyHC antibody, diluted 1:2, was added to experimental wells and 50 μl BSA/MEM added to control wells. Slides were incubated for 1 h and washed 3 × 10 min in BSA/MEM. TRITC-conjugated Goat anti Mouse IgG was diluted 1:100 and 50 μl was added to all wells and incubated in a dark humidified box for 1 h. Finally, slides were rinsed 3 × 10 min in BSA/MEM, then mounted in Gelvatol containing DABCO.

Desmin staining. The protocol for this is exactly as described under Sarcomeric Myosin Heavy Chain Staining. Desmin antibody was

diluted to 1:50, and goat anti Mouse IgG TRITC was diluted to 1:100.

Staining for β-gal activity. Cells were washed for 2 min in warmed PBS containing calcium and magnesium, and then fixed in cold 0.5% glutaraldehyde in PBS for 15 min on ice. All steps after this were carried out using cold reagents, and on ice. Slides were washed in 2 mM magnesium chloride in PBS for 30 s, and then for 10 min. Cells were then incubated for 15 min in detergent solution (1 mL 1M magnesium chloride, 0.5 ml 10% sodium desoxycholate, 1 ml 10% NP-40 in 500 mls PBS). X-Gal solution was prepared by diluting stock X-Gal to a final concentration of 1 mg/ml in stain solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM magnesium chloride, 0.01% sodium desoxycholate, 0.02% NP-40 in PBS, pH 7.4). Cells were incubated in X-Gal solution for 3 h at 37°C wrapped in foil. After this incubation, slides were washed 2 × 5 min in PBS and then mounted in Gelvatol containing DABCO.

Where dual staining was carried out, the fixation protocol for β-gal preceded that for desmin/myosin. Staining for MyoD also came first in any dual staining procedure involving desmin or myosin.

All slides were observed under bright-field, phase contrast, and epifluorescence optics using an Olympus BX50 microscope or, where appropriate, an Olympus OM-U2 inverted microscope. Photographs were taken using T-max 400 film and selected negatives printed on Ilford Multigrade III paper.

RESULTS

Monocultures of Dermal Fibroblasts and Myoblasts

In order to determine the normal behaviour of fibroblasts and myoblasts when cultured under the regime described above, we examined the confluent monolayers of both cells types grown in isolation. Both cell types reached confluency by day 3 after seeding (see Materials and Methods) and were left a further 4 days before examination. Under phase contrast optics, confluent fibroblasts exhibited the well-documented standard morphology characteristic of this cell type [Simon et al., 1989]. In particular we never observed multinucleate cells, save for a few binuclear cells, nor was there any indication of syncytial masses in the cultures. Cells remained β-gal positive (Fig. 1A), but immunocytochemistry failed to detect any MyoD-positive cells, or cells expressing desmin or MyHC (Fig. 1B–D). Confluent C2C12 myoblasts largely fail to form myotubes under these conditions (Table I and Fig. 2A), but express MyoD and desmin (Fig. 2B,C). All of the few myotubes also express MyHC, but only some 20% of the mononuclear cells gave positive staining for MyHC using our protocol (Fig. 2D, Table I).

Myogenic Conversion of Dermal Fibroblasts

To investigate whether the presence of myoblasts induced dermal fibroblast conversion to a myogenic lineage we examined the expression of the muscle-specific markers, desmin, MyoD, and MyHC in fibroblasts which had been co-cultured in a 1:1 volume ratio with C2C12 myoblasts for 7 days in medium containing 10% serum. Fibroblasts were identified as β-gal positive, confirming their origin from ROSAβ-geo26 mice. The proportion of myotubes to mononuclear cells counted in these co-cultures (5%) did not appear to be significantly reduced from that measured for confluent cultures of C2C12 monocultures (Table I). This was a surprising result given that approximately half the cells in the wells were fibroblasts. In addition, some myotubes were weakly β-gal positive in patches, confirming that a proportion of the myotube nuclei must be of fibroblastic origin. Most of the cells in these cultures remained mononuclear however, and many were positively stained for MyoD and were of C2C12 morphology. A small but significant proportion of the mononuclear cells which were classified as myogenic by this staining pattern were also β-gal positive. We encountered difficulty in calculating accurate estimates of these β-gal positive MyoD-expressing cells because of the extremely high cell densities obtained in these cultures, but repeated sampling of wells gave an estimate of some 6% of the total mononuclear population.

Dermal fibroblasts cultured with myoblasts in the Lab Tek chambers according to the design outlined in Materials and Methods (paired cultures) are shown in Figure 3. As with the monocultures (Fig. 2A), C2C12 myoblasts did not generally fuse into myotubes even when in dense cultures (Fig. 3A, Table I). However, cultures of fibroblasts appeared very different from the pattern seen in monoculture (Fig. 1). In this case, structures reminiscent of myotubes as well as rounder syncytial masses were to be found at a high frequency within a background of mononuclear fibroblasts (Fig. 3B, Table I). In addition, some of the mononuclear fibroblasts ex-

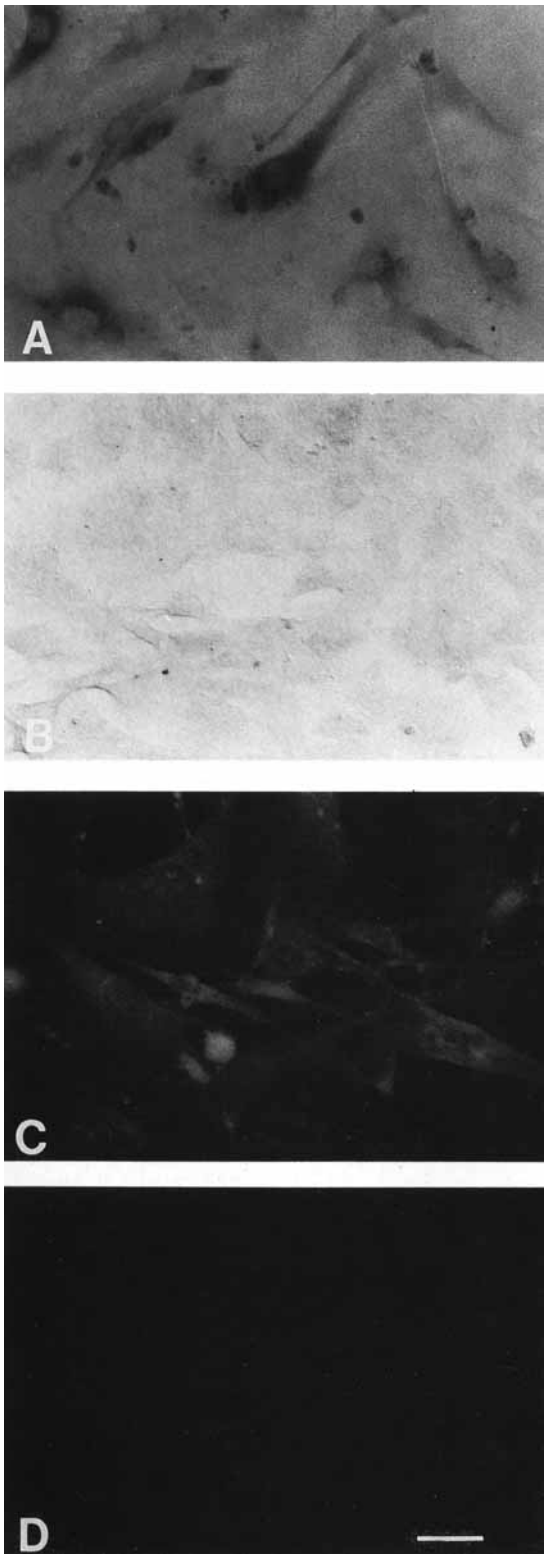

Fig. 1. Dermal fibroblast monocultures. ROSA β-geo26 mouse dermal fibroblasts cultured for 7 days form confluent cell monolayers typical of fibroblast cultures. For ease of demonstration, photographs were taken from the periphery of culture wells. Fibroblasts were β-gal positive (**A**), but negative for MyoD (**B**), desmin (**C**), and MyHC (**D**). Multinucleate cells (more than 2 nuclei per cell) were not seen, nor any rounded cells on or within the monolayer. Bar = 30 μm.

pressed significant levels of the muscle-specific transcription factor MyoD (Fig. 3C,D, Table I), though a few also expressed MyHC (Fig. 3E,F and Table I). We also noted that the frequency of "myotube" formation in dermal cultures was highest in those wells located within 2 cm distance of the C2C12 cultures on the chamber slides. However, even at the most distal regions of the slide some fibroblast conversion to a myogenic phenotype was seen (Table I).

In order to further validate these results and to begin a characterisation of the basis of myogenic conversion, we examined whether close cellular association was a prerequisite for fibroblast conversion. We prepared conditioned medium from confluent cultures of C2C12 myoblasts as described in Materials and Methods (MCM). Dermal fibroblasts grown to confluence in Lab-Tek chambers were incubated in MCM for 7 days. In the presence of MCM, confluent dermal fibroblasts (Fig. 4) again show some conversion to a myoblastic lineage as demonstrated by evidence of MyoD expression (Fig. 4A), cell alignment as a precursor to fusion (Fig. 4B), and for positive desmin staining (Fig. 4C). It was extremely difficult to obtain consistently accurate data on the proportion of fibroblasts so converted by counts of cell populations at the centre of the well because the cell density was so high, but counts recorded from the less dense perimeter of the chamber wells indicated that approximately 5% of the cells become myogenic under these culture conditions. This is a low value; nevertheless this statistic compares with a value of zero obtained when confluent dermal fibroblast monocultures are maintained for 7 days in fibroblast-conditioned medium (Fig. 1, Table I). In some cases conditioned medium (MCM) was added to cultures of low-density, rapidly proliferating fibroblast populations. Arrest of cell proliferation follows within 2 days (data not shown). Under these conditions MCM was less successful at forcing fibroblast conversion to a myogenic phenotype as assayed by MyoD expression after 7 days. In addition, no syncytia were observed. Approximately 3% of nuclei were positive for MyoD under these conditions (data not shown).

Fibroblasts cultured from neonatal mouse muscle were also tested for their capacity for myogenic conversion when grown in the paired culture arrangement. We did not observe any tendency for muscle fibroblasts to behave in a similar manner to dermal fibroblasts (Table I). Similarly we could not demonstrate the forma-

tion of "myotubes" in fibroblast wells, nor any positive staining for desmin. In addition, we have not been able to observe any effect of MCM on 7 day confluent monocultures of muscle fibroblasts (Table I).

TABLE I. Myogenic Conversion of Fibroblasts: Effects of Culture With Myoblasts and of Myoblast-Conditioned Medium*

Culture	% Myogenic mononuclear cells			% Myotubes
	MyoD	Desmin	MyHC	
Monoculture				
C2C12	>90	>95	20–25	4–5
DF	0	0	0	<1
MF	0	0	0	<1
Paired culture				
C2C12	>90	>95	18–25	4–5
DF within 2 cm	10–12	nd	3–5	12–14
DF beyond 2 cm	6–8	nd	1–2	7–8
MF (any well)	0	0	0	<1
Conditioned medium				
DF	4–5	3–4	nd	4–5
MF	0	0	0	<1

*Mononuclear cell counts were taken from 4 wells per treatment. In each well, 3 areas delineated by an eyepiece graticule were scored to give a total cell count. The same areas were then re-scored for positively stained cells and a percentage calculated from the total of 12 measurements. Because of uncertainties in the total cell count, caused by high cell densities, these figures should be regarded as best estimates rather than exact values. Calculation of % myotubes was based upon a count of the number of nuclei residing within any cell containing more than 2 nuclei divided by the total nuclear count in a field of view. Five separate fields of view for each treatment were scored. DF = dermal fibroblasts; MF = muscle fibroblasts.

DISCUSSION

The availability of tissue-specific markers and the ease of culturing primary cells has contributed greatly to the establishment of skeletal muscle as a model system in which to study differentiation. The discovery of a class of genes, the MyoD family which can convert many non-muscle cells to the myogenic lineage, has served to stimulate a recent upsurge of work into myogenesis which has led to rapid progress in our

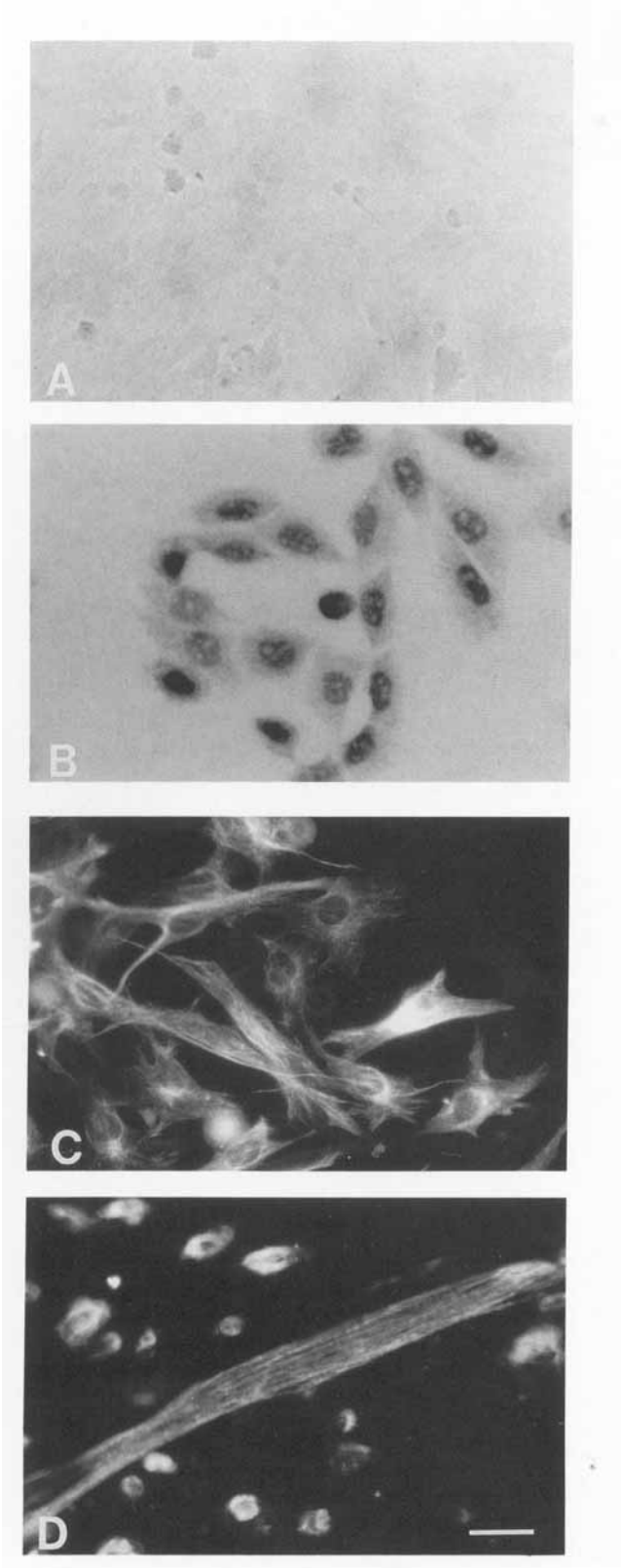

Fig. 2. C2C12 myoblast monocultures. Confluent myoblasts cultured in medium containing 10% serum show very low fusion rates even at 7 days. The cells were β-gal negative (**A**) as expected but stained well for the myogenic-specific marker MyoD (**B**), where two types of staining patterns were often recorded. A dense nuclear stain was most abundant within the confluent monolayer, while a proportion of the cells at the periphery of the well (as in this figure) show a paler staining pattern. Desmin (**C**) was abundant in all cells, but MyHC could only be detected in the few myotubes present plus some 20% of the mononuclear myoblasts (**D**). Bar = 30 μm.

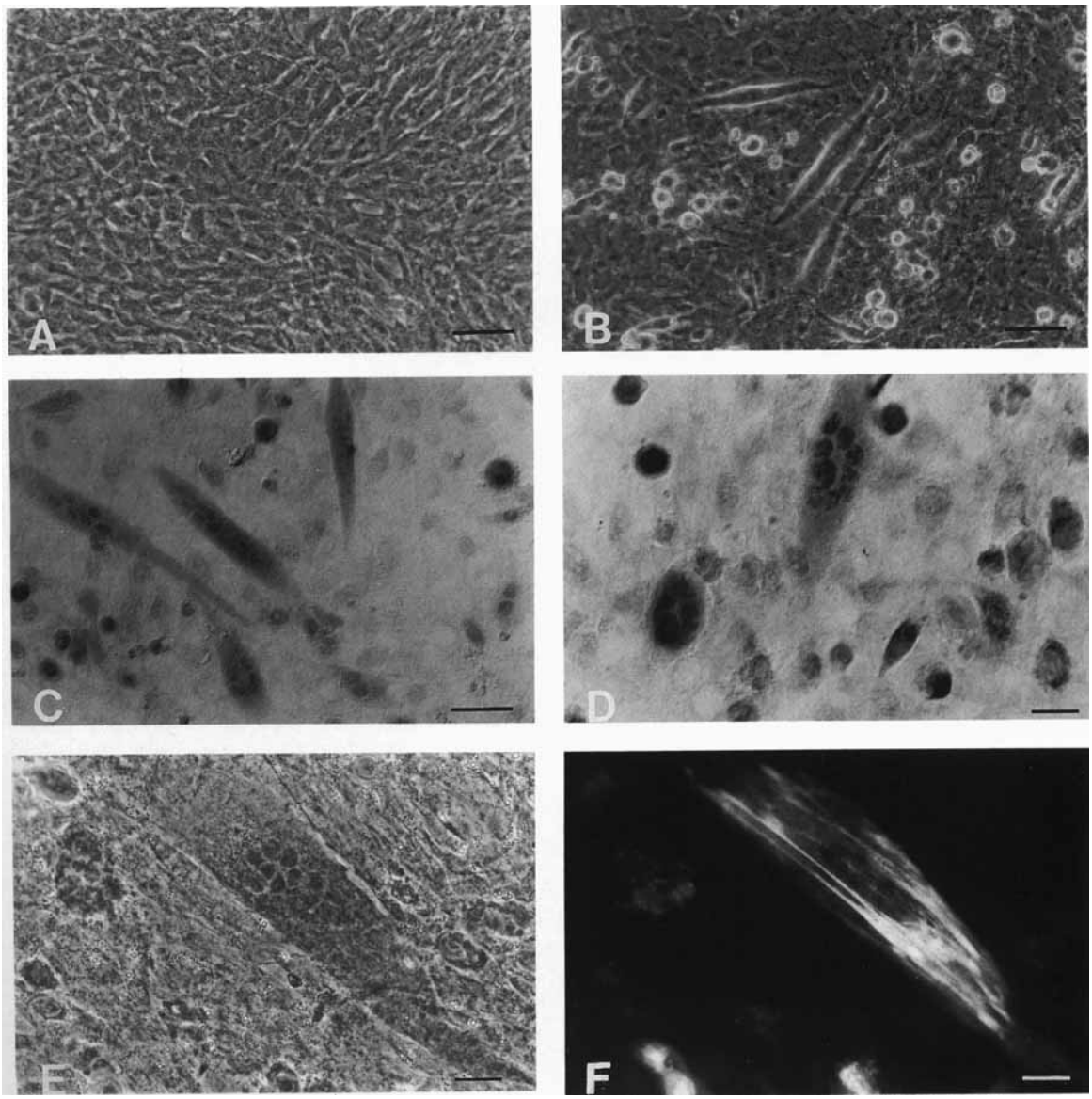

Fig. 3. Paired cultures of myoblasts and dermal fibroblasts. After 7 days, C2C12 myoblasts have formed dense confluent monolayers with little evidence of myotube formation. The cultures are mitotically quiescent and have no rounded mononuclear cells in or above the monolayer (**A**; phase contrast micrograph of live culture). In contrast, dermal fibroblasts have an abundance of rounded mononuclear cells amongst the confluent monolayer, and myotube-like structures are visible (**B**; phase contrast micrograph of live culture). Myotube-like structures stain positive for MyoD as do many of the rounded cells (**C**). Under greater magnification (**D**) the fibroblast monolayer can be seen to also contain rounded syncytial masses containing up to 10 MyoD-positive nuclei as well as a proportion of MyoD-positive nuclei in the mononuclear population. Sarcomeric MyHC expression is seen in the myotube structures, but as is shown in comparisons of micrograph **E** (phase contrast) with micrograph **F** (immunofluorescence), many of the rounded syncytia do not stain well for MyHC and very few mononuclear cells express MyHC. Bar = 50 μm for A–C and 20 μm for D–F.

understanding of myogenic specification in embryogenesis [Buffinger and Stockdale, 1995]. Evidence from in vitro studies has shown that formation of axial skeletal muscle is the product of inductive interactions between notochord, neural tube, and the paraxial mesoderm during early development [Kenny-Mobbs and Thorogood, 1987; Buffinger and Stockdale, 1995; Stern and Hauschka, 1995]. During subsequent stages, it has been generally accepted that skeletal muscles of the vertebrate body are derived from the dorsal portion of somites, the dermomyotome [Christ et al., 1977], which gives rise to two main classes of cell types, the myoblasts and the dermal fibroblasts of the dorsal region of the body [Buckingham, 1992]. It has always been assumed that at this late stage of tissue specification, myoblasts would be the only possible

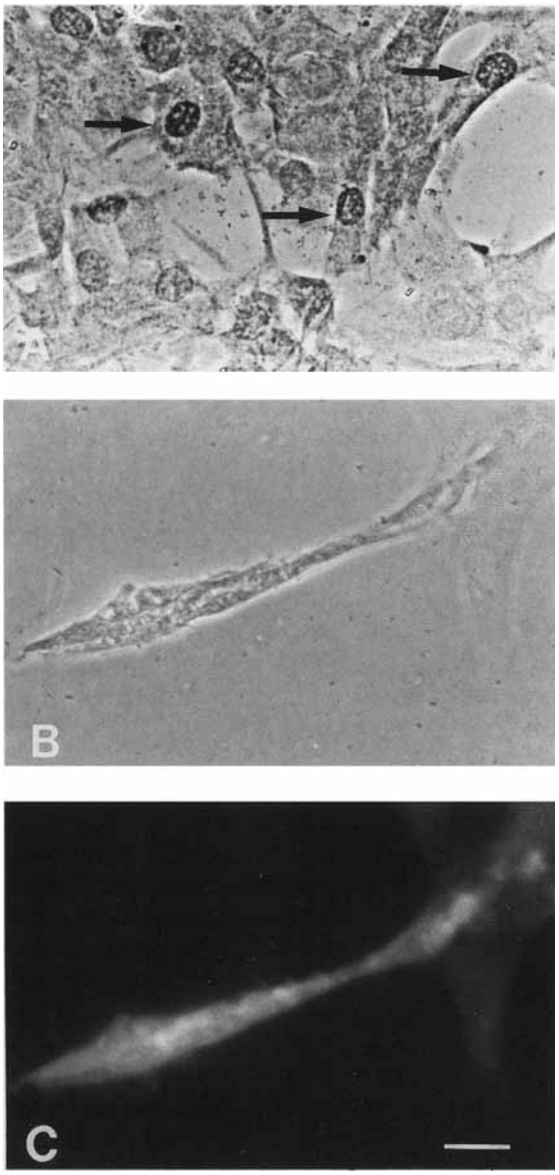

Fig. 4. Effects of myoblast conditioned medium on dermal fibroblasts. Confluent monolayers of dermal fibroblasts left for 7 days in MCM can be seen to contain MyoD positive nuclei (**A**). The 3 nuclei that are indicated in this micrograph (arrows) were scored as positive for the purpose of obtaining the quantitative data given in Table I. This may be an underestimate of the incidence of MyoD-expressing fibroblasts as at least 3 other more lightly stained nuclei are visible in this micrograph in a background of negative nuclear staining. Micrograph **B** (phase contrast) shows the alignment and possible fusion of a group of fibroblasts resident at the edge of a confluent monolayer, together with an (out of focus) group of mononuclear cells. The cells making up the aligned array of fibroblasts stain brightly for desmin, while the group of mononuclear cells does not (**C**, immunofluorescence). Bar = 30 μm.

source of skeletal muscle, so it was some time before the significance of the results reported by Chaudhari et al. [1989] and Courbin et al. [1989] became fully appreciated. Their work on the fusion of fibroblast nuclei into forming myotubes, taken together with a knowledge of their origin in the dermomyotome, suggested to us that at least some fraction of dermal fibroblasts might be capable of myogenesis. This we found to be the case in the in vivo situation where we reported the in vivo conversion of mouse dermal fibroblasts to a myogenic pathway [Gibson et al., 1995]. Using an X-irradiated mdx mouse model [Wakeford et al., 1991] in which muscle fibres are deficient in the cytoskeletal protein dystrophin [Partridge et al., 1989], we showed that implantation of normal dermal fibroblasts into mdx muscle, the genetic homologue of human Duchenne muscular dystrophy, resulted in the formation of dystrophin-positive muscle fibres. Since X-irradiation removes host muscle satellite cells, it was concluded that the only source of newly formed fibre myonuclei capable of allowing dystrophin expression were the implanted dermal fibroblasts. These results were further supported by the work of Breton et al. [1995] who showed that skin fibroblasts carrying an introduced lacZ gene driven by a myogenic promoter, which could only express β-gal in myogenic cells, did indeed express this product in the mononuclear state. This result was further evidence for the conversion of fibroblasts to myogenesis rather than merely their fusion into already existing myotubes. In the present study we set out to examine the possible conversion of dermal fibroblasts to myoblasts using in vitro methods which allow more exact manipulation of experimental variables.

Our data on monocultured cells show that confluent fibroblasts left in isolation for a week can survive without medium exchange, having reached confluency by 3 days in culture. The flattened morphology of the cells (Fig. 1) suggest that the population is mitotically quiescent and may be showing signs of starvation, but the cells retain all the characteristics of fibroblasts. Monocultures of C2C12 myoblasts (Fig. 2) grown in serum-rich media also reach confluency by day 3 from the seeding density we use, and are able to maintain a viable confluent culture for at least another 4 days. Despite the inevitable depletion of nutrients, very few multinucleate myotubes are formed in these cultures. In cocultures where cell mixing was forced, we were able to confirm the findings of Chaudhari et al. [1989], Courbin et al. [1989], and Breton et al. [1995] that a degree of cell fusion between fibroblasts and myoblasts is seen, though at quite a low frequency (Table I). The major observation reported here relates to the results obtained from paired cultures of fibroblasts and myoblasts,

where segregated colonies of cells are maintained in isolation whilst still able to share a common culture medium. The C2C12 cells retain the same attributes demonstrated in monoculture (Fig. 3A, Table I), as do fibroblasts isolated from skeletal muscle (Table I). Dermal fibroblasts show a complex set of alterations from that seen in monoculture; syncytial (multinuclear) cell masses are formed, many of which resemble myotubes in shape (Fig. 3B, Table I). In addition, there appear to be many rounded cells within the fibroblast cell mass (Fig. 3B,C) suggestive of a pattern often seen in cultures of satellite cells. The nuclei present within the syncytia are positive for MyoD, as are a number of nuclei residing within mononuclear cells (Fig. 3C,D), and sarcomeric myosin heavy chain is expressed to variable degrees within the syncytia (Fig. 3E,F). By all these criteria, a small proportion of dermal fibroblasts are thus capable of conversion to a myogenic lineage when sharing a muscle-conditioned environment. These data also show that fusion of fibroblasts, or physical contact with myoblasts, is not a pre-requiste for myogenic conversion. The subtleties of this effect were further demonstrated by our observation that fibroblast islands more distant from myoblasts were slightly less efficient at conversion (Table I). Trials with drops of methylene blue showed that the convection currents generated within the tissue culture incubator was sufficient to ensure some mixing of the medium in the Quadriperm containers within 24 h. Thus it was unlikely that simple diffusion of a putative soluble factor produced by the myoblasts was responsible for the graded efficiency of myogenic conversion. We also noted that little if any effect could be seen in the paired cultures until days 6 to 7 of culture. Before this period (specifically at day 4) dermal fibroblasts retained the characteristics seen in monoculture. Although we did not stain for MyoD at this stage, there was no evidence of the "myotube" formation or the rounded cells found in later cultures. By day 7, the fibroblast cultures show some signs of deterioration which we attribute to lack of nutrients, but the degree of conversion to a myogenic phenotype was also greatest. We could not successfully extend the cultures beyond 8 days so we cannot comment on the theoretical potential of dermal fibroblasts to convert. These data on the time-course of visible conversion do not imply that fibroblasts need constant exposure to myoblast-derived factors, as it may merely reflect on the time required to initiate muscle-specific gene activation and expression. Taken together with the observations concerning the efficiency of fibroblast conversion with distance, it is possible to predict that the myogenic activity of any putative diffusible factor is rather short-lived, such that lower levels of active agent tend to be found distant from the source, but that exposure for a short duration is sufficient to initiate the complex cascade of myogenic conversion seen in some of these fibroblasts.

As a beginning to the characterisation of the biological basis of the fibroblast conversion factor we asked whether this factor was truely a soluble factor by collecting conditioned medium from C2C12 cultures and using it immediately on cultures of fibroblasts. No effect was seen on muscle fibroblasts, but as shown in Figure 4 and Table I, dermal fibroblasts were responsive to myoblast-conditioned medium. Proliferating fibroblasts responded by ceasing mitoses and activating MyoD expression, as do fibroblasts maintained as confluent cultures (Fig. 4A). Confluent fibroblasts also showed a degree of cell alignment and desmin expression (Fig. 4B,C). We conclude that a secreted differentiation factor is produced by confluent myoblasts, but we suspect that this factor is not very stable in standard tissue culture medium. Apart from the tentative suggestions put in the discussion of the paired culture results, the only evidence we have for this conclusion is the finding that myoblast conditioned medium stored for 24 h at 4°C fails to elicit a response from dermal fibroblasts. This finding adds to our current hypothesis that the putative differentiation factor acts rapidly upon responsive fibroblasts and that there may be no requirement for constant exposure to the factor. This diffusible factor thus acts to switch cell differentiation from one pathway to another, a concept familiar to many developmental pathways [for a review see Tickle, 1991; Davidson, 1993].

While this report was being drafted, a paper appeared [Salvatori et al., 1995] which provides data in general support of the findings on co-cultures that we present here. Conversion of dermal fibroblasts was found to be stimulated by the presence of myoblasts, much as has also been reported by Breton et al. [1995]. A major difference between the two studies is that Salvatori and co-workers failed to detect fibroblast conversion in their construction of the paired culture system. The two approaches differ in many respects. They chose to co-incubate the

two cell types in medium containing low levels of serum (2%, in comparison to 10% used here). While this is routine for forcing differentiation of confluent myoblasts, low serum levels may not be the ideal choice when considering the solution stability of soluble proteins. Another source of the discrepancy could be the timespan of the paired incubation of C2C12 myoblasts with fibroblasts; these workers terminated their experiments after only 4 days of culture. We found no evidence of fibroblast conversion by day 4 in our experiments, but did so by days 6 and 7. Salvatori and co-workers also attempted to characterise the nature of the myogenic factor using the 10T½ fibroblast cell line [Taylor and Jones, 1979], rather than dermal fibroblast secondary cultures. They observed no case of fusion or differentiation of cells exposed to conditioned medium prepared from C2C12 myoblasts. We cannot comment on this result as they do not provide sufficient data or details of their protocols, but it is likely that variations in procedures are again the source of this discrepancy.

The finding that fibroblasts from the connective tissues of neonatal muscles appear to be totally unresponsive to the myoblast-derived differentiation factor not only provides strong support for our data on the conversion of dermal fibroblasts, but also demands a discussion of the embryological origin of various fibroblast populations. Dermal fibroblasts arise from a domain of the dermomyotome, the dermatome [Wachtler and Christ, 1992], whilst fibroblasts of skeletal muscle (at least the hypaxial and limb musculature) are thought to be derived from the somatopleure [Chevallier et al., 1977]. Thus it appears that all cells derived from the dermomyotome may originally have the potential to develop into muscle, and recent evidence has shown that cells of the dermatome and myotome share a novel homeobox gene expression pattern [Oliver et al., 1995]. Even after separation of the myoblast and dermal fibroblast lineages during later development, our results suggest that at least some dermal fibroblasts retain a myogenic potential into later life.

This finding could be of major therapeutic potential in disorders where skeletal muscle is grossly affected. Muscle satellite cells have long been considered an ideal vehicle for gene therapy in muscle disorders such as Duchenne muscular dystrophy [Partridge, 1991; Watt and Jones, 1996]. It has been suggested that a patient's own myogenic cells could be genetically engineered to carry the normal dystrophin gene prior to reintroduction into sites of muscle necrosis. Unfortunately, there now seems little likelihood that muscle satellite cells recruited from patients will serve as vehicles because of the limited replicative abilities of such cells [Webster and Blau, 1990]. On the other hand, dermal fibroblasts from such patients are not compromised by the disease process; they retain a high proliferative potential and are readily harvested in large numbers [Jones, 1990]. Dermal fibroblasts have also proven most amenable to genetic manipulation in many systems [Garver et al., 1987; St Louis and Verma, 1988; Axelrod et al., 1990]. In a possible therapeutic intervention for Duchenne muscular dystrophy, dermal fibroblasts could be harvested from a patient's own skin, the correct dystrophin gene inserted, and the population expanded rapidly in culture. Fibroblasts could then be implanted into the patient's muscle where they would participate in muscle regeneration. This hypothesis has already been successfully tested in one of our laboratories [Gibson et al., 1995] using the mdx mouse model and work is now in progress using human fibroblasts.

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