

Ectopic skeletal muscles derived from myoblasts implanted under the skin

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Accepted 11 September; published on WWW 28 October 1998

SUMMARY

We investigated the potential of cultured myoblasts to generate skeletal muscle in an ectopic site. Myoblasts from a clonal cell line or from expanded primary cultures were injected under the skin of the lumbar region of adult syngenic Balb/c mice. One to 7 weeks after injection, distinct muscles, of greater mass in mice injected with clonal myoblasts (6-78 mg, $n=37$) than in mice injected with primary myoblasts (1-7 mg, $n=26$), had formed between the subcutaneous panniculus carnosus muscle and the trunk muscles of host animals. These ectopic muscles exhibited spontaneous and/or electrically-evoked contractions after the second week and, when stimulated directly in vitro, isometric contractile properties similar to those of normal muscles. Histological, electron microscopical and tissue culture examination of these muscles revealed their largely mature morphology and phenotype. The fibres, most of which were branched, were contiguous, aligned and capillarised, exhibited normal sarcomeric protein banding patterns, and expressed muscle-specific proteins, including desmin, dystrophin, and isoforms of developmental and adult myosin heavy chain. Enveloping each fibre was a basal lamina, beneath which lay quiescent satellite cells, which could be stimulated to produce new muscle in culture.

Presence of endplates (revealed by α -bungarotoxin and neurofilament staining), and the eventual loss of expression of neural cell adhesion molecule and extrasynaptic acetylcholine receptors, indicated that some fibres were innervated. That these muscle fibres were of implanted-cell origin was supported by the finding of Y-chromosome and a lack of dystrophin in ectopic muscles formed after subcutaneous injection of, respectively, male myoblasts into female mice and dystrophin-deficient (mdx) myoblasts into normal C57Bl/10 muscle. Our results demonstrate that an organised, functional muscle can be generated de novo from a disorganised mass of myoblasts implanted in an extramuscular subcutaneous site, whereby the host contributes significantly in providing support tissues and innervation. Our observations are also consistent with the idea that myogenic cells behave like tissue-specific stem cells, generating new muscle precursor (satellite) cells as well as mature muscle. Subcutaneous implantation of myoblasts may have a range of useful applications, from the study of myogenesis to the delivery of gene products.

Key words: Myoblast, Myogenesis, Skeletal muscle, Subcutaneous transplantation

INTRODUCTION

Murine models have effectively demonstrated the potential and limitations of myoblast transplantation as a means of repairing or replacing muscle fibres damaged by disease (Partridge, 1991; Partridge and Davies, 1995). On the one hand, normal myoblasts from primary cultures implanted into mouse muscles can produce functional new muscle, on the other, such success is achieved only after radical treatment of the host, which may include immunosuppression of the animal, and irradiation and/or damage of the muscle into which the cells are to be implanted (Morgan et al., 1990; Kinoshita et al., 1994; Irintchev et al., 1997a). Even more remarkable results can be achieved with myoblasts from permanent clonal lines, but the period of investigation is limited by late neoplastic growth (Wernig et al., 1991, 1995). The reasons for the variable success under these different conditions remain obscure but the

results clearly indicate that there are manipulatable factors in the host muscle environment that can either promote or inhibit formation of new muscle from myogenic cells.

With this idea in mind we considered using, as a control injection site for our ongoing work on intramuscular myoblast transfer, an ectopic environment lacking the cellular, extracellular and physical topographic characteristics of mature skeletal muscle. The subcutaneous space was chosen for this purpose since it not only satisfies these requirements, but it is also easily accessible and previous experiments with minced skeletal muscle grafts have shown it can support growth and differentiation of muscle cells (Carlson, 1972). Using physiological, immunocytochemical, ultrastructural and cell culture methods, we show here that myoblasts can form, in a subcutaneous environment, distinct skeletal muscles bearing many of the morphologic and phenotypic features of normal muscle in vivo. Subcutaneous implantation could prove

valuable in evaluating the myogenicity of different myoblast cultures, as well as the immunological reactions of the host, cell survival and tumour formation (Wernig et al., 1991; Watt et al., 1991; Morgan et al., 1993; Roy et al., 1993; Huard et al., 1994; Guerette et al., 1995; Wernig and Irintchev, 1995; Fan et al., 1996), which are sometimes unpredictable when muscle serves as host (Irintchev et al., 1997b).

MATERIALS AND METHODS

Experimental design and rationale

Mice were purchased from Charles River Wiga (Sultzfled, Germany) or bred in the laboratory and kept under standard laboratory conditions. They were treated in accordance with the German law for protection of animals.

In the main set of experiments, formation of subcutaneous muscle by myoblasts derived from a clonal line and from expanded primary cultures was compared. This comparison was of interest since previous work has shown that cells from these two types of culture differ in myogenicity and tumorigenicity after intramuscular implantation (Wernig et al., 1991; Irintchev et al., 1997a). Syngenic female Balb/c mice were used as hosts in order to genetically monitor the behaviour of the primary cells, which had been derived from male donors. Likewise, male Balb/c mice were used as hosts to the clonal myoblasts, which had been derived from a female donor, as well as to the primary myoblasts. In another set of experiments, to assess the contribution of host myogenic cells to the formation of subcutaneous muscle, myoblasts derived from muscles of a dystrophin-deficient mdx mouse were injected subcutaneously into C57Bl/10 mice, which have normal dystrophin expression, and the expression of dystrophin in fibres of the new muscles which subsequently formed was used as a measure of host contribution. Finally, in a group of Balb/c animals, non-histocompatible C2nlsBAG clonal myoblasts were implanted. This was done to test whether the injected cells are rejected, and, if so, whether it is due to a cell-mediated cytotoxic reaction, as is the case after intramuscular injection.

Myoblast cultures

Myoblasts from a clonal cell line (F8B61) were previously derived from hindlimb muscles of a neonatal female Balb/c mouse (Wernig et al., 1991). They were grown on uncoated plastic Petri dishes in Waymouth's medium (Gibco BRL, Eggenstein, Germany) supplemented with 10% foetal bovine serum (FBS, PAN Systems, Aidenbach, Germany) and incubated in a CO₂-enriched (5%) humid atmosphere at 37°C.

Primary myoblast cultures (designated i28, B8, B13 and B14) were previously derived from hindlimb muscles of 4- to 14-day-old male Balb/c mice (Irintchev et al., 1997a). The cells were grown on collagen type I-coated Petri dishes in Ham's nutrient mixture F-10 (Gibco) supplemented with 20% FBS, penicillin/streptomycin (100 U/100 µg/ml, Sigma) and either with (B8, B13 and B14) or without (i28) basic fibroblast growth factor (human recombinant, 2.5 ng/ml, Pharma Biotechnology, Hannover, Germany). After 2-5 passages, the expanded primary cultures, which consisted of 95-100% desmin-positive cells, were injected subcutaneously into mice.

Cultures of mdx myoblasts were derived from approximately 120 living single fibres explanted from the extensor digitorum longus, soleus and tibialis anterior superficialis muscles of a 7-week-old mdx male mouse (Rosenblatt et al., 1995, 1996). The muscles were incubated for 1.5 hour in 0.2% w/v collagenase type I (Sigma) in Dulbecco's modified Eagle medium (DMEM). The fibres were then freed by mechanical dissociation with a wide-mouth Pasteur pipette, isolated in 24-well Falcon Primaria plates (Becton Dickinson, Heidelberg, Germany) coated with 0.1% Matrigel (Becton Dickinson) in DMEM, and incubated in DMEM containing 20% FBS (Gibco), 10% horse serum (PAA, Durham, UK) and 0.5% chick embryo extract

(Imperial, Hampshire, UK), a medium in which the myogenic cells are induced to emigrate from their muscle fibre and proliferate. Four days later, when 50-250 mononucleate cells surrounded each of the fibres, the fibres were eliminated from the culture with a drawn Pasteur pipette and the mononucleate cells were trypsinised, pooled and expanded in the same culture conditions as described for the primary myoblasts.

Finally, non-histocompatible C2nlsBAG clonal myoblasts were used. This is a β-galactosidase-expressing subclone of the established C2C12 cell line (Hughes and Blau, 1992). In muscles of histocompatible hosts (C3H or CBA/J mice, major histocompatibility complex H-2^k) the cells differentiate and express β-galactosidase for up to two years post-transplantation (Wernig and Irintchev, 1995). These cells were cultured similar to the F8B61 myoblast clone.

Cell implantations

Myoblasts were injected into 2- to 7-month-old male and female Balb/c and C57Bl/10 mice. After being expanded in culture, the cells were trypsinised, centrifuged and resuspended in Hanks' balanced salt solution (HBSS) without Ca²⁺ and Mg²⁺. Typically, 10⁶ cells, suspended in 4-20 µl HBSS, were injected into each site. Subcutaneous injections do not require the host animal to be anaesthetised, only restrained by an assistant. About half of the animals were injected in this way; the other mice were to receive, in addition to subcutaneous injections, intramuscular injections of myoblasts, and so were anaesthetised, by intraperitoneal injections of 0.4 mg/kg fentanyl (Fentanyl-Janssen, Janssen, Neuss, Germany), 10 mg/kg droperidol (Dehydrobenzperidol, Janssen) and 5 mg/kg diazepam (Valium 10 Roche, Roche, Grenzach-Wyhlen, Germany). The cell suspensions were injected subcutaneously with a 26S-gauge needle connected to a 10-µl or 100-µl Hamilton syringe (Hamilton, Bonaduz, Switzerland). The skin near the tail shaft was lifted with forceps, and the needle was then introduced into the skin about 0.5 cm from the tail and inserted 1-2 cm cranially, with care being taken to prevent the tip of the needle from penetrating the panniculus carnosus muscle above and the trunk muscles below. The cells were then injected. Often two separate injections into the same animal were made, one on either side of the midline.

Dissection of implants

At various times ranging from 7 to 300 days after injection, the animals were anaesthetised and the new tissue, when present, removed. Under a dissecting microscope, the skin with the underlying panniculus carnosus muscle on the extreme right side of the dorsal surface was cut from the hip to the rib cage. Two transverse cuts were then made through the whole dorsal surface, one just cranial to the tail, the other just caudal to the rib cage. The resultant skin-panniculus flap was bluntly separated from contact with the underlying tissues, turned like a book page from right to left, stretched, and pinned to the wax pad on which the animals had been placed. The implants were situated either on the ventral surface of the flap or on the fasciae of the trunk muscle, and were encased in a translucent, epimysium-like sheath. They were distinct, elongated masses of variable size, of which only the larger ones were attached at both ends to the host fasciae. The implants were removed and placed in small Petri dishes filled with physiologic saline where they were electrically stimulated (25 V, 0.5 milliseconds, 1 Hz) with two field electrodes to test excitability. Immediately afterwards, the implants were weighed and then prepared for physiological assessment, frozen on pieces of liver for histological and immunocytochemical analyses, embedded in synthetic resin (Epon) for electron microscopical analysis, or dissociated in collagenase for analysis of whole individual fibres.

Isometric tension measurements

The contractile properties of 5 subcutaneous muscles dissected from 5 animals 28-52 days after injection of clonal myoblasts were tested *in vitro*. The muscles were harvested without removing the

epimysium-like connective tissue in which it was embedded. A 6-0 surgical silk thread (Ethicon, Norderstedt, Germany) was tied to the connective tissue at each end of the long axis of the muscle, which was then mounted in a lucite chamber perfused with aerated Tyrode's solution heated to 25°C. One silk thread was connected to a force transducer, the other to a metal hook firmly attached to the chamber wall. Contractile measurements were made as previously described (Wernig et al., 1990, 1995; Irintchev et al., 1990). Muscles were adjusted to optimal length and stimulated with suprathreshold electrical pulses (20-25 V, 0.5 milliseconds) via silver electrodes in the bath. Variables measured included isometric twitch and tetanic (20, 50 and 100 Hz for 2 seconds) tension, twitch contraction and half-relaxation time. Acetylcholine (ACh) sensitivity was tested by rapid exchange of the normal perfusion solution with Tyrode's solution containing 50 mg/l ACh perchlorate. The amplitude of the evoked contracture was expressed as a fraction of the amplitude of a preceding 100 Hz tetanus. For comparison, the isometric contractile properties of 5 soleus muscles isolated from 5 untreated 7-month-old female Balb/c mice were also determined. Upon completion of the tension recordings, the muscles were weighed.

Histology

Six μm thick serial cryostat sections were cut parallel or transverse to the long axis of the implants. Unfixed sections were stained with aqueous Toluidine Blue-borax (both at 1% w/v) to reveal general morphology. For all other assays, sections were fixed in pure acetone or methanol. Synaptic and extrasynaptic acetylcholine receptors were stained with rhodamine-conjugated α -bungarotoxin, alone or in combination with immunostaining for either neurofilaments or the neural cell adhesion molecule (NCAM, see below; Wernig et al., 1991). Staining of DNA with bis-benzimide (Hoechst No. 33258, Sigma) was used to reveal cell nuclei. β -Galactosidase activity was assayed only in implants formed after implantation of C2nlsBAG cells according to the method of Sanes et al. (1986a).

Immunofluorescence identification of a variety of cell and tissue-specific marker molecules was achieved as described previously (Irintchev et al., 1993, 1994, 1995, 1997a,c; Wernig and Irintchev, 1995). The following primary antibodies were used: anti-desmin (clone D33, Dako, Hamburg, Germany), anti-NCAM (clone H-28, Hirn et al., 1981), anti-dystrophin (clone DYS2, Novo Castra Labs, Newcastle upon Tyne, UK), anti-laminin (clone LAM-1, ICN, Meckenheim, Germany), two antibodies which recognise T-cell subsets, anti-Ly2/CD8 (clone 53-6.7; Ledbetter and Herzenberg, 1979) and anti-L3T4/CD4 (GK 1.5; Dialynas et al., 1983), an antibody specific for macrophages, anti-MAC-1 (M1/70, Boehringer Mannheim, Mannheim, Germany), an antibody against neurofilaments (4142, Sigma) and antibodies against embryonic (clone F1-652, ATCC, Maryland, USA), slow type I (clone A4-840, DSM, Braunschweig, Germany), and fast types IIa (clone SC-71, DSM) and IIb (clone BF-F3, DSM) myosin heavy chain isoforms. In addition, an affinity-purified polyclonal antibody against M-cadherin, produced in our laboratory, was used to identify satellite cells (Irintchev et al., 1994). The antibody was raised in rabbits as described by Rose et al. (1994) and its specificity tested by western blotting, ELISA and immunocytochemistry, which showed no cross-reactivity with other adhesion molecules (NCAM, N-cadherin and E-cadherin).

Fixed sections were overlaid with normal goat serum, incubated with primary antibodies overnight at 4°C and washed in PBS. The first antibody was then indirectly labelled either with 5-((dichlorotriazin-2-yl)amino)-fluorescein (DTAF)- or rhodamine-conjugated IgG raised against the appropriate species, or with biotinylated IgG and DTAF- or rhodamine-conjugated streptavidin. All secondary antibodies were affinity purified and purchased from Jackson ImmunoResearch Laboratories (Dianova, Hamburg, Germany).

Electron microscopy

Ten muscles, removed from 7 animals either 40 or 48 days after

implantation, were prepared for electron microscopy. The muscles were fixed in 5% glutaraldehyde in 0.1 M phosphate buffer for 1 hour at room temperature and divided into small pieces (approximately $2 \times 1 \times 1$ mm) which were kept in fixative for a second hour. Following several rinses in buffer, the tissue blocks were fixed in 1% osmium tetroxide for 2 hours at room temperature, washed again, stained en bloc with 4% w/v uranyl acetate in water for 20 minutes at room temperature, and embedded in Epon (Roth, Karlsruhe, Germany). Transverse and longitudinal ultrathin sections were stained with lead citrate and observed in a Philips CM 100 electron microscope.

Analysis of individual muscle fibres and myogenic cell cultures derived from them

Individual fibres were explanted from 6 ectopic muscles removed from 5 animals 69 days after injection of myoblasts. The muscles were incubated in collagenase type I at 37°C for 3.5 hours and then their fibres liberated by repeated pipetting with a wide-mouth Pasteur pipette. Immediately after, some fibres were fixed in 4% paraformaldehyde in PBS at 37°C for 5 minutes, washed in PBS and then permeabilised by incubation in 0.3% Triton X-100 (Sigma) in PBS for 5 minutes. Following another wash in PBS, the fibres were incubated in 20% normal goat serum for 30 minutes at room temperature to reduce non-specific binding of antibody. Expression of desmin and M-cadherin in individual fibres was immunocytochemically assayed by suspending the fibres in 0.7% carrageenan (Sigma) containing M-cadherin and desmin antibody for 20 hours at 4°C, followed by incubation first with a Cy3-conjugated goat anti-rabbit IgG (for detection of M-cadherin) and then DTAF-conjugated rabbit anti-mouse IgG+IgM (for detection of desmin) for 1.5 hours each at room temperature. All antibodies were optimally diluted and each antibody incubation was followed by six washes in PBS. The fibres were then placed on microscope slides, allowed to air-dry, and mounted in Vectashield Mounting Medium with DAPI (Vector, Boehringer Ingelheim Bioproducts Partnership, Heidelberg, Germany), a DNA counterstain. Other single fibres were isolated in culture as described above for the derivation of mdx myoblasts from single fibres, grown for 1 week in 10% horse serum and 0.5% chick embryo extract in DMEM, and then fixed in equal parts ice-cold methanol-acetone for 5 minutes and immunofluorescently assayed for expression of desmin.

Determination of the origin of the ectopic muscles

In situ hybridisation with the Y-chromosome-specific DNA probe Y1 (or 145SC5; Nashioka, 1988) was used to identify male donor cells that had been implanted into female mice as previously described (Irintchev et al., 1997a). Labelling of the probe and detection of the hybridised DNA in tissue sections was performed with digoxigenin-labelling and detection kit (non-radioactive, Boehringer Mannheim). Nuclei were stained with bis-benzimide.

RESULTS

General observations

Large amounts of tissue resembling skeletal muscle had formed by 1 week after subcutaneous injection of myoblasts from clone F8B61, with little further growth during the following 6 weeks (Figs 1 and 2). Newly formed tissue was found in 96% (53/55) of the implantation sites. The implants were located between the fascia of the underlying trunk muscles and the fascia of the subcutaneous panniculus carnosus muscle, to which it was firmly attached by white aponeurosis-like ends. The muscle bellies were elongated and had oval cross-sectional profiles, but in some cases part of the implant was flat and spread in a fan-like fashion (Fig. 1). The muscles were vascularised, and they often appeared to contain

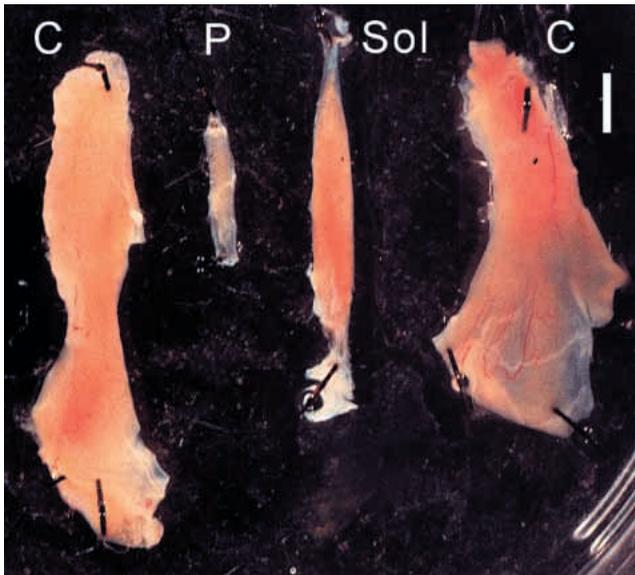


Fig. 1. Three subcutaneous muscles isolated 40 days after injection of clonal (C) or primary (P) Balb/c myoblasts. Also shown, for comparison, is an intact soleus muscle (Sol) of an adult Balb/c mouse in which the subcutaneous muscle derived from primary myoblasts (P) had grown. Muscles have been fixed with glutaraldehyde for subsequent electron microscopy (Fig. 1). Bar, 2 mm.

branches of the segmental nerves supplying the panniculus carnosus muscle (Fig. 1). They contracted *in situ* either spontaneously or when touched with the tips of forceps, and *in vitro* upon electrical stimulation with field electrodes. All of the implants which had been removed at times longer than three months after injection contained tumours (Fig. 2).

Similar results were obtained with myoblasts from expanded primary cultures, with five notable exceptions. First, these muscles were significantly smaller than those derived from clonal myoblasts (Figs 1 and 2), a result which accords with our findings from previous experiments in which these cells were injected into muscles (Wernig et al., 1991; Irintchev et al., 1997a), and which may reflect an inherently lower myogenic capacity of these cells. Second, contraction of these muscles did not occur spontaneously (but could be electrically-induced *in vitro*). Third, these muscles did not form any firm attachments with the host fasciae nor did they appear to have aponeurosis-like ends. Fourth, while implants were frequently found during the first 2 months after injection (50/68 implantation sites), at recovery times longer than 2 months implants could not be found in any of the injection sites (Fig. 2), indicating that the muscles present at earlier times after implantation eventually degenerate. Fifth, there were no tumours in any of the muscles or implantation sites, which is consistent with the behaviour after intramuscular implantation of expanded primary myoblasts (Irintchev et al., 1997a).

Contractile properties

Ectopic muscles formed 28–52 days after injection of clonal myoblasts produced large isometric twitch and tetanic tensions *in vitro*, amounting to 30–50% of those produced by normal soleus (slow-twitch) muscle from adult mice (Fig. 3). There was a deficit in tension per unit muscle mass, an indication of

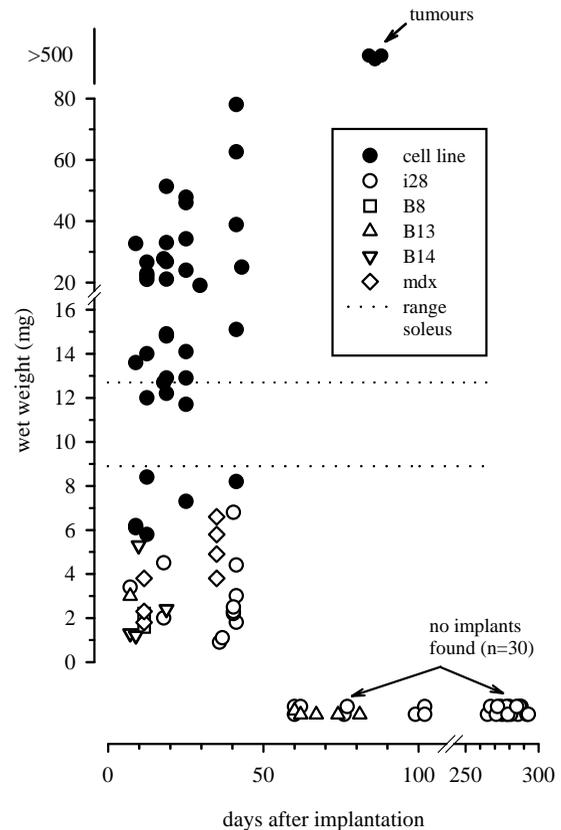


Fig. 2. Wet weight of ectopic muscles derived from clonal myoblasts (filled circles) and from expanded primary cultures (open symbols) isolated at different times after subcutaneous cell injection. Symbols below 0 on the y axis indicate implantation sites at which no implants were present. The clonal myoblasts, and the primary myoblasts from 4 different cultures (i28, B8, B13 and B14), were injected into syngenic Balb/c mice. Myoblasts derived from muscles of a mdx mouse were injected into histocompatible C57Bl/10 mice. The implants were defined as muscles on the basis of contractile activity (spontaneous or evoked) and/or histological analysis. Tumours were verified histologically. Weights of additional implants used for ultrastructural analysis or in tissue culture experiments were not measured.

a relatively large connective tissue component, and perhaps the presence of immature or misorientated fibres. The duration of peak isometric tetanic force (2 seconds), the twitch-to-tetanus ratio, and the twitch contraction and half-relaxation times were comparable to those of normal soleus muscle (Fig. 3).

The amplitudes of the contractures evoked by application of ACh, which reflect presence of extrasynaptic ACh receptors, varied largely in individual ectopic muscles (0–29% of maximum tension).

Histological features

Between one and seven weeks after injection, the formation of muscle derived from the injected cells resembled the regeneration of adult skeletal muscle *in vivo*. There was a progressive decrease with time in the number of myoblasts, small mononucleate cells expressing embryonic MyHC, desmin, NCAM (Fig. 4A) and M-cadherin, accompanied by a progressive increase in the number of myotubes and mature muscle fibres. The latter had larger cross-sectional diameters

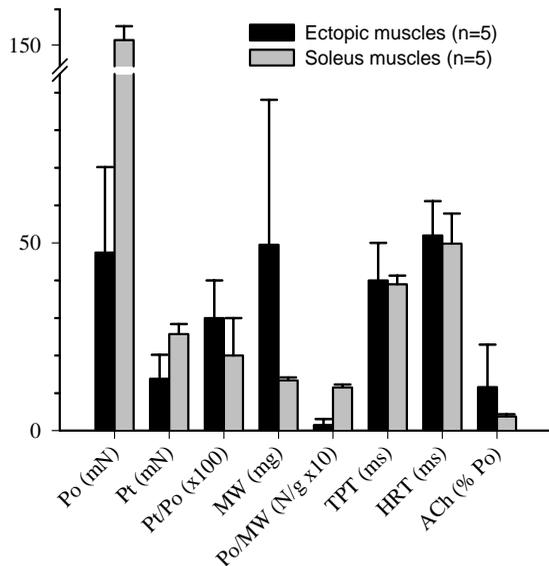


Fig. 3. Isometric contractile properties and weights of subcutaneous muscles of clonal myoblast origin. Data for untreated soleus (slow-twitch) muscles of adult female Balb/c mice are presented for comparison. The maximum twitch (Pt) and tetanic (Po) tensions produced by the subcutaneous muscles, although significant, were not commensurate with the large muscle weight (MW); this is indicated by a relatively low normalised Po (Po/MW). The twitch-to-tetanus ratio (Pt/Po), time to peak twitch tension (TPT), twitch half-relaxation time (HRT) and peak contracture evoked by ACh (expressed as a percentage of Po) were all comparable to the normal soleus muscle. Values are means + s.d.

and a characteristic pattern of staining (desmin- and dystrophin-positive, M-cadherin-negative, and NCAM-positive or negative, Figs 4B,D and 5A), and were individually enclosed by basal lamina (identified by laminin staining, Fig. 5A). Some fibres continued to express embryonic MyHC, while most others expressed adult isoforms of MyHC (types I, IIa and IIb, Fig. 6A-C). There was mingling among the different types of fibre, but, generally, there was a prevalence of one type of fibre in a given area in a cross-section of muscle. Despite being well-differentiated, the fibres in the implants were a half to a third the diameter of fibres in most normal muscles of adult mice. Their small size can be ascribed in part to functioning at a level that is insufficient to induce muscle hypertrophy (Ferry et al., 1997; Yang et al., 1997). But this is not the only reason, for we have previously observed that donor-derived muscle fibres in host skeletal muscles are also of small calibre, despite being innervated and functional (Wernig et al., 1991; Irintchev et al., 1997a); it could be that implanted myogenic cells can only produce fibres of a limited size even in the best conditions.

There were several indications that the more mature muscles were innervated. Some of the fibres did not express NCAM, a molecule known to be down-regulated in muscle fibres after innervation (Sanes et al., 1986b; Wernig et al., 1991). While there were extrasynaptic ACh receptors in some fibres, in others they were absent (Fig. 5B), which also suggests an innervation-dependent loss of extrasynaptic receptors. Staining of receptors with α -bungarotoxin revealed end-plates (Fig. 5B), and combined receptor-neurofilament staining confirmed the presence of axons, that is, innervation of the end-plates

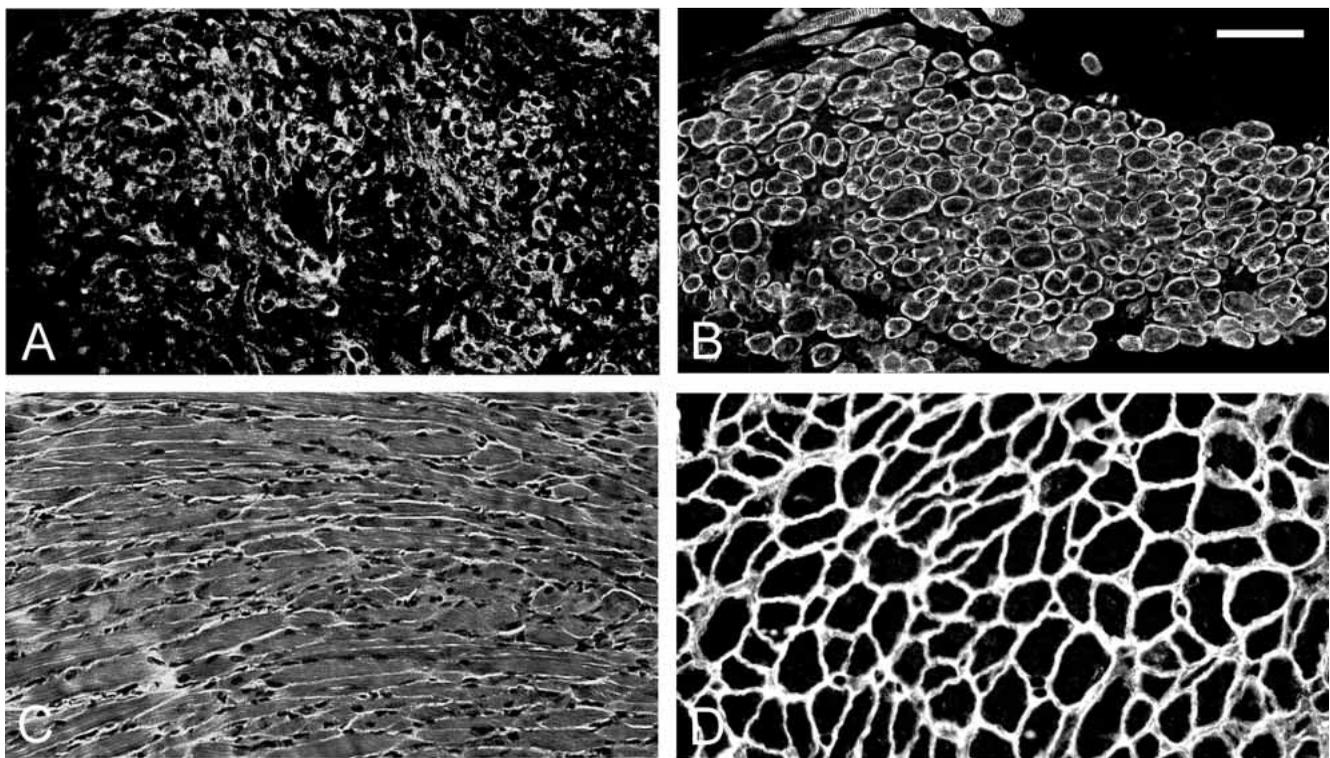
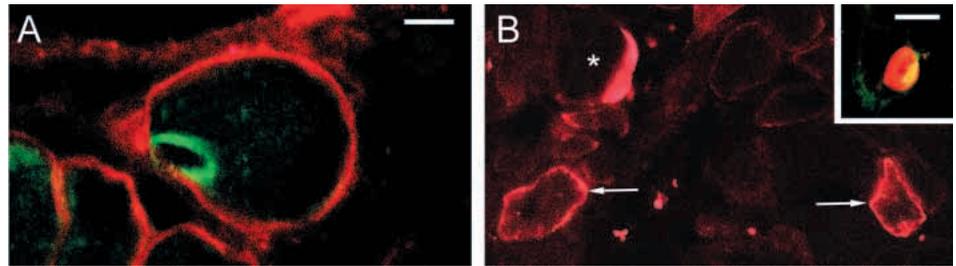


Fig. 4. Histological features of subcutaneous muscles derived from primary (A,B) and from clonal myoblasts (C,D). Transverse section from an 8-day-old implant stained with antibody against NCAM reveals loosely packed immature myoblasts and myotubes (A). Transverse sections from 45-day-old implants stained for desmin (B) and dystrophin (D), and a longitudinal section from a 46-day-old implant stained with Toluidine Blue (C) reveal contiguous and aligned muscle fibres. Bars: 40 μ m (A-C); 20 μ m (D).

Fig. 5. Satellite cells and ACh receptors in cross-sections from muscles of clonal myoblast origin, 46 days after injection. (A) Double immunofluorescence labelling showing an M-cadherin positive (green) mononucleate cell in the satellite cell position under the laminin-positive (red) basal lamina. (B) ACh receptor labelling with rhodaminated α -bungarotoxin shows two muscle fibres with extrasynaptic receptors (arrows) and another fibre bearing receptors only at an end-plate-like plaque (asterisk). The inset, made by superimposing α -bungarotoxin staining and neurofilament staining (green fluorescence) on a single frame of film, revealed that such receptor plaques are indeed innervated end-plates (axonal staining here appears yellow). Bar, 5 μ m.



(Fig. 5B, insert). Consistent with these observations was the finding of intramuscular nerve branches in tissue sections.

Mac-1-positive macrophages were present in all muscles 1-7 weeks after injection. Initially (1-2 weeks) these cells were numerous and concentrated at the surface of the muscles. At later times after injection, there were fewer Mac-1-positive cells, and they were dispersed among muscle fibres. It is not clear whether this persistence of Mac-1-positive cells is indicative of ongoing damage/regeneration of the mature implants, or whether these cells were simply resident macrophages, which also occur in normal adult muscles (Irintchev et al., 1995; Pimorady-Esfahani et al., 1997). At all times after injection, CD8- and CD4-positive cells (killer and helper T-lymphocytes, respectively) were only occasionally observed. Similar observations have been made after injection of clonal and primary myoblasts into muscles of syngenic animals (Irintchev et al., 1995, 1997b; Wernig and Irintchev, 1995).

Numerous M-cadherin-positive mononucleate cells were seen between the sarcolemma and basal lamina of muscle fibres in sections of muscles removed after longer recovery times (Fig. 5A), and on freshly isolated single fibres explanted from these muscles (Fig. 7). When living single fibres were put into tissue culture for 1 week, mononucleate cells, which expressed desmin, detached, divided and fused to form myotubes (Fig. 8). In addition, in muscles formed from clonal myoblasts, but not primary myoblasts, clusters of M-cadherin-positive mononucleate cells were observed in circumscribed areas not associated with muscle fibres or basal laminae. Similar clusters have previously been found during the early stages of tumour growth after intramuscular

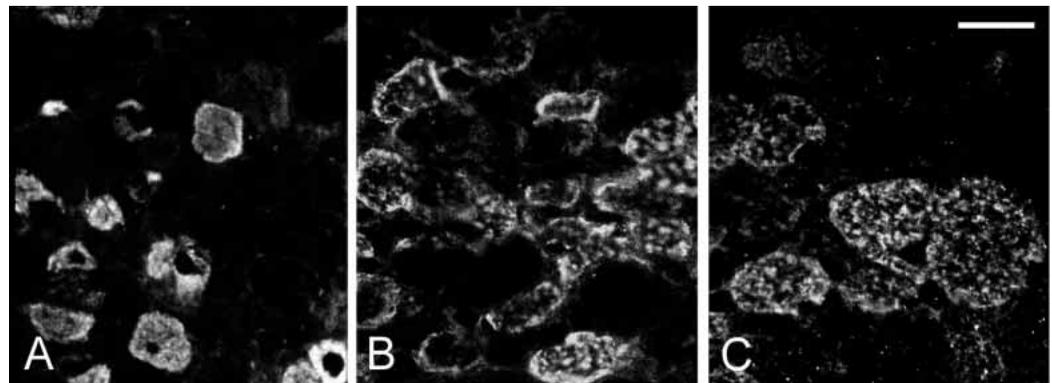
implantation of clone F8B61 myoblasts (A. Irintchev, A. Wernig, unpublished results). It would appear, therefore, that the formation of tumour by clone F8B61 cells is independent of the type of host environment in which the cells were injected.

Muscle fibre morphology

The gross structure of isolated single muscle fibres was not unlike that of normal or regenerated muscle fibres. Their length approximated the length of the muscle from which they derived and they had clearly defined regular cross-striations and subsarcolemmal nuclei, some of which bulged from the fibre surface. Most of the fibres among the population of fibres sampled were branched (Fig. 9), a feature also common in regenerated, dystrophic and even aged muscle of mice (Ontell and Feng, 1981; Irintchev and Wernig, 1987; Wernig et al., 1990; Bockhold et al., 1998). There is no evidence that branched fibres would have contributed to the functional deficits of the muscle; on the contrary, contractile properties of regenerated muscles are often normal or close to normal (Wernig et al., 1990, 1995; Irintchev et al., 1997c).

In the electron microscope, muscle fibres at different levels of maturation were observed. The majority of the muscle fibres were well-developed with defined sarcomeres showing clear A-bands, I-bands, Z-lines and, frequently, M-lines (Fig. 10A). T-tubules and their associated sarcoplasmic reticulum at the triads could be seen in longitudinal orientation in the immature fibres but in their normal transverse orientation in the majority of fibres (Fig. 10A). This finding is consistent with the reorientation observed during normal development, the T-tubules starting with a mainly longitudinal orientation and

Fig. 6. Myosin heavy chain isoforms in sections of a muscle derived from clonal myoblasts (45 days after injection). Cross-sections stained with antibodies against embryonic (A), slow (B) and fast IIa isoforms (C). The pictures show different parts of the muscle. Bar, 20 μ m.



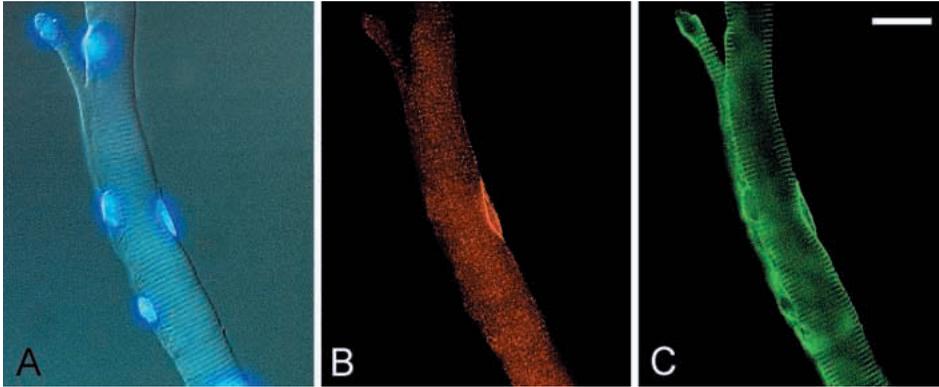


Fig. 7. Immunofluorescence detection of a satellite cell on a muscle fibre explanted from a subcutaneous muscle 69 days after injection of primary myoblasts. The fibre in A, whose nuclei are stained with DAPI, has on it a satellite cell, identified by its characteristic expression of M-cadherin (B) and desmin (C). Bar, 25 μ m.

gradually reorientating to their normal transverse position at the A-band/I-band junction. Satellite cells, with a typical quiescent appearance with minimal cytoplasm, were frequently seen in the muscle fibres in their defining position between the plasma membrane and basal lamina (Fig. 10B). Capillaries were frequently observed between muscle fibres, and fibroblasts were also positioned in the extracellular space along with small bundles or thin layers of collagen fibrils (Fig. 10B). The fibroblasts apparently originate from the host, as do capillaries, since most if not all of the injected cells were myogenic (desmin-positive).

Origin of the ectopic muscles

Contribution of the injected cells to the formation of the ectopic muscles was verified by the experiments in which myoblasts from expanded primary cultures originating from male animals were injected subcutaneously into female hosts. In situ hybridisation of tissue sections with a Y chromosome-specific DNA probe revealed numerous nuclei of implanted cell origin (Fig. 11A,B). In mature implants, most labelled nuclei were located at the periphery of muscle fibres, as in normal fibres (Fig. 11B), and not centrally, as in regenerating muscle fibres. Nuclear labelling was not observed in blood vessels and connective tissue septa indicating no contribution of the implanted cells to host-derived support tissues.

In addition to the injected cells, host cells from exogenous sites (neighbouring muscles or dermal fibroblasts, Morgan et al., 1987; Gibson et al., 1995) might also have participated in the formation of the ectopic muscle fibres. To test this possibility, dystrophin-deficient mdx myoblasts from expanded primary cultures were injected subcutaneously into C57Bl/10 mice (6 mice, 11 implants), whose muscle fibres express dystrophin (see Fig. 4D). Dystrophin was not expressed in the mdx implants isolated at 13 or 39 days after injection, times at which dystrophin was expressed in ectopic muscles formed

from dystrophin-positive myoblasts (see Fig. 4D). This clearly indicates that host myogenic cells do not contribute to muscle fibre formation in the ectopic muscles.

Implantation of non-histocompatible myoblasts

Ten days after subcutaneous injection of C2nlsBAG myoblasts into non-histocompatible Balb/c mice (MHC H-2^k and H-2^d, respectively) implants were found in all four injection sites ($n=4$ animals). However, morphological examination and immunostaining for muscle-specific marker molecules revealed that these implants lacked muscle fibres. Nor was the activity of β -galactosidase, the enzyme encoded by the lacZ reporter gene in C2nlsBAG myoblasts, histochemically detectable. The implants consisted of fibrotic and fatty tissue in which there were Mac-1-positive macrophages and T-cells of the helper (CD4-positive) and killer (CD8-positive) subsets. These findings are consistent with the observation of rapid (less than 2 weeks) and complete rejection of the same cells via a cell-mediated cytotoxic reaction when injected into the muscles of non-compatible hosts (Wernig and Irintchev, 1995). In four other animals, which were allowed to recover for 3 weeks after subcutaneous injection of C2nlsBAG myoblasts, implants could not be found ($n=8$). This indicates that the fatty and fibrotic tissue, which is present 10 days after the injection of cells, is eventually resolved.

DISCUSSION

A number of different cell and tissue types can survive in a subcutaneous compartment. Neoplastic cells thrive in this space and subcutaneous implantations are widely used in oncological studies (Manzotti et al., 1993). Parathyroid tissue autotransplanted under the skin of patients with renal hyperthyroidism also remains viable and retains its function

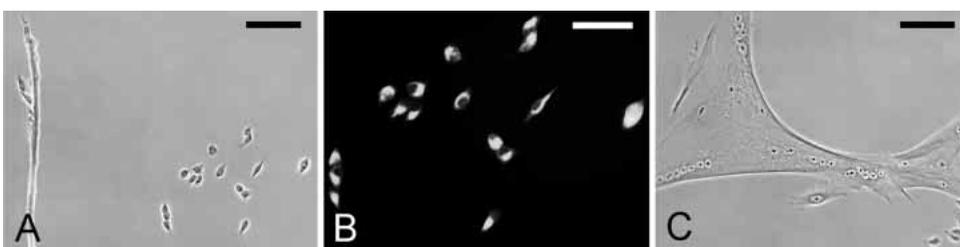


Fig. 8. Fibres explanted from subcutaneous muscles produced new muscle in culture. (A) A fibre after 5 days in culture. The cells which migrated from it and divided expressed desmin (B), a marker of myogenic cells. (C) Another culture, in which the mononucleate cells have differentiated into myotubes. Bars: 40 μ m (A,C); 70 μ m (B).

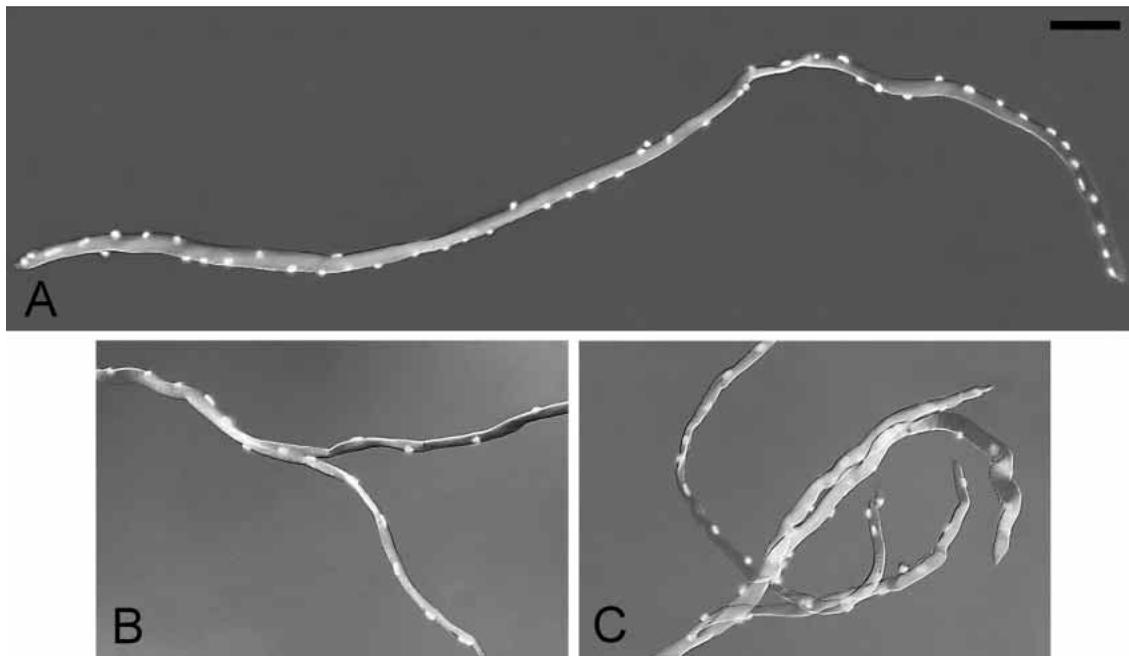


Fig. 9. Gross morphology of individual fibres isolated from 69-day-old muscles. Only a small number of the fibres were unbranched (A). 'Exploded' views revealed that most fibres appeared split (B) or were multiply branched (C). Nuclei are stained with DAPI. Bar, 75 μ m.

(Kinnaert et al., 1993). Even myogenic cells, extracted from foetal or neonatal rat heart muscle (Li et al., 1996), or trapped in a mince of rat skeletal muscle (Carlson, 1972), form functional new muscle after subcutaneous implantation. Thus, it should not be surprising that myoblasts from primary or clonal cultures can produce new muscle in an ectopic, subcutaneous environment. But just how much muscle? In view of the results of low efficiency of intramuscular myoblast transplantation (Hoffman, 1993; Partridge and Davies, 1995; Grounds, 1996), it might be expected to be small. We recently found that there is no increase in either the mass or force of mouse soleus muscle into which 10^6 myoblasts from primary cultures had been injected 1-4 months earlier (Irintchev et al., 1997a); we estimated that only 15,000 cells, 1.5% of the number injected, had been incorporated into the host muscle. Yet, in the present study, cells from the same primary cultures and of the same starting number produced, in some cases, nearly 7 mg of muscle – just under the average weight of a mature mouse soleus muscle. A possible explanation for the successful growth under the skin is the lack of host myogenic cells which might have an advantage over the foreign cells. It has been shown that when the proliferative capacity of the satellite cells in a host muscle is reduced or eliminated by X-ray irradiation or severe damage, the efficiency of subsequent implantation of primary myoblasts is significantly improved (Morgan et al., 1990; Kinoshita et al., 1994; Irintchev et al., 1997a). Though the amount of new muscle under these conditions has been estimated to be as high as 10 mg per million myoblasts (Morgan et al., 1996), which compares favourably with the present results, the yield following intramuscular injection typically is less than achieved subcutaneously. This indicates that other factors, unrelated to muscle cells (e.g. fibroblasts, extracellular matrix) in the intact, and to a lesser extent in the regenerating, muscle, inhibit

intramuscularly implanted myogenic cells. Removal or attenuation of this inhibition, by damage or irradiation of the host muscle, or avoidance of it altogether, by ectopic implantation, may reveal more fully the 'intrinsic' myogenic potential of implanted cells *in vivo*.

There are some interesting parallels in the behaviours of clonal and primary myoblasts when injected subcutaneously and into skeletal muscles (Wernig et al., 1991; Irintchev et al., 1997a). Myoblasts from the clonal line produced during the first 7 weeks after subcutaneous injection large and well-differentiated muscles which were later (at about 3 months) destroyed by neoplastic growth. When these same cells were injected into soleus muscles, they behaved similarly, producing during the first 4 months after injection large amounts of morphologically normal and functional muscle which was then destroyed by neoplastic (rhabdomyosarcoma) growth (Wernig et al., 1991). The amount of muscle produced subcutaneously by primary myoblasts, although appreciable, was 75-80% less by mass than the amount produced subcutaneously by clonal myoblasts. Intramuscular injection of these cells results in a similar disparity in the amount of muscle produced and, as in the subcutaneous compartment, an absence of neoplastic growth (Irintchev et al., 1997a). Somewhat surprising, however, in view of the finding that donor-derived muscle fibres produced after intramuscular injection of primary myoblasts survive for at least 4 months (Irintchev et al., 1997a), was the absence of subcutaneous muscles in all of the animals examined at times longer than 3 months after injection. Though the reason for this was not studied, we believe that the muscles had degenerated rather than never formed, since subcutaneous muscles were present during the first two months after injection.

Of fundamental interest is that highly organised and functional three-dimensional arrays of muscle fibres developed

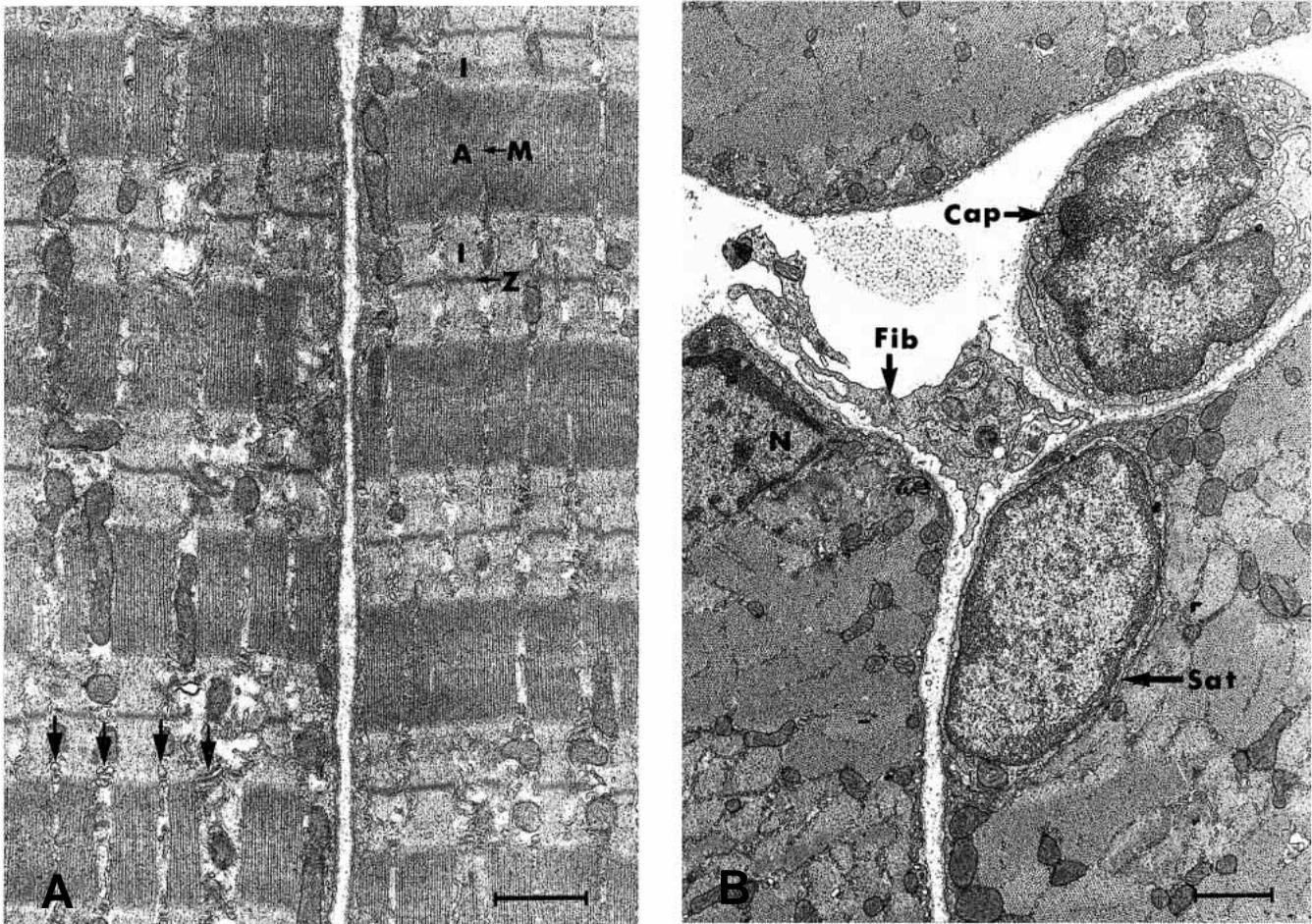


Fig. 10. Ultrastructural features of a mature implant (40 days) derived from clonal myoblasts. (A) Longitudinal section of part of two adjacent differentiated fibres. The myofibrils are composed of well-defined sarcomeres with clear A-bands (A), I-bands (I), Z-lines (Z) and faint M-lines (M) in the centre of the A-bands. The triads (arrows) have adopted the transverse orientation seen in mature fibres. (B) Transverse section showing the convergence of three adjacent fibres, one of which contains a satellite cell (Sat) which, judging by the very small content of cytoplasm, is in a quiescent phase. Also contained in this field is a myonucleus (N), capillary (Cap) and fibroblast (Fib) with associated collagen fibrils. Bars, 1 μ m.

from injected suspension of disorganised myoblasts in a non-muscle environment which must have been achieved through interactions with the host. In standard cell culture conditions devoid of exogenous morphogenetic stimuli, myoblasts display

only a rudimentary organogenic behaviour. Streams of aligned cells are characteristic of confluent myoblast cultures, and a similar local orientation of myotubes is also a feature of differentiated muscle cultures. However, when a morphogenetic

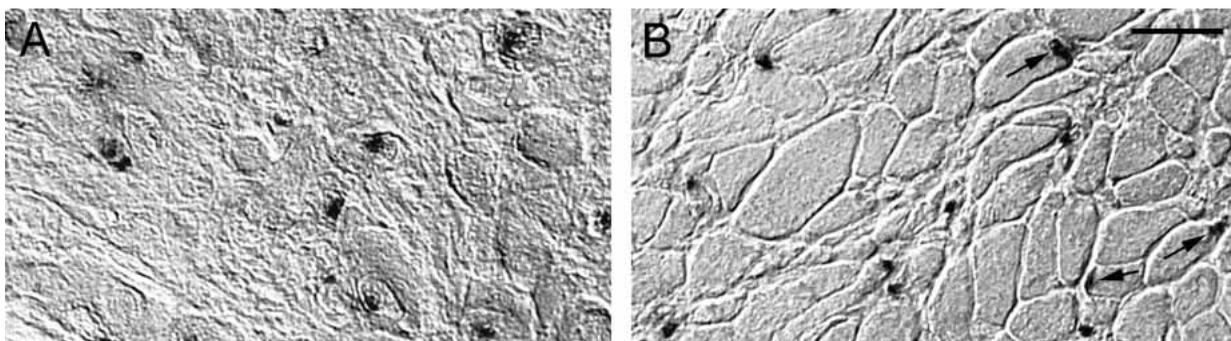


Fig. 11. Stained donor-derived (male) nuclei in two muscles formed from primary myoblasts implanted 8 (A) and 45 (B) days earlier into female hosts. The male nuclei were detected by in situ hybridisation with a Y-chromosome specific DNA probe. Note immature appearance of the early implant (A) and the peripheral localisation of labelled nuclei (arrows) in the mature implant (B). Bar, 20 μ m.

stimulus such as mechanical stretch or a substratum of orientated extracellular matrix is applied to myoblast cultures, highly organised and differentiated skeletal muscle masses, more akin to their mature counterpart in vivo, can be generated (Vandenburgh, 1988; Swadison and Mayne, 1992). The importance of stretch on the regeneration of muscle in vivo from implanted muscle fragments has also been demonstrated (Carlson, 1972). Subcutaneously, unidirectional stretch of the tissue on which the injected cells developed could have been produced simply by the continuous heaving of the diaphragm. Such a stimulus could orientate the developing myoblasts and at later times, when the muscle had formed firm attachments to host fasciae, induce hypertrophy of the fibres (Vandenburgh, 1983; Yang et al., 1997). Apart from mechanical stimuli, the host contributed, via unknown mechanisms, to the implants by supplying vascular, connective and nervous tissue. Sufficient vascularisation, and in particular capillarisation, is an obvious requirement for survival, differentiation and functional activity of muscle. Equally important for the establishment of organised muscle is the presence of connective tissue cells which are required for the formation of muscle fibre basal laminae and provide other extracellular matrix proteins and growth factors (Sanderson et al., 1986; Grounds, 1991; Swadison and Mayne, 1992). Finally, the advanced development of the ectopic muscles was certainly promoted by the presence of innervation, a factor of well known significance in muscle development and regeneration (Gutmann, 1962; Anzil and Wernig, 1989).

There is now abundant evidence to support the contention that satellite cells in vivo appear capable of not only forming new muscle but of self-renewal, characteristics which, in addition to them being quiescent under normal conditions, make them candidates for tissue-specific stem cells (see Morrison et al., 1997, for a recent review on stem cell properties). Satellite cells retain these stem-cell-like properties even when extracted from muscle and put into tissue culture, where they will divide, differentiate and form myotubes as well as produce quiescent mononucleate cells capable of re-entering the cell cycle (Quinn et al., 1984; Baroffio et al., 1996). When returned to muscle, some of these cells will form new muscle fibres or fuse with existing host fibres, and some will integrate with fibres as quiescent satellite cells (Morgan et al., 1994; Irintchev et al., 1997a). Our results demonstrate that this also occurs in the subcutaneous space. While most of the progeny of the injected cells fused to form differentiated muscle fibres, a small fraction became satellite cells. In cross-sections, these cells could be seen between the basal lamina and sarcolemma, exhibited a quiescent morphology and expressed M-cadherin, a satellite cell marker. Cells expressing M-cadherin and desmin, another marker of myogenic cells (Kaufman and Foster, 1988), were also seen on isolated single fibres. These apparently quiescent cells retained their myogenic potential in tissue cultures, where they could be stimulated to migrate from the muscle fibre, divide and form myotubes.

The most salient feature of the subcutaneous model is its simplicity. Cells can be easily and quickly implanted, and large amounts of new muscle are rapidly generated without the need to pretreat the site, as is the case when a muscle serves as host. Although still in its infancy, the model has a range of potential applications. It should enable the study of myogenesis using myoblasts isolated from normal or diseased animals, as well as testing the effects of various treatments on the development of

these cells in vivo. Since the behaviour of cells in the subcutaneous space qualitatively mimic their behaviour when injected into muscle, the model could be used as an in vivo testing ground for the myogenic, tumourigenic and immunogenic properties of cultured myoblasts prior to their therapeutic use in myoblast transfer therapy (Partridge, 1991). Myoblasts can also be genetically engineered to form muscles which synthesise and secrete therapeutic non-muscle proteins into the systemic circulation (Partridge and Davies, 1995). The subcutaneous space could be an appropriate site for the development of such modified cells and the delivery of their protein product. Finally, muscle generated subcutaneously could be used as a graft for the functional restoration of damaged or missing small muscles.

This study was supported by grants from the European Commission (BMH4-97-2767) to A.W. and M.J.C. and the Deutsche Forschungsgemeinschaft (We859/7) to A.W. J. David Rosenblatt is a Fellow of the Alexander von Humboldt Foundation. The authors gratefully acknowledge the technical assistance of Stefanie Briel, Jennifer Heckroth, Jutta Krischler, Michael Bone and John Walsh.

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