Functional improvement of damaged adult mouse muscle by implantation of primary myoblasts

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1. Myoblasts from expanded primary cultures were implanted into cryodamaged soleus muscles of adult BALB/c mice. One to four months later isometric tension recordings were performed *in vitro*, and the male donor cells implanted into female hosts were traced on histological sections using a Y-chromosome-specific probe. The muscles were either mildly or severely cryodamaged, which led to reductions in tetanic muscle force to 33% ($n = 9$ muscles, 9 animals) and 70% ($n = 11$) of normal, respectively. Reduced forces resulted from deficits in regeneration of muscle tissue as judged from the reduced desmin-positive cross-sectional areas (34 and 66% of control, respectively).

2. Implantation of 10^6 myogenic cells into severely cryodamaged muscles more than doubled muscle tetanic force (to 70% of normal, $n = 14$), as well as specific force (to 66% of normal). Absolute and relative amounts of desmin-positive muscle cross-sectional areas were significantly increased indicating improved microarchitecture and less fibrosis. Newly formed muscle tissue was fully innervated since the tetanic forces resulting from direct and indirect (nerve-evoked) stimulation were equal. Endplates were found on numerous Y-positive muscle fibres.

3. As judged from their position under basal laminae of muscle fibres and the expression of M-cadherin, donor-derived cells contributed to the pool of satellite cells on small- and large-diameter muscle fibres.

4. Myoblast implantation after mild cryodamage and in undamaged muscles had little or no functional or structural effects; in both preparations only a few Y-positive muscle nuclei were detected. It is concluded that myoblasts from expanded primary cultures – unlike permanent cell lines – significantly contribute to muscle regeneration only when previous muscle damage is extensive and loss of host satellite cells is severe.

It has long been known that whole muscles and muscle minces can be transplanted from animal to animal with considerable success (for review see Carlson, 1972). In contrast, transplantation of cultured myogenic cells (MCs) has been generally much less efficient in both humans and animals (Hoffman, 1993; Partridge & Davies, 1995). This difference may be due to the effects of incubation on the behaviour and viability of cultured myoblasts *in vivo* as it has been found that the vast majority of cultured myoblasts die soon after implantation, whilst non-cultured myoblasts from muscle slices survive for long periods after transplantation (Huard, Acsadi, Jani, Massie & Karpati, 1994; Fan, Beilharz & Grounds, 1996).

Implantation of normal MCs has been considered as possible therapy for genetic muscular disorders, and, in addition, genetically engineered myoblasts might deliver missing gene products (for reviews see Partridge, 1991; Hoffman, 1993; Partridge & Davies, 1995). In all cases, the fate of the implanted cells in terms of degrees of survival and proliferation, fusion capacity, immunogenicity and the possibility of malignant growth in animal models are of interest, especially in view of the inefficacy of myoblast implantations revealed in clinical trials with Duchenne dystrophy patients (Gussoni *et al.* 1992; Huard, Roy, Bouchard, Malouin, Richards & Tremblay, 1992; Karpati *et al.* 1993; Tremblay *et al.* 1993; Mendell *et al.* 1995). Previous studies with permanent mouse muscle cell lines in our laboratory have provided evidence for formation of muscle tissue *in vivo*, innervation of the newly formed muscle fibres and contribution to the contractile strength of the implanted muscles, but also formation of tumours at later stages (Wernig, Irintchev, Härtling, Stephan, Zimmermann & Starzinski-Powitz, 1991*a*). MCs from primary cultures have been shown to survive and differentiate after implantation in the mouse (Watt, Lambert, Morgan, Partridge & Sloper,

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1982; Morgan, Hoffman & Partridge, 1990; Huard, Labrecque, Dansereau, Robitaille & Tremblay, 1991; Rando & Blau, 1994; Huard *et al.* 1994) but functional effects have not been investigated. Only one study shows improvement of contractile parameters of damaged and X-ray irradiated muscles in the rat (Alameddine, Louboutin, Dehaupas, Sebille & Fardeau, 1994). It should be pointed out that in most previous studies in the mouse, mixtures of different cell types, rather than pure myoblast cultures, have been implanted. Methods of obtaining mouse myoblast cultures with high purity have only recently been developed and used for implantation (Rando & Blau, 1994; present study). The present investigation concerns the question of whether, and under what circumstances, implanted primary MCs contribute to contractile force in adult muscle. In particular, we have examined the role that existing muscle damage plays in promoting the incorporation of new cells into regenerating muscle.

METHODS

Animals and animal care

Inbred BALB/c mice, purchased from Charles River Wiga (Sultzfeld, Germany) or bred in the laboratory and kept under standard laboratory conditions, were used for experiments. Donor satellite cells were isolated from 4- to 13-day-old male animals, grown in culture and then implanted into female animals aged 2–7 months. The sex of the donor animals was determined first by examination (longer anogenital distance in males) and later verified by *in situ* hybridization of cultured cells or cryostat sections from donor liver with a Y-chromosome-specific probe (see below). All animal procedures were performed in accordance with the German law for protection of experimental animals.

Preparation of primary myoblast cultures

Young animals were killed by cervical dislocation, dipped briefly in cool alcohol (4 °C, 70% v/v), fixed on a disinfected pad and washed again with alcohol, then with cool (4 °C) sterile phosphate-buffered saline (PBS; composition (mM): 137 NaCl, 2·7 KCl, 8·1 Na_2HPO_4, 1·5 KH_2PO_4) containing penicillin G–streptomycin (1000 U ml^{-1} and 1000 $\mu g\ ml^{-1}$, respectively; Sigma), and amphotericin B (1·25 $\mu g\ ml^{-1}$, Fungizone; Gibco). Under a dissecting microscope, the hindlimb skin was removed, the musculature dissected out and placed in a 60 mm diameter plastic tissue culture dish (Falcon, Becton Dickinson, Heidelberg, Germany) containing 5 ml Hanks' balanced salt solution (HBSS; composition (mM): 137 NaCl, 0·5 KCl, 0·3 Na_2HPO_4, 0·4 KH_2PO_4, 4·2 $NaHCO_3$, 5·0 D-glucose, 25 Hepes) supplemented with Ca^{2+} (0·1 mM $CaCl_2$) and Mg^{2+} (0·5 mM $MgCl_2$, 0·4 mM $MgSO_4$). The tissue was minced with fine scissors under a lamina flow hood to a diameter of 1 mm or less, then dissociated in a mixture of 2 ml collagenase solution (0·07% w/v in HBSS, 385 U ml^{-1}, Type Ia; Sigma) and 6 ml Ham's nutrient mixture (F10 medium; Gibco) supplemented with penicillin–streptomycin (100 U ml^{-1} and 100 $\mu g\ ml^{-1}$, respectively; Sigma) for 30 min in a cell culture incubator (37 °C, 5% CO_2). The suspension was transferred to a 50 ml plastic tissue culture tube (Greiner, Solingen, Germany), and triturated using a 10 ml plastic pipette (30 times in and out) and then allowed to settle. The supernatant, containing mostly desmin-negative (non-muscle) cells (see Quality control of myoblast cultures), was discarded. The pellet was resuspended in 5 ml crude trypsin solution (1 : 250; Serva, Heidelberg, Germany) diluted to 0·25% (w/v, i.e. 5·5 U ml^{-1} with HBSS), incubated in a shaking water-bath (37 °C; shake frequency, ~2 s^{-1}) for 10 min and triturated with a plastic pipette as above. After trypsin activity was blocked by addition of 5 ml F10 medium supplemented with 20% fetal bovine serum (FBS; PAN Systems, Aidenbach, Germany) and penicillin–streptomycin (100 U ml^{-1} and 100 $\mu g\ ml^{-1}$, respectively), the cell suspension was filtered through sterile 40 μm polyamide nylon mesh (neoLab, Heidelberg, Germany), centrifuged (250 *g* for 15 min at room temperature, 20–25 °C) and the pellet resuspended in 3 ml F10–FBS–antibiotic solution. Cells were seeded in 60 mm diameter dishes coated with collagen Type I (3 mg ml^{-1}, collagen S; Boehringer Mannheim, diluted 1 : 10 with 0·1 N HCl) and incubated at 37 °C in CO_2 (5%)-enriched air.

Myoblast culture growth

Initially, growth medium consisted of Eagle's minimum essential medium supplemented with D-valine (80%; Gibco), FBS (20%) and antibiotics. More recently, F10 medium (80%) supplemented with FBS (20%), antibiotics and basic fibroblast growth factor (bFGF, human recombinant, 2·5 ng ml^{-1}; Serva, Heidelberg or Pharma Biotechnology, Hannover, Germany) has been used (Rando & Blau, 1994).

Sixteen to twenty-four hours after initial plating most of the myogenic cells (desmin positive and small in diameter; see below and also Baroffio, Aubry, Kaelin, Krause, Hamann & Bader, 1993) remain unadhered whilst numerous fibroblast-like cells (desmin negative and large in diameter) spread on the bottom of the dish. The non-adherent cells were collected by aspiration and replated. The culture medium was changed every 2–3 days and if numbers of fibroblast-like cells appeared to increase preplating (Richler & Yaffe, 1970) was performed. Within 7–10 days cultures typically consisted of >90% desmin-positive myogenic cells and the cell numbers derived from the hindlegs of each neonatal mouse amounted to 10^6. During the first 10 days culture growth was not apparently dependent on presence of bFGF or the type of medium. After this period cell proliferation ceased or was much reduced, but after 2–3 weeks active proliferation resumed and the culture was expanded to more than 10^7 cells. The period of restrained culture growth appeared to be shorter when bFGF was present in the medium and we now routinely use F10 medium supplemented with bFGF (Rando & Blau, 1994) for culture growth. Unless otherwise indicated, the cells used for implantations were passaged 2–6 times. The potential for expansion of these cell cultures was large: three preparations were cultured up to passage 40 without loss of their proliferative capacity.

Quality control of myoblast cultures

Culture purity. Double staining of samples for desmin and cell nuclei (see below) was routinely performed to control purity of myogenic cell cultures from the point of tissue dissociation to the time of implantation. Quantification was performed as follows: fields containing cell nuclei (bis-benzimide staining, UV filter) were selected at random at high magnification (×500, Axiophot; Zeiss) and counted, after which the fluorescence filter was changed to visualize desmin staining (rhodamine excitation) and desmin-positive cells were counted. Whenever possible, at least 100 (typically 150–200) cell nuclei per preparation were evaluated. The cell cultures used in this study consisted of 94–99% desmin-positive cells.

Differentiation. To test the potential of the cultured cells to form myotubes, prior to each implantation a fraction of the cells was seeded into 35 mm diameter collagen-coated Petri dishes and cultured in growth medium until 70–80% confluency was reached. Growth medium was then replaced by differentiation medium consisting of 98% Dulbecco's modified Eagle's medium (4·5 g l^{-1}

glucose; Gibco), 2% horse serum (PAN Systems), and penicillin–streptomycin. Between 3 and 7 days after medium switch, cultures were examined daily with phase-contrast optics (×400, Axiovert; Zeiss) for the presence of multinucleated cells. In all cultures numerous myotubes, some of which contracted spontaneously, were observed.

Control for micro-organism contamination of cultures. Cultures were routinely checked under the microscope for signs of contamination with micro-organisms. In addition, cells from each culture were grown for 2 weeks without antibiotics to reveal possible latent bacterial infection. Bis-benzimide (Hoechst 33258; Sigma) staining of monolayer cultures was used to detect mycoplasma contaminations.

Cell implantations

Cell implantations were performed as previously described (Wernig *et al.* 1991*a*; Wernig, Irintchev & Lange, 1995). Surgery was done under neuroleptanalgaesia with fentanyl (Fentanyl-Janssen; Janssen, Neuss, Germany), droperidol (Dehydrobenzperidol; Janssen) and diazepam (Valium 10 Roche; Roche, Grenzach-Wyhlen, Germany) at doses of 0·4, 10·0 and 5·0 mg kg^{-1} I.P., respectively. The soleus muscle of the right limb was exposed along its entire length and frozen by a single application of the flat end (3·0 mm × 0·7 mm) of a copper rod pre-cooled in liquid nitrogen (cryode) onto the muscle surface midway between the tendons for 10 s (mild cryodamage). Immediately after thawing each muscle was implanted with myoblasts (4 μl cell suspension containing 1×10^6 cells in HBSS without Ca^{2+} and Mg^{2+}; $n = 13$ muscles, 13 animals) or injected with HBSS (4 μl, control, $n = 11$). After implantation wounds were closed with 7-0 Ethilon polyamide threads (Ethicon, Norderstedt, Germany). The animals were kept warm for several hours on a thermostatically controlled plate (37 °C) and allowed to recover.

In two other groups freeze–thawing of the soleus muscle was repeated 3 times to induce severe muscle damage. During each freezing stage, the cryode was placed first on the proximal half of the muscle for 10 s and then immediately moved to the distal half for 10 s. After the final thawing of the muscle, the animals of one group ($n = 14$) were each implanted with 10^6 myoblasts and those in the other (control) group ($n = 9$) were injected with HBSS only.

Finally, in some animals ($n = 6$) undamaged soleus muscles were injected with myoblasts.

In vitro isometric tension measurements

Contraction measurements were performed 1–4 months after cryodamage and cell implantation or cryodamage alone as previously described (Irintchev, Draguhn & Wernig, 1990; Wernig, Irintchev & Weisshaupt, 1990; Wernig *et al.* 1991*a*). Under neuroleptanalgaesia (see above), soleus nerve–muscle preparations were dissected out. Muscles were mounted in Lucite® chambers perfused with aerated Tyrode solution (composition (mM): 125 NaCl, 1·0 $MgCl_2$, 1·8 $CaCl_2$, 5·4 KCl, 24 $NaHCO_3$, 10 D-glucose) and connected to a force transducer. Muscle contractions were evoked either directly via a pair of silver electrodes in the bath or indirectly via nerve suction electrodes. Muscle length was adjusted so that maximal twitch tension was produced upon single stimuli. Voltage amplitudes were set to twice the lowest values sufficient for maximum twitch stimulation (final values 20–25 V for direct and 4–6 V for indirect muscle stimulation). Single pulses (0·5 ms duration for direct and 0·1 ms for indirect stimulation) and tetani (20, 50 and 100 Hz for 2 s) were used for stimulation.

The temperature was kept at 25·0 ± 0·5 °C throughout the measurements. Muscles and nerves were stimulated alternately with single pulses and tetani in a sequence which was kept constant in all experiments. A minimum of 3 min was allowed for muscle recovery between stimulations. Signals were stored in a digital oscilloscope (model HM 208; Hameg, Frankfurt, Germany) and plotted on paper.

ACh sensitivity was tested by rapid exchange of the normal perfusion solution with ACh-containing Tyrode solution (5 and 50 mg l^{-1} acetylcholine perchlorate; Sigma). The amplitude of the evoked contracture was expressed as a fraction (%) of the amplitude of a preceding tetanus (100 Hz).

After the contraction measurements muscles were gently blotted and weighed with parts of the distal and proximal tendons present.

Histological procedures

Both soleus muscles of each animal were fixed at approximately resting length on a piece of formalin-fixed turkey liver and frozen in isopentane pre-cooled in liquid nitrogen (Irintchev, Zweyer & Wernig, 1995; Wernig *et al.* 1995). Serial cross-sections (6 μm thickness) were collected on chrome–gelatin or silane-coated slides. Cell smears or cells grown on coverslips were used for staining of cultured cells.

Histology and histochemistry. For general histology sections were stained with an aqueous solution of Toluidine Blue (1% w/v) and borax (1% w/v). To visualize endplates, stainings for Acetylcholinesterase (AChE) activity and ACh receptors were performed (Wernig *et al.* 1991*a*). Staining of DNA with bis-benzimide was used to reveal cell nuclei, and, in cultured cell preparations, mycoplasma contamination.

Immunofluorescence. The following primary monoclonal antibodies were used: anti-desmin (clone D33; Dako, Hamburg, Germany; purified mouse IgG diluted to 1 μg ml^{-1}), anti-NCAM (neural cell adhesion molecule; clone H-28; Hirn, Pierres, Deagostini-Bazin, Hirsch & Goridis, 1981; rat IgG, hybridome supernatant diluted 1 : 100), anti-dystrophin (clone DYS2; Novo Castra Labs, Newcastle-upon-Tyne, UK; mouse IgG, 1 : 20) and anti-laminin (clone LAM-1; ICN Biomedicals, Eschwege, Germany; purified rat IgG, 5 μg ml^{-1}). Affinity-purified polyclonal antibody against M-cadherin was raised in rabbits as described by Rose *et al.* (1994) and its specificity was proved using Western blots, enzyme-linked immunoabsorbent assays and immunofluorescent stainings (data not shown). No cross-reactivity with other adhesion molecules (NCAM, N-cadherin and E-cadherin tested) was detected.

Affinity-purified biotin-conjugated goat anti-rat, anti-mouse and anti-rabbit IgG; rhodamine-conjugated anti-mouse and anti-rat IgG; normal sera and IgGs; and 5-((dichlorotriazin-2-yl)amino)-fluorescein (DTAF)–streptavidin were purchased from Jackson Immunoresearch Laboratories (Dianova, Hamburg, Germany).

Immunofluorescent staining procedures have been described previously in detail (Wernig *et al.* 1991*a*; Irintchev, Salvini, Faissner & Wernig, 1993; Irintchev, Zeschnigk, Starzinski-Powitz & Wernig, 1994; Irintchev *et al.* 1995). Briefly, after acetone or methanol fixation and blocking of non-specific binding with normal serum, sections were incubated with primary antibodies (overnight, 4 °C), and after washing with PBS reacted with either rhodamine-conjugated second antibody or with biotin-conjugated second antibody followed by DTAF-streptavidin.

***In situ* hybridization.** *In situ* hybridization with the Y-chromosome-specific DNA probe Y1 (or 145SC5; Nashioka, 1988) was used to identify male donor cells implanted into female animals (Grounds, Lai, Fan, Codling & Beilharz, 1991). Y1/pGEM7 plasmid DNA expressed in *Escherichia coli* was digested with *Eco*RI

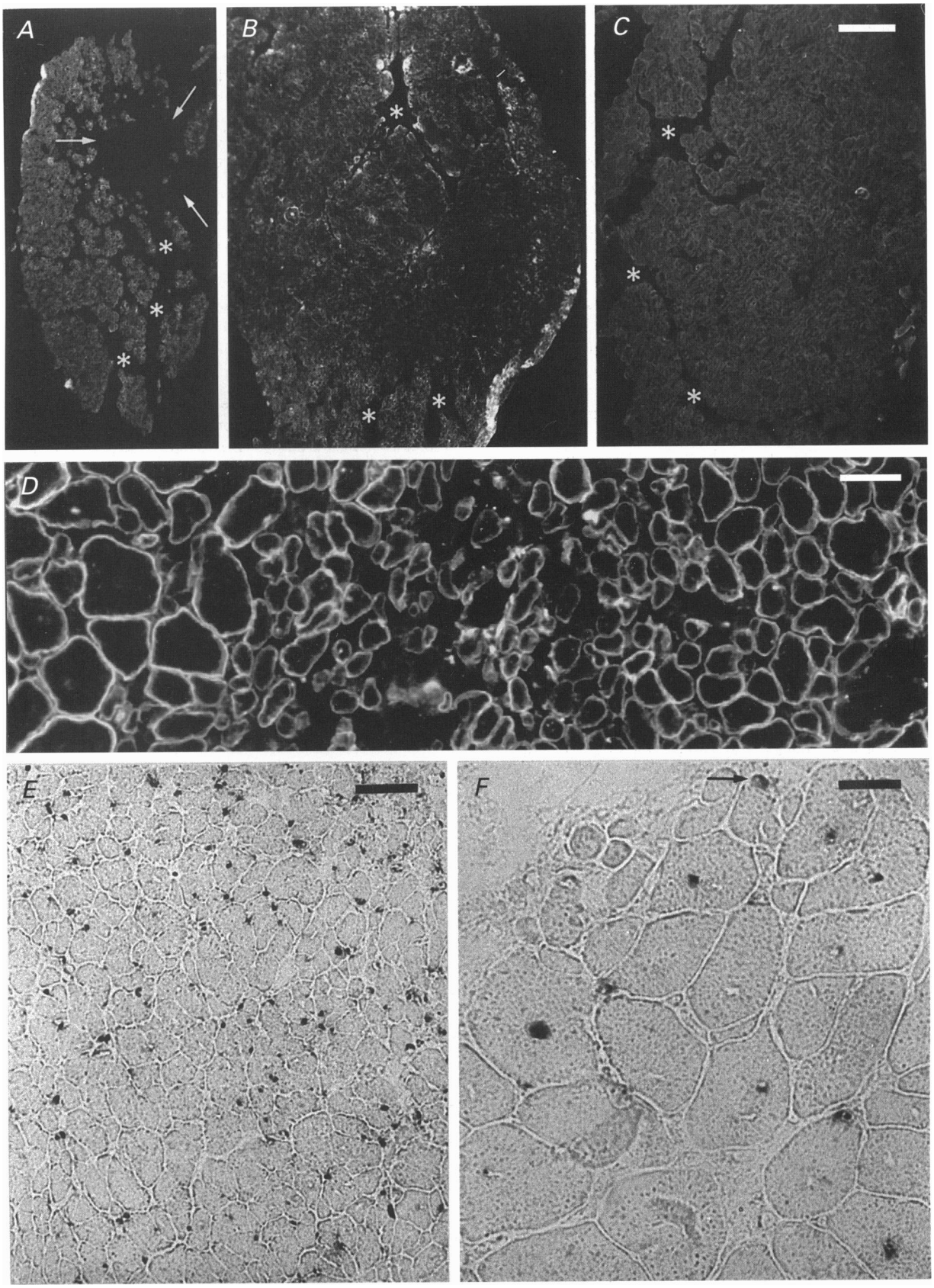

Figure 1. For legend see facing page.

restrictase (Boehringer Mannheim), the 1·5 kb Y1 fragment was isolated using agarose gel electrophoresis and digoxigenin labelled with a labelling kit as suggested by the manufacturer (DIG DNA labelling and detection kit, non-radioactive; Boehringer Mannheim). Tissue sections were mounted on silane-coated slides, air dried, fixed with absolute ethanol (10 min, room temperature), rehydrated through a 70, 50 and 30% (v/v) ethanol series, washed with PBS containing 5 mM $MgCl_2$, digested with proteinase K (0·1 μg ml^{-1} in PBS for 15 min at 37 °C; Sigma), washed with 0·2% glycine–PBS (10 min, room temperature), and fixed in 4% (w/v) paraformaldehyde in PBS (10 min, room temperature). Culture cells, grown on uncoated glass slides, were washed with PBS, air dried, fixed in paraformaldehyde, washed with PBS and air dried again.

To reduce non-specific hybridization, sections were overlaid with prehybridization solution composed of 4× saline–sodium citrate buffer (SSC), 50% (v/v) amberlite–deionized formamide, 1× Denhardt's solution, 5% (w/v) dextran sulphate and 0·5 mg ml^{-1} salmon testes DNA (all from Sigma) and incubated for 15 min at 37 °C. Cell preparations were only rehydrated with 2× SSC. Hybridization was performed with prehybridization solution containing labelled Y1-probe DNA (0·4–0·7 ng μl^{-1}) overnight (16 h) at 37 °C. After thorough washing in buffers with increasing stringency (twice for 15 min in 4× SSC–45% formamide, at 42 °C; twice for 5 min in 2× SSC, at 42 °C; 15 min in 0·2× SSC, at 50 °C; 15 min in 0·1× SSC, at 60 °C), labelled DNA was visualized with the digoxigenin detection kit as suggested by the manufacturer (Boehringer Mannheim). Finally, bis-benzimide staining of nuclei was performed.

In the course of establishment of optimal staining conditions, specificity controls included omission of labelled probe (negative), omission of the antibody to digoxigenin (negative), staining of sections from intact solei of female (negative) and male (positive) animals. Routinely, sections from normal muscles of male and female animals were added onto each slide with sections from experimental muscles. The reaction product in male control muscles was confined to nuclei as revealed by nuclear bis-benzimide staining and, in most cases, covered only a fraction of the nucleus. Labelling efficiency of nuclei in control muscles varied from 16 to 50% (see below).

Quantification of the labelling efficiency in sections from male muscles was performed after each staining. Several fields were selected at random using first the bis-benzimide filter and then the fraction of Y-positive nuclei was counted using bright-field optics (×500, Axiophot; Zeiss; 100–250 nuclei quantified per muscle). Counting of Y-positive nuclei in implanted muscles was performed on two or three complete good quality sections made through the endplate region of each muscle (at ×500). The whole cross-sectional area of each muscle was scanned with the photographic frame of the microscope seen in the visual field so that no overlapping of frames, and thus double counting of nuclei, occurred. All positive nuclei in the section were counted. Correction of absolute numbers was made in accordance with the staining efficiency determined in the concomitantly stained sections of male muscles.

Cross-sectional areas and numbers of muscle fibre profiles. Cross-sectional areas occupied by muscle (desmin positive) and non-muscle tissue (desmin negative) were measured on a single complete section from the endplate region of individual muscles as described previously (Wernig *et al.* 1995). Briefly, low-power videoimages (×6·25 objective lens) were taken with a high-sensitivity video-camera and enhanced with an image processor system. Areas were measured directly on the video monitor using the software of the image processor.

The total number of muscle fibre profiles was evaluated from complete muscle cross-sections reconstructed from videoprints (Toluidine Blue staining, final magnification ×382) as described previously (Wernig *et al.* 1995). Due to split and branched fibres in regenerated muscles (see Wernig *et al.* 1990, 1995) the number of profiles is bound to be higher than the number of muscle fibres; no attempt was made to correct for this (Wernig *et al.* 1990).

Statistical analysis

One-way analysis of variance (ANOVA) and a subsequent Tukey's test were performed to compare mean values of more than two groups (see Wernig *et al.* 1995). The accepted level of significance was 0·05 or less. Throughout the text mean group values are given with standard deviations.

RESULTS

Incomplete muscle regeneration after severe cryodamage

Soleus muscles were mildly or severely cryodamaged (see Methods). At 4 weeks after spontaneous regeneration the amount of contractile force was particularly small after severe cryodamage (Table 1) and there was no further improvement after the first month (not shown): directly and indirectly evoked maximum tetanic and twitch tension

Figure 1. Soleus muscles 1·0–2·5 months after severe cryodamage with and without MC implantation

A–C, low power magnification of desmin-stained cross-sections through the endplate region of non-implanted muscle (*A*), MC-implanted muscle (*B*), and intact muscle contralateral to that shown in *A* (*C*). The regenerated non-implanted muscle has a much smaller cross-sectional area than the other two muscles and, in addition, a fibrotic area devoid of desmin-positive profiles (arrows). The implanted muscle (*B*) has a cross-sectional area roughly comparable to that of the intact muscle (*C*) and only perimyseal septa (asterisks) are desmin negative, as also seen in the other two muscles. The scale bar in *C* represents 100 μm and applies also to *A* and *B*. *D*, dystrophin staining 2·5 months after severe cryodamage and MC implantation delineates large-diameter (>20 μm, left) and numerous small-diameter (<20 μm) muscle fibre profiles. Scale bar represents 20 μm. *E* and *F*, *in situ* hybridization with the Y-chromosome-specific probe reveals numerous donor-derived male nuclei (1 month post implantation, *E*). *F*, Y-positive nuclei in large-diameter fibres are more often localized centrally (6 of 8 seen in the figure), whilst the typical localization in small-diameter fibres (arrow) is peripheral (2 months post implantation). Scale bars represent 40 μm (*E*) and 20 μm (*F*).

Table 1. Functional recovery of soleus muscles after severe, mild or no cryodamage

	Group no.	Maximum tetanic tension: Direct stim. (mN)	Maximum tetanic tension: Indirect stim. (mN)	Maximum twitch tension: Direct stim. (mN)	Maximum twitch tension: Indirect stim. (mN)	Wet muscle weight (mg)	Specific tension (N g^{-1})	ACh (50 mg l^{-1}) contracture (%)	No. of muscles
Severe cryodamage									
Non-implanted	1	48 ± 24*	49 ± 16*[a]	9 ± 3*	9 ± 3*[a]	11 ± 3†[b]	4·3 ± 2·0*[b]	8·4 ± 3·4*[b]	9
MC-implanted	2	102 ± 30*	88 ± 27*[c]	19 ± 7*	18 ± 6*[c]	14 ± 3	7·3 ± 2·5*	4·0 ± 2·6	14
Contralateral	3+4	145 ± 15	143 ± 14[d]	27 ± 6	25 ± 5[d]	13 ± 2	11 ± 1·9	3·3 ± 1·5	23
Mild cryodamage									
Non-implanted	5	102 ± 23‡	99 ± 18‡	20 ± 5‡	19 ± 3‡	13 ± 3	7·9 ± 1·6‡	7·3 ± 2·8	11
MC-implanted	6	120 ± 31‡	114 ± 35‡	22 ± 7‡	20 ± 8‡	14 ± 3	9·0 ± 2·3‡	8·2 ± 4·3	13
Contralateral	7+8	145 ± 18	142 ± 19	28 ± 6	28 ± 6	13 ± 2	11 ± 1·7	5·2 ± 2·5[e]	23
No cryodamage									
Contralateral	9	156 ± 32	—	31 ± 5	—	14 ± 1	12 ± 1·8	4·7 ± 1·9	6
MC-implanted	10	156 ± 16	—	30 ± 4	—	14 ± 2	11 ± 0·9	4·7 ± 1·3	6

Contractile parameters and muscle weights of regenerated non- and MC-implanted, intact MC-implanted and intact contralateral muscles of BALB/c mice studied 1–4 months after treatment. Direct stim. and Indirect stim., direct and indirect stimulation of muscle contraction as defined in the text. Values are means ± S.D. The two groups of contralateral control muscles (one for non-implanted, the other for MC-implanted) in the two categories (severe cryodamage, groups 3 and 4, and mild cryodamage, groups 7 and 8) had very similar values and therefore, for brevity, overall means are shown here. In the statistical analysis, however, each group was treated independently. Statistical analysis (one-way ANOVA–Tukey's test, independently performed for groups 1–4 and 5–8): *$P<0·05$ compared with all other groups; †$P<0·05$ compared with MC-implanted muscles; ‡$P<0·05$ compared with contralateral controls. Groups 9 and 10 were compared using Student's t test and no significant differences were found. [a]$n=7$; [b]$n=8$; [c]$n=11$; [d]$n=18$; [e]$n=21$.

amounted to about one-third that of undamaged contralateral control muscles (Table 1). Muscle wet weight was only slightly diminished but specific tension (in N g^{-1}) was markedly smaller. Staining muscle cross-sections for the muscle-specific protein desmin revealed loss of muscle tissue, and large areas of desmin-negative tissue not present in undamaged muscles (Fig. 1*A–C*). Total muscle cross-sectional areas were reduced to less than one-half of intact muscles and one-third was occupied by desmin-negative tissue (Table 2). Force deficit obviously resulted from limited regeneration of muscle tissue (force 33% of control, desmin-positive cross-sectional area 34% of control) and not from functional deficits of the regenerated tissue since force production per unit of muscle-specific area was normal (Table 2) and twitch : tetanus ratios (indicative of the degree of muscle activation) were similar to those of undamaged muscles (data not shown).

After mild cryodamage muscle regeneration was more pronounced but still incomplete, maximum tetanic tension reaching 70% (Table 1) and desmin-positive cross-sectional area 66% of normal on average (Table 2).

Mean values of most parameters measured in severely cryodamaged muscles (twitch and tetanic tension, muscle weight, specific tension, number of profiles, total and desmin-positive area) were significantly lower ($P < 0·05$, Student's two-tailed t test) than in mildly cryodamaged solei (Tables 1 and 2).

It is noteworthy that regeneration of soleus in other mouse strains (CBA/J, C57Bl/6, C57Bl/10) following the same amount of cryodamage (mild) is considerably better and muscle strength reaches near-normal values (Wernig & Irintchev, 1995; Wernig *et al.* 1995; Irintchev, Zweyer & Wernig, 1997).

MC implantation improves severely cryodamaged muscles

MC implantation after severe cryodamage caused significantly larger tetanic and twitch tensions than regeneration after cryodamage with no cell implantation (Table 1). On average, the amount of tension remained below that for untreated controls, but single values were comparable to undamaged muscles (not shown). Also specific tension (tetanic tension per unit tissue weight) was higher than that in non-implanted cryodamaged muscles though still lower than that in untreated control muscles, whilst wet muscle weight and ACh (50 mg l^{-1}) contracture reached normal values. Obviously, MC implantation had caused significantly better muscle regeneration due to building of additional functional muscle tissue (increased numbers of fibre profiles, total area and absolute and relative desmin-positive cross-sectional area; Table 2). The additional muscle tissue apparently produced similar tension (tetanic tension/desmin-positive area; Table 2) indicating an overall good integration of the new muscle fibres (see Discussion).

Table 2. Structural recovery of soleus muscles after severe, mild or no cryodamage

	Group no.	No. of fibre profiles	Total muscle cross-sectional area (mm^2)	Desmin-positive cross-sectional area (mm^2)	Desmin-positive/ total area (%)	Tetanic tension/ desmin-positive area ($mN\ mm^{-3}$)	No. of muscles
Severe cryodamage							
Non-implanted	1	542 ± 208	0·35 ± 0·14*	0·24 ± 0·12*	67 ± 12*	199 ± 61	7
MC-implanted	2	1562 ± 295*	0·77 ± 0·14*	0·70 ± 0·13	91 ± 3·9	154 ± 38	6
Contralateral	3+4	811 ± 60[a]	0·77 ± 0·08	0·71 ± 0·07	92 ± 2·3	202 ± 17	12
Mild cryodamage							
Non-implanted	5	857 ± 93	0·51 ± 0·07†	0·45 ± 0·06†	89 ± 3·2	214 ± 27	5
MC-implanted	6	1124 ± 212*	0·62 ± 0·08	0·57 ± 0·08	93 ± 2·4	185 ± 41	6
Contralateral	7+8	807 ± 66	0·76 ± 0·15	0·68 ± 0·14	90 ± 3·4	229 ± 55	8
No cryodamage							
Contralateral	9	842 ± 85	0·79 ± 0·11	0·72 ± 0·10	90 ± 1·3	218 ± 35	6
MC-implanted	10	911 ± 64	0·76 ± 0·07	0·69 ± 0·06	90 ± 1·9	228 ± 27	6

Number of muscle fibre profiles, cross-sectional areas and tension per unit area in regenerated non- and MC-implanted, intact MC-implanted muscles and intact contralateral muscles of BALB/c mice studied 1–4 months after treatment. Values are means ± S.D. Groups and statistical analysis as in Table 1. * $P < 0·05$ compared with all other groups; † $P < 0·05$ compared with contralateral controls. Mean values for groups 9 and 10 do not differ significantly from each other (Student's two-tailed t test). [a] $n = 9$.

Table 3. Donor-derived cells in intact, mildly or severely cryodamaged muscles

	No cryodamage	Mild cryodamage	Severe cryodamage
Absolute numbers			
Median	2	16	459
Range	0–8	5–79	309–600
Corrected numbers			
Median	14	112	918
Range	0–56	35–553	618–1200
No. of sections	8	12	8
No. of muscles	3	4	4

Numbers of Y-positive nuclei in cross-sections from non-, mild or severely cryodamaged muscles implanted with 10^6 myoblasts from the same culture. Corrected numbers are absolute numbers multiplied by factors reciprocal to the staining efficiency (no. of positive nuclei/(no. of positive + negative nuclei)) of individual stainings. The efficiency was determined in sections from male muscles stained concomitently with the experimental muscle cross-sections.

Histological observations

Cross-sections of MC-implanted muscles show larger desmin-positive areas interrupted by smaller areas of desmin-negative tissue than those in non-implanted cyrodamaged muscles (Fig. 1*A–C* and Table 2). Immunostaining for dystrophin, a muscle fibre-specific membrane-bound protein, reveals many small-diameter muscle fibres (Fig. 1*D*). *In situ* hybridization with Y-chromosome-specific DNA labels numerous nuclei predominantly in small muscle fibres (Fig. 1*E*) indicating their origin from the implanted Y-positive cells (see also Table 3). Less frequently, labelled nuclei are found in large-diameter muscle fibres (Fig. 1*F*). Incidentally, nuclei are often in a central position in large-diameter fibres but in a peripheral position in small-diameter fibres (Fig. 1*F*).

Labelling of synaptic features like ACh receptors, ACh-esterase and NCAM on spaced serial sections reveals that donor-derived small- and large-diameter fibres bear endplates (Fig. 2*A–G*). This is in line with the finding that most of the muscle can be activated by stimulation of the supplying nerve (Table 1, no significant differences).

It is important to note that implanted myogenic cells not only form new muscle fibres but also contribute to the satellite cell pool in the regenerated muscles; this can be seen from Fig. 3*A–C*, where M-cadherin immunostaining

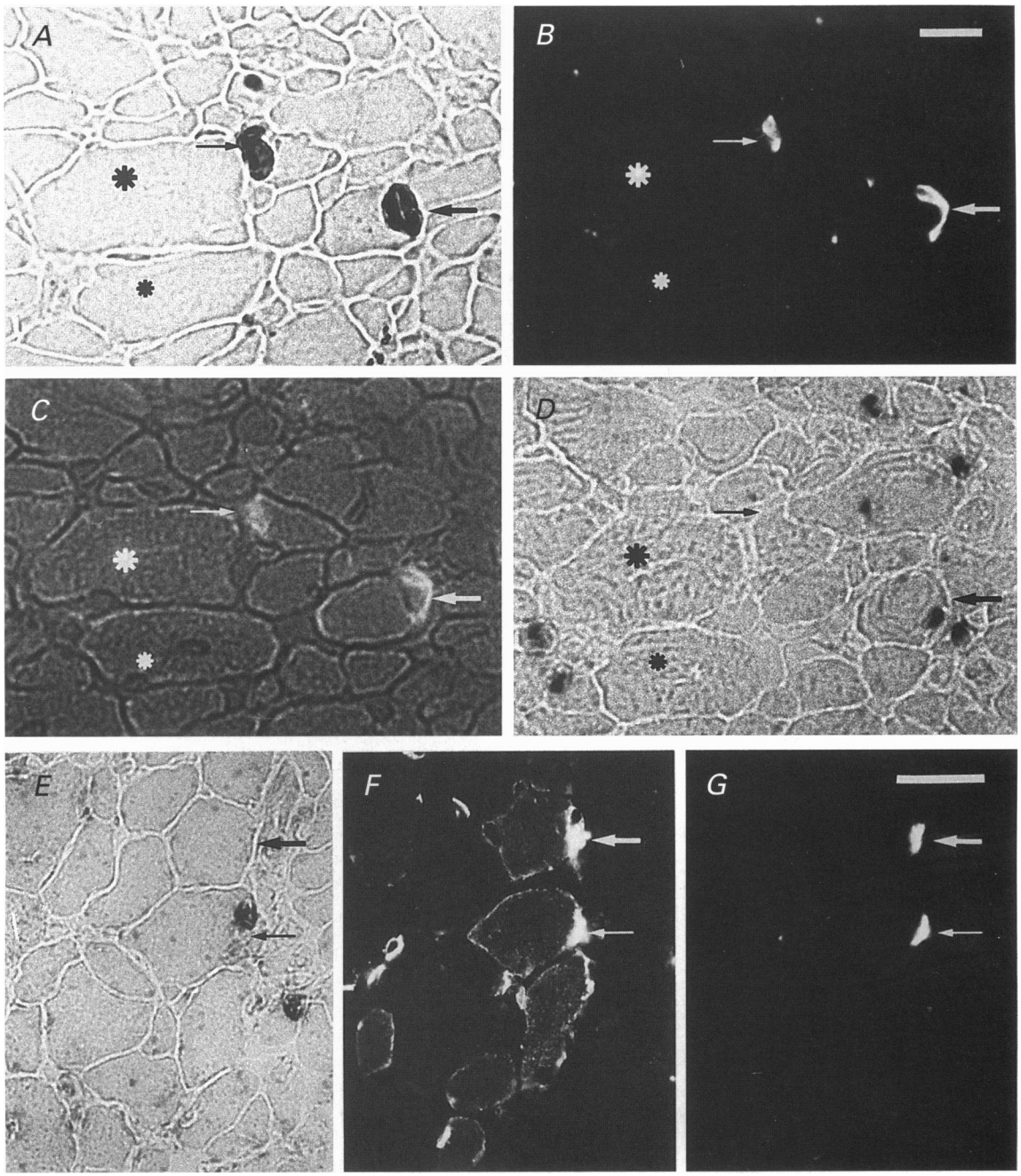

Figure 2. Endplates on donor-derived muscle fibres

A–D, 34 days after repeated freezing and MC implantation. Individual muscle fibres traced in spaced serial cross-sections stained for synaptic markers (*A*, acetylcholinesterase; *B* and *C*, α-bungarotoxin staining of ACh receptors) and using the Y-chromosome-specific probe to identify donor nuclei (*D*). *C*, the same section as in *B* with additional bright-field illumination to outline the contours of the fibres. A Y-positive small-diameter muscle fibre (*D*, thick arrow) has an endplate containing both esterase (*A*) and receptors (*B* and *C*). Another small-diameter fibre (*D*, thin arrow) also carries an esterase- (*A*) and receptor-positive (*B* and *C*) endplate but does not contain Y-positive nuclei in this and other nearby sections (not shown). Two larger fibres (small and large asterisk) were positive for synaptic markers and the Y-probe at other levels (not shown). The scale bar in *B* represents 10 μm and applies also to *A–D*. *E–G*, two spaced serial cross-sections, 72 μm apart, from a regenerated MC-implanted muscle 2·5 months post implantation. One section is stained with the Y-chromosome-specific probe (*E*); the other is double-stained for NCAM (*F*) and ACh receptors (*G*). Two muscle fibres (arrows), one Y-positive (*E*, thin arrow) bear endplates (*G*) at which accumulation of NCAM (*F*) is present. At and around the endplate level, the entire cross-sectional area of the fibres is NCAM positive (*F*). The scale bar in *G* represents 20 μm and applies also to *E* and *F*.

typical for satellite cells (Irintchev *et al.* 1994) is associated with a Y-positive cell (thin horizontal arrow). Also, such cells are located within the basal lamina sheath of the parent muscle fibre (not shown).

MC implantation into untreated and mildly cryodamaged muscles

Single freezing of the muscle (mild cryodamage) was followed by better spontaneous recovery (Tables 1 and 2). Surprisingly, implantation of MCs had no significant effect on contractile force (Table 1) and cross-sectional areas (Table 2), though the number of profiles was increased (Table 2). Moreover, MC implantation into undamaged muscles caused no detectable functional or structural effect (Tables 1 and 2). Most impressively, the numbers of Y-positive nuclei were lowest in undamaged muscles, clearly higher after mild cryodamage and highest after severe cryodamage (Table 3). This correlated well with the numbers of 'surplus' fibre profiles formed (Tables 2 and 3).

DISCUSSION

Whilst previous investigations have shown that cultured myogenic cells may survive after implantation into adult muscles of humans and other vertebrates, their proliferative capacity *in vivo* and their contribution to contractile force are far from clear (Watt *et al.* 1982; Law *et al.* 1990; Morgan *et al.* 1990; Huard *et al.* 1991, 1994; Wernig *et al.* 1991*a*; Gussoni *et al.* 1992; Karpati *et al.* 1993; Tremblay *et al.* 1993; Alameddine *et al.* 1994; Rando & Blau, 1994; Mendell *et al.* 1995; Wernig & Irintchev, 1995). So far only permanent cell lines have been demonstrated to contribute significantly to the contractile force of host muscles (Wernig *et al.* 1991*a*, 1995). Such implants form mature muscle fibres which extend for at least several millimetres in the long axis of the host muscle and become innervated. However, after this, rhabdomyosarcomas inevitably form in the implanted muscles (Wernig *et al.* 1991*a*). The myogenic capacity of primary cultures *in vivo* is clearly smaller than that of permanent cell lines: whilst implantation of the latter into cryodamaged or even toxin-paralysed muscles led to muscle weight/force exceeding control muscles (Wernig *et al.* 1991*a*), the former did not (present results). Also, Alameddine *et al.* (1994) found no functional effect of MC implantation unless the damaged muscle was X-ray irradiated prior to implantation (see also Morgan *et al.* (1990) who found a higher yield of donor-derived cells after X-ray irradiation of *mdx* muscles). The present findings add that a clear functional improvement of cryodamaged muscles may be achieved by MC implantation. They also demonstrate that the survival and/or amount of proliferation of the implanted myoblasts depends on the amount of previous damage.

The limited capacity of primary cultures to produce muscle tissue *in vivo* may be due to the death of many cells in the first days after implantation (Huard *et al.* 1994; Fan *et al.* 1996) and a lower proliferative capacity compared with permanent lines (see also below). In undamaged muscle the total number of donor cells after 1–4 months is clearly smaller than the number of cells implanted (10^6): assuming the best case, i.e. even distribution of implanted Y-positive nuclei along the length of the soleus muscle, fourteen positive nuclei per section (from Table 3) amount to about 19 000 nuclei in the whole muscle given a muscle fibre length of 8 mm (Brooks & Faulkner, 1988) and a section thickness of 6 μm. Clearly then, undamaged muscle provides a poor surrounding for implanted MCs and most cells presumably die. After mild cryodamage 150 000 nuclei remained, which

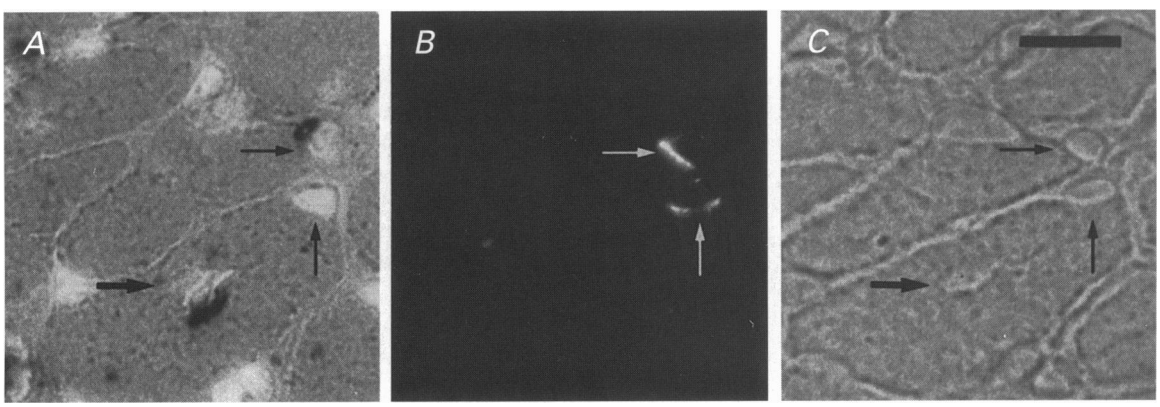

Figure 3. Implanted myogenic cells also form satellite cells

Muscle cross-sections 2 months after repeated freezing and MC implantation. *A*, donor nuclei double-stained with the Y-chromosome-specific probe (dark spots) and bis-benzimide (fluorescent label) to visualize all nuclei. *B*, serial section to that shown in *A* stained for M-cadherin and nuclei (bis-benzimide, not shown). *C*, same section as in *B*, using bright-field optics to show the outlines of the fibres. The upper M-cadherin-positive satellite cell in *B* has a Y-positive nucleus (horizontal arrow) which indicates its donor-derived origin. A Y-positive muscle cell nucleus and a muscle cell nucleus is pointed out by the thick arrow in *A* and *C*. The scale bar in *C* represents 10 μm and applies also to *A* and *B*.

is higher than in undamaged muscles, but still less than the number of cells implanted. In contrast, the population of Y-positive nuclei after severe muscle damage would be around 1 200 000, i.e. higher than the number of initially implanted cells.

These observations suggest that MCs from primary cultures, unlike permanent cell lines, depend to a high degree for survival and proliferation *in vivo* on growth factors and/or adhesion molecules provided by damaged muscle tissue. One molecule released from damaged muscle fibres is bFGF (Clarke, Khakee & McNeil, 1993). bFGF was used in some of our culture media so that such MCs might have become bFGF dependent. However, MCs grown without bFGF also showed the same dependence on damaged muscle tissue, indicating that bFGF dependence is not the cause for poor survival in undamaged muscles. Some other molecules associated with muscle damage and thus potentially involved in muscle regeneration are transforming growth factor beta, platelet-derived growth factor, insulin-like growth factor I, NCAM, M-cadherin, tenascin-C (Yablonka-Reuveni & Seifert, 1993; Irintchev *et al.* 1993, 1994; Kinoshita, Vilquin & Tremblay, 1995; Lefaucheur & Sebille, 1995). Besides coming from damaged muscle cells such factors might also originate from fibroblasts (e.g. tenascin-C, Irintchev *et al.* 1993), phagocytes (Cantini, Massimino, Bruson, Catani, Dalla Libera & Carraro, 1994) and other non-myogenic cells and be identical to those causing proliferation of resident satellite cells after focal damage of muscle fibres (Irintchev & Wernig, 1987; Wernig *et al.* 1990, 1991*a*, *b*).

There is still another factor to be considered. Host satellite cells apparently are competitors for the proliferative signals in muscle regeneration. The closer vicinity of MCs to the source of release and their position within basal lamina sheaths of damaged myofibres provide better conditions for their involvement in fibre repair than for MCs implanted as a bulk along the track of the needle. Better survival of implanted MCs was indeed found after inactivating host cells by previous X-ray irradiation (Morgan *et al.* 1990; Alameddine *et al.* 1994), strongly supporting the above notion. Thus severe muscle damage might not only be effective in causing larger amounts of proliferating signals but also in reducing the number of competing host satellite cells such that implanted cells survive and grow in larger numbers.

In summary, it appears that MCs from primary cultures are capable of forming functional muscle tissue which becomes innervated and significantly contributes to contractile force. It is equally clear that sufficient growth-stimulating factors need to be present and it will be necessary in future experiments to define them. This in turn explains why implanted minced muscle tissue gave good functional results. It is important that implanted MCs form not only muscle fibres but also satellite cells, allowing later repair processes and muscle fibre hypertrophy to occur. Several other problems will need to be solved, e.g. the definition of culture conditions to exclude formation of immunogenicity and tumorgenicity, which have both been recently observed to occur upon change in the culture medium (A. Irintchev, M. Zweyer & A. Wernig, unpublished observations). If these problems can be solved it seems feasible to build new muscle tissue from cultured cells, which might be of importance in orthopaedic or plastic surgery. For replacement of genetically defective muscle in, for example, dystrophies, both suppression of host satellite cells and initial provision of growth factors seem mandatory.

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