

Migration of adult myogenic precursor cells as revealed by GFP/nLacZ labelling of mouse transplantation chimeras

Harald Jockusch* and Sylvana Voigt

Developmental Biology and Molecular Pathology, W7, University of Bielefeld, D-33501 Bielefeld, Germany

*Author for correspondence (e-mail: h.jockusch@uni-bielefeld.de)

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Summary

We studied the migratory behaviour of adult muscle precursor cells in the mouse into and from skeletal muscle grafts using green fluorescent protein (GFP) and nuclear LacZ transgenes as complementary and double markers of the cell's origin. Owing to the small molecular mass and extreme solubility of GFP, this label provided a drastically increased sensitivity for detection compared with the markers that had been used previously. During the first six weeks after the operation, the graft/host border was well defined, with only occasional local intermingling and co-fusion of host and donor myogenic cells. Seven to eleven weeks after the operation we found that the host myogenic cells had migrated into the graft, and graft myogenic cells

had migrated into the adjacent host muscle, with integration of donor nuclei into pre-existing myotubes or muscle fibres. There was no indication of an origin of, or target for, these myogenic cells besides neighbouring muscles. Our observations indicate migration of these cells through solid muscle tissue, over a distance of several millimetres. The migratory activity of adult myogenic precursor cells can be stimulated by traumatic events in either the target muscle or the muscle of origin.

Key words: Stem cells, Migration, Muscle transplantation, GFP, nLacZ, Nude mouse

Introduction

In vertebrates, clonal expansion, successive determination and migration of stem cells are the major events in organogenesis. As shown by the analysis of chimeras, the importance of migration and intermingling relative to clonal expansion and coherence differs widely among organ systems (McLaren, 1976; West, 1999). In melanoblasts, clonal coherence is retained despite migration, whereas migration and intermingling (plus co-fusion) is typical for the myogenic stem cells of limb muscles, and extensive intermingling of cells is observed in the brain. In adult higher vertebrates, stem cell migration has previously not been considered to be of great importance, at least not in the neuromuscular system. Recent work on adult stem cells, however, has not only demonstrated migration in the adult but also transdetermination, for example, from neural (Galli et al., 2000) and bone marrow cells (Ferrari et al., 1998) to muscle cells. In the latter case, the transdifferentiated bone marrow cells were transported through the blood stream and assumed a muscle phenotype in a muscle environment. The issue of more localized migration of myogenic cells has been studied repeatedly in the past years. Often the cells to be studied were permanent myogenic cell lines, and in common with experiments on embryo chimeras, such experiments require a label of origin, for example, a chromosomal sex difference (Grounds et al., 1991; Smythe and Grounds, 2001), a species difference (Le Douarin and Teillet, 1974), a cell-autonomously expressed allelic difference or, as in the case in most experiments with cell lines, a transgene. As a result of these experiments, migration of injected permanent

cells into neighbouring muscle (Hughes and Blau, 1990; Watt et al., 1994) and a limited exchange of cells between resident and implanted muscles (Watt et al., 1987; Morgan et al., 1993) have been reported. With myogenic lines it was noted that the cells seem to be preferentially attracted to damaged and regenerating host muscles (Watt et al., 1994; Grounds and Davies, 1996). On the basis of model experiments, a role as a chemoattractant has been assigned to transforming growth factor β and hepatocyte growth factor (Bischoff, 1997). Delayed differentiation (Smythe and Grounds, 2001) and the activity of metalloproteinases (El Fahime et al., 2000) seem to promote the migratory activity of myogenic precursor cells.

Here we use a new type of marker of origin, a combination of an enhanced GFP transgene (Okabe et al., 1997), driven by the β -actin promoter, a CMV enhancer and a nuclear-localized bacterial β -galactosidase (nLacZ) knock-in, driven by the endogenous desmin promoter (Li et al., 1996). In addition, we used a novel fixation method for GFP (Jockusch et al., 2003). The β -actin promoter would be expected to lack cell-type specificity, whereas the desmin promoter conveys specificity of expression for smooth, cardiac and skeletal muscle. Although the latter expression pattern was confirmed (Li et al., 1996), the GFP pattern was not homogeneous (Jockusch et al., 2003), and levels of expression were especially high in smooth, cardiac and skeletal muscle. In skeletal muscle, mature muscle fibres had a higher GFP level than myotubes, and among mature fibres the oxidative ones were richer in GFP than the glycolytic fibres. In cross-striated muscle all fibres expressed GFP in the transgenic mouse, and the fluorescence level was

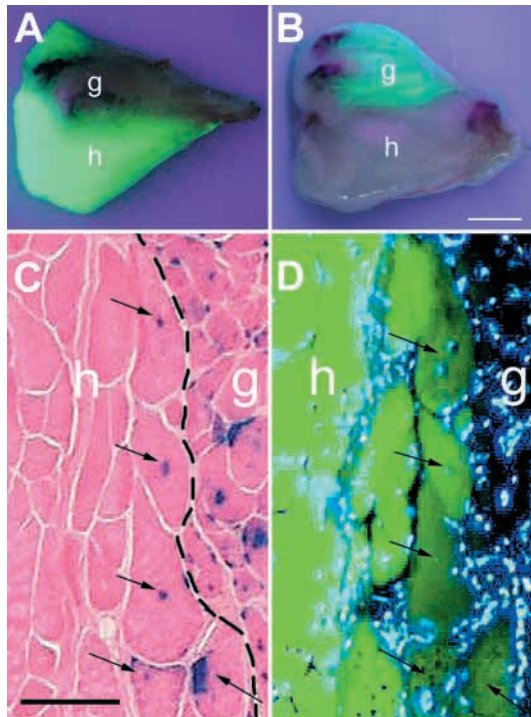


Fig. 1. Complementary muscle grafts with GFP-labelled host, 0 to GFP (A,C,D) and GFP labelled donor, GFP to 0 (B). (A,B) Macroscopic appearance under UV light of grafts of tibialis anterior (TA) muscle to the TA bed. (A) 49 days p.o., (B) 70 days p.o. Bar in B, 2 mm. (C,D) Complementary labelling in a 0/nLacZ graft, 50 days p.o. Cross-section at graft(g)/host(h) border. (C) X-gal plus eosin staining, (D) GFP (green) and Hoechst (blue) nuclear fluorescence. The donor was homozygous for the nLacZ knock-in (and for DesKO). There are several GFP-positive muscle fibres that have nLacZ-positive nuclei (arrows), indicating co-fusion of donor myoblasts with host muscle fibres. Bar in C, 100 μ m.

always well above any autofluorescence levels (Jockusch et al., 2003).

In conjunction with muscle grafting, we have studied the migratory behaviour of resident myogenic stem cells, in both directions, from host to graft and vice versa. Owing to the sensitivity of the GFP-labelling method we visualized hitherto unrecognised migratory activities of myogenic stem cells through solid muscle tissue.

Preliminary reports on these experiments have been published elsewhere (Jockusch et al., 2000; Eberhard and Jockusch, 2002).

Materials and Methods

Mouse strains

The following original mouse strains were used: A2G-*adr* (cf. Füchtbauer et al., 1988; Steinmeyer et al., 1991), 129 – Des^{-/+} with the pDes-nLacZ knock-in on chromosome 1 (Li et al., 1996); C57BL with the p β actE-GFP transgene on chromosome 15, line C57BL/6 Cr Slc TgN(act-EGFP)OsbC15-001-FJ001 (Okabe et al., 1997); NMRI-nu, obtained from Harlan Winkelmann, Borcheln, Germany.

We bred the eGFP (designated GFP) and nLacZ transgenes into the NMRI-nu strain to obtain nude mice as recipients, either GFP or GFP and nLacZ (GFP/nLacZ) labelled. The absence of a transgene (i.e. wildtype) is indicated by '0'.

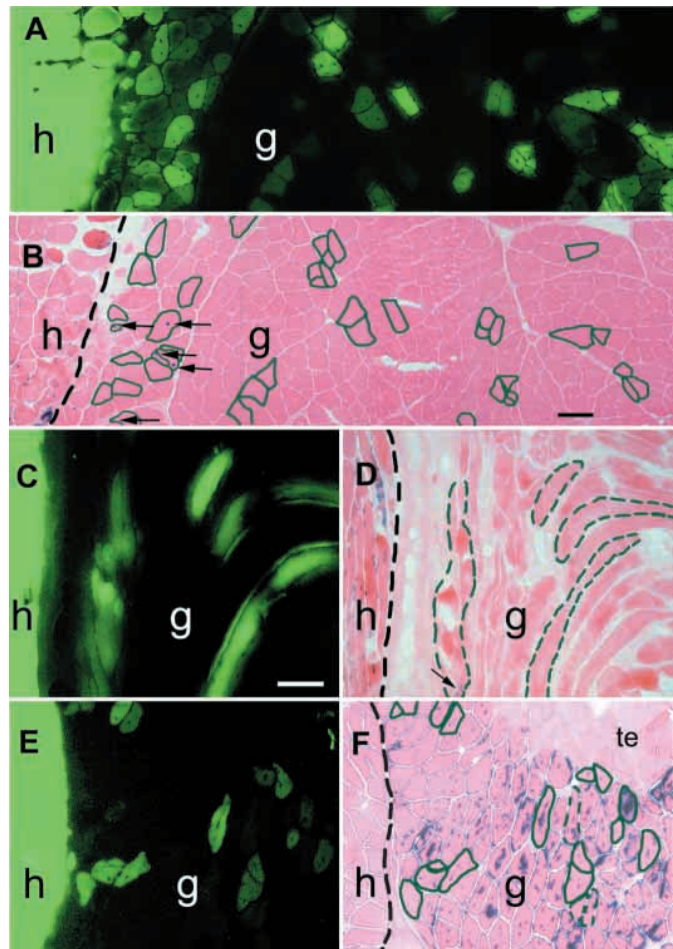


Fig. 2. Unlabelled graft to a double-labelled host, 0/0 to GFP/nLacZ, 77 days p.o. (A-D), and nLacZ-labelled graft to a GFP host (complementary label), 0/nLacZ to GFP/0, 75 days p.o. (E,F). Migration of host cells into the graft. The same region of the host(h)/graft(g) border in adjacent sections is shown in fluorescence (A,C,E) and after Xgal/eosin staining (B,D,F). (A,B,E,F) Cross-sections of proximal parts, (C,D) longitudinal sections of distal parts of the graft site. (A,E) Within the graft there are numerous fibers with host label (GFP) that can be followed through serial sections, as indicated by green contours in B,F. In B,D, a few nLacZ-positive nuclei are found in GFP-positive fibres close to the host (arrows). In F, nearly all of the GFP-positive fibres show blue nuclei. C,D,F indicate immigration of host stem cells and co-fusion with graft muscle fibres rather than ingrowth of host muscle fibres. In C, a green margin along bent muscle fibres is due to a local GFP overspill during thawing of the section (Jockusch et al., 2003). Bar, 100 μ m.

Surgery, organ preparation and histochemistry

Ages of donors ranged from 103 to 172 days (average 117 days), and those of recipients from 47 to 74 days (average 70 days). Transplantation of anterior tibial muscles with the attached extensor digitorum longus was performed as described previously (Füchtbauer et al., 1988) but with ketasetel-5/xylazine (Selectavert, Weyarn-Holzolling, Germany; Animedica, Senden-Bösensell, Germany) anaesthesia and without bupivacaine pretreatment of the graft. Animals gained weight and the operated leg was used normally after two weeks. All animal experiments were performed according to the German laws for the protection of animals, and a permit was obtained from the local authorities.

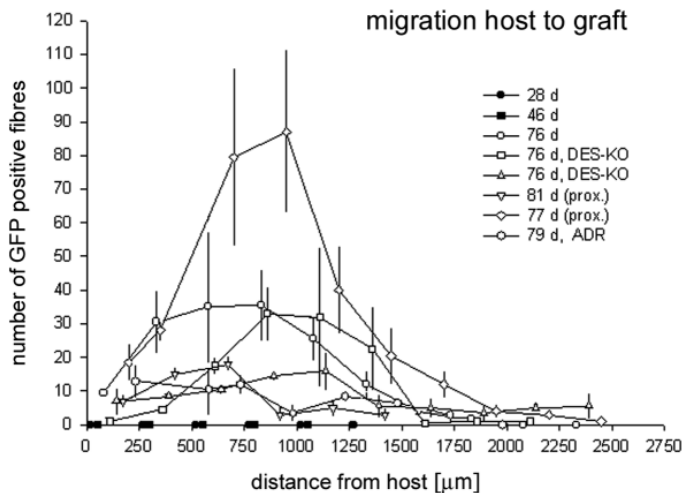


Fig. 3. Quantitative evaluation of the colonization of muscle grafts by host myogenic cells: results of six independent 0 to GFP transplantation experiments. Numbers of GFP-positive fibres in the graft were counted in eight to 10 sections in segments of 250 μm across the section and plotted as a function of the distance from the host/graft (h/g) border; symbols indicate average numbers between proximal and distal counts, with ends of bars indicating proximal and distal averages. The experiment with 77 days regeneration time is identical to the one shown in Fig. 2A-D. No immigration was observed in short-term experiments (filled symbols).

Between 4 and 11 weeks after operation (p.o.), animals were killed by cervical dislocation, and the grafted anterior tibial muscle (TA) with the adjacent M. gastrocnemius and M. soleus were removed in one piece, but without the tibia, and mounted either as proximal or distal halves supported by a chunk of hard-boiled egg white for cross sectioning (starting with the cut surface) or, in some cases, longitudinal sectioning. The blocks were shock frozen in liquefied propane (-190°C) followed by liquid N_2 . Frozen sections were cut on a Leitz cryostat microtome to 8 to 10 μm and processed for the subsequent staining. Owing to its extreme solubility, GFP cannot be fixed with conventional aqueous fixatives in frozen cross-sections of

the muscle. We have developed a fixation method that avoids loss of the highly soluble GFP. In brief, this consisted of the exposure of the section to formaldehyde (FA) vapour from a pad soaked with 37% FA at -20°C for at least 2 hours (Jockusch et al., 2003).

The FA-fixed sections were counterstained with Hoechst stain (Sigma, B2261) to visualize nuclei. Parallel sections (conventionally fixed or unfixed) were stained for β -galactosidase and subsequently with eosin. The immuno- and enzyme-histochemical methods used here (Jockusch et al., 2003) worked only on unfixed sections so that muscles could not be fixed as a whole. For GFP fluorescence, sections were embedded in Elvanol (10 g Mowiol 4-88 + 40 ml PBS + 20 ml glycerol); conventionally stained sections were embedded in Entellan (Merck, Darmstadt, Germany).

Evaluation and documentation

Serial sections of grafts with neighbouring gastrocnemius and soleus muscles were photographed within a few days after fixation on a Zeiss Axiophot microscope equipped with UV (and Nomarski) optics using an Olympus digital camera DP10.

Processing of images was done with Photoshop 5.5 (Adobe Systems Inc., San José).

Results

Regeneration of muscle grafts

To distinguish donor from host muscle we employed the highly diffusible cytoplasmic label, enhanced GFP [henceforth abbreviated GFP (Okabe et al., 1997)], and the localized marker *E. coli* β -galactosidase with a nuclear localization signal [nLacZ (Li et al., 1996)] that labels nuclei close to a transgenic nucleus. These markers were used either in double-label (GFP/nLacZ versus 0/0) or in complementary (GFP/0 versus 0/nLacZ) modes. Whereas the GFP signal is less intense in immature than in mature skeletal muscle (Jockusch et al., 2003), the opposite is true for the nLacZ transgene, which, in comparison with adult muscle, is expressed at a higher level in myotubes and at sites of regeneration. The nLacZ transgenic mice were usually heterozygous for the nLacZ knock-in at the desmin locus, and their muscle structure and function was not affected (Li et al., 1996). In some experiments, muscles from homozygous desmin-deficient (DES-KO) (and thus homozygous nLacZ knock-in) and from myotonic ADR mice (cf. Füchtbauer et al., 1988; Steinmeyer et al., 1991) were used. There was no indication that these relatively mild myopathic phenotypes had a significant effect on the migration of myogenic precursor cells.

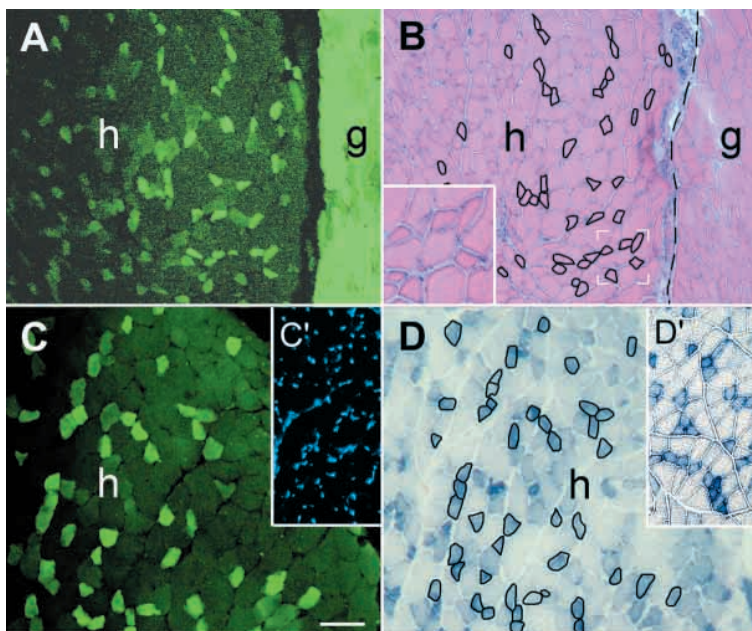


Fig. 4. Emigration of graft cells into the host: GFP to 0, 55 days p.o. The border between the host gastrocnemius (h, left) and the grafted anterior tibial muscle (g, right) is shown. (A) Fluorescence shows GFP. (B) The adjacent section is haematoxylin/eosin-stained, with contours to show GFP-positive fibres. Inset, enlargement of the framed region to show that there are no central nuclei in the GFP-positive fibres. (C) Region of host with GFP-positive fibres. (D) Same region as in C, with SDH staining to show fibre types and black contours to indicate GFP-positive host muscle fibres. Insets in C and D, with comparable regions in the contralateral leg to which no GFP-positive TA had been grafted, to show lack of autofluorescence of oxidative fibres. In C', blue fluorescence indicates Hoechst-stained nuclei, and in D', SDH enzyme histochemistry indicates a fibre-type pattern. Bar, 100 μm .

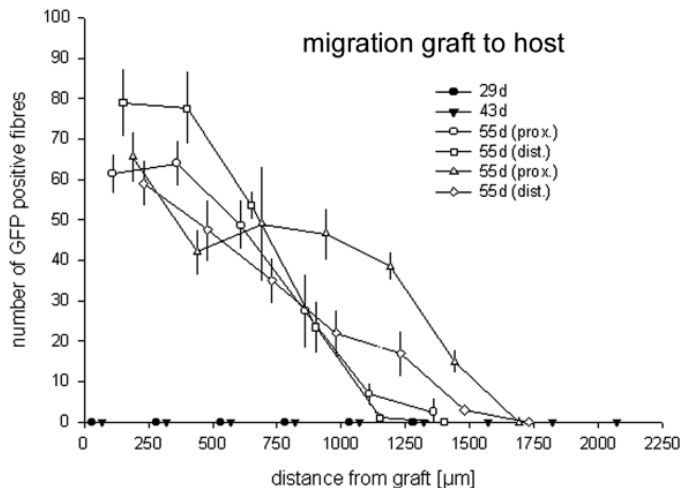


Fig. 5. Quantitative evaluation of the migration of graft myogenic cells into adjacent host muscle: results of four GFP to 0 experiments. In cross-sections through adjacent host and graft muscles, GFP-positive fibres in the host were counted. Numbers are given as a function of the distance from the graft. Averages (symbols) and highest and lowest counts (ends of bars) are given separately for proximal and distal sections (cf. Fig. 3 for additional explanations). Empty circles correspond to the experiment shown in Fig. 4. No emigration was observed in short-term experiments (filled symbols).

Three to ten weeks after transplantation, grafts of the *M. tibialis anterior* (TA) into the TA bed of the host had regenerated and were innervated and functional (Füchtbauer et al., 1988). UV illumination confirmed that the regenerated anterior tibial muscle was overtly of the donor type, that is, GFP positive when the donor was GFP positive and the host negative, and vice versa (Fig. 1A,B), although GFP fluorescence appeared to be less compact in the case of the GFP to 0 grafts, probably because of the heterogeneity of the maturation state, owing to ongoing regeneration. Expectedly, the grafted muscles showed signs of regeneration, such as central nuclei (Fig. 1C), and higher proportions of oxidative fibres than unoperated muscle do.

In general the lateral border between the host and the graft appeared to be sharp, with a separation of the grafted TA and the host gastrocnemius by a thin lining of connective tissue, the perimysium. At some places, however, some intermingling was observed, and this led to co-fusion between host and graft myogenic cells as demonstrated by GFP-positive fibres with LacZ-positive nuclei (Fig. 1C,D).

Immigration of myogenic stem cells from host to graft

Following longer regeneration periods for 0 to GFP transplantations, GFP-positive fibres appeared in the graft, often in small groups of two to five (Fig. 2). Parallel staining with standard methods of muscle histochemistry revealed nothing special for the GFP-positive fibres in comparison to their GFP-negative neighbours in the graft. With the host double labelled, nLacZ-positive nuclei in GFP-positive fibres were usually found close to the host. The finding that GFP-positive fibres in the graft contained only a minority of host nuclei was corroborated by a complementary labelling

experiment of the type nLacZ to GFP (Fig. 2E,F). At distances of >0.25 mm from the host muscle, blue nuclei were present in GFP-positive fibres at apparently the same density as in GFP-negative fibres.

In total, 10 transplantation experiments of the type 0 to GFP have been performed, in various combinations with the nLacZ label. Cross-sections of grafted TAs proximal and distal from the centre were quantitatively evaluated by counting GFP-positive fibres as a function of the distance from host muscle (Fig. 3). A maximal number of GFP-positive fibres (single or in small groups) was observed at distances between 0.25 and 1.5 mm from the host muscle, with maximal distances of 2 mm.

Emigration of myogenic cells from graft to host

In experiments of the type GFP to 0, migration of graft cells into host muscle can be monitored after 7 weeks of regeneration (Fig. 4). Central nuclei, which would indicate previous regeneration, were not observed in GFP-positive host fibres with Hoechst fluorescence or in HE-stained parallel sections (Fig. 4A,B). Oxidative fibres of the host were found to be preferentially converted to GFP-positive ones (Fig. 4C,D). In the contralateral leg, no GFP fluorescence or green autofluorescence was found in corresponding regions of the gastrocnemius and soleus muscles (Fig. 4C,D insets).

Quantitative evaluation of the colonization of adjacent host muscle by graft myogenic cells shows gradients of GFP-positive fibres from the graft border extending nearly 2 mm into the host (Fig. 5). Surprisingly, with grafts from neonatal mice, extremely poor regeneration and no migration of myogenic cells from graft to host was observed (data not shown).

Initial stages of the emigration of graft cells could be observed in GFP/nLacZ to 0/0 grafts (Fig. 6). Apparently donor myogenic cells, as recognized by both the GFP and the nLacZ label, detach from the graft and migrate along the endomysial connective tissue into the adjacent muscle, over distances of several hundred microns (Fig. 6A-D). In one case, an nLacZ-positive, GFP-negative cell was observed; this was probably a very immature muscle precursor cell in which the GFP expression was below the threshold of detection. In longitudinal sections, regenerated immature fibres can be distinguished from mature fibres by their lower GFP content and very high β -galactosidase activity. As in the complementary case 0 to GFP, single GFP-positive fibres are seen in a GFP-negative environment (Fig. 6E,F), but, in contrast to the myogenic cells at initial stages of emigration, nLacZ-positive nuclei were not observed, indicating that few myogenic precursor cells had fused into pre-existing host muscle fibres so that the nuclei of the latter were in excess of the donor nuclei, which provided the GFP gene and its highly diffusible product.

Discussion

In the present work, the GFP label of origin, in combination with an nLacZ labelling in the same tissue or nLacZ as a complementary label, has revealed a surprising migratory activity of myogenic cells, both from host to graft and from graft to host muscles. Despite extensive spreading within muscle fibres, the GFP label remains strictly cell autonomous as shown

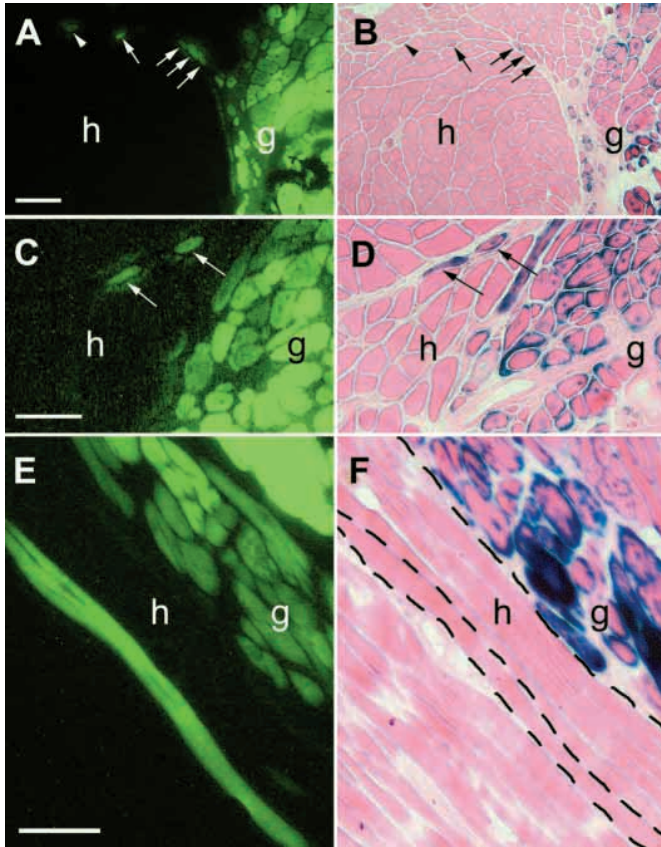


Fig. 6. Early stages of graft cell migration: double-labelled donor grafted into unlabelled host, GFP/nLacZ to 0/0, two different experiments (A-D and E,F), both 52 days p.o. The same region of the host (h)/graft(g) border is shown in the adjacent cross (A-D) and longitudinal (E,F) sections. (A,C,E) Fluorescence to show donor GFP; (B,D,F) Xgal plus eosin, to show donor-labelled nuclei. (A-D) Arrows indicate GFP/nLacZ-positive donor cells 'on their way' into host muscle; arrow heads indicate a cell in which no blue nucleus was seen. (E,F) GFP-positive fibres in the host muscle with no nLacZ-positive nucleus visible. In the graft, low GFP level and strong β -galactosidase activity indicate myotubes and immature muscle fibres. Bars, 100 μ m.

by single GFP-positive fibres in a GFP-negative muscle environment (e.g. Fig. 6C). The use of GFP as a cell marker in muscle transplantation has drastically increased the sensitivity of the marker in comparison to others previously used in this context, for example, the biochemical marker glucose phosphate isomerase alloenzyme (cf. Partridge et al., 1978; Füchtbauer et al., 1988), the nuclear marker chromosomal sex (in situ hybridisation using a Y probe) (cf. Grounds et al., 1991; Augustin et al., 1998; Smythe and Grounds, 2001), the transgenes LacZ and nLacZ, and dystrophin. The reason is probably the efficient longitudinal distribution of GFP owing to its low molecular mass and extremely high diffusibility in a cytoplasmic environment in comparison with, for example, bacterial β -galactosidase, the product of the LacZ transgene (Arrio-Dupont et al., 2000). Furthermore, the diffusion of the modified β -galactosidase in this study is probably retarded by its nuclear localization signal. This explanation predicts that at larger distances from a double-labelled donor or host, the

probability of detecting 'blue nuclei' in a 'green cytoplasm' should decrease. This is indeed the case. Because in the transgenic strain used the level of GFP in connective tissue is very low in comparison with that in muscle (Jockusch et al., 2003) both GFP and nLacZ are muscle-specific markers at the graft site, unlike markers such as glucose phosphate isomerase or the Y chromosome. The observation of muscle fibres containing the cytoplasmic marker GFP in immigrant cells and the pseudonuclear marker nLacZ in the surrounding tissue excludes the possibility that ingrowth of muscle fibres, for example, from the suture at the knee, can account for the results shown here, and strongly supports the alternative – migration of myogenic cells and co-fusion with resident fibres.

In most experiments with transgenic labels permanent cell lines have been used as donors, often in combination with short (a few days) regeneration times. In such experiments, the role of matrix metalloproteinases during the migration of myogenic cells has been demonstrated (El Fahime et al., 2000). However, the use of grafted muscle tissue creates a situation closer to natural conditions for cell migration (cf. Fan et al., 1996). In previous experiments using muscle grafts no or very low migratory activity of myogenic precursor cells has been observed (Watt et al., 1987; Moens et al., 1996). This difference in our results may not only be due to the use of markers of origin with a low diffusion coefficient, like dystrophin, but also to a lack of an initial traumatic stimulus (which was provided by replacing one muscle by another in our experiments). The induction of degeneration/regeneration and elimination of host myogenic cells by irradiation has proved to be essential to obtain even low yields of circulating stem cells that integrated into skeletal recipient muscle (Ferrari et al., 1998). In more recent experiments, Smythe and Grounds transplanted longitudinally sliced EDL muscles into a slit of the anterior tibial muscle of the mouse (Smythe and Grounds, 2001) but did not pharmacologically induce muscle degeneration or irradiate the host to suppress its contribution to regeneration. Here, the 'double wounding', that is, local destruction of both host and donor perimysium, would have provided a strong wound stimulus and removed an important barrier to migration. Using the Y chromosome as a marker, Smythe and Grounds showed that immigration of graft cells into host muscle was enhanced by a delayed differentiation owing to the inactivation of the *MyoD* gene.

Origin of myogenic cells

The appearance of donor markers in host tissue and vice versa may be due to circulating pluripotent stem cells (Ferrari et al., 1998; Galli et al., 2000). However, in the present transplantation experiments the evidence points to a local and muscular origin of migrant myogenic cells (cf. Grounds et al., 2002). Host cells in a graft, and graft cells in a host, appear only after a delay of about 7 weeks. Graft cells in the host are distributed in a gradient from their presumed origin, the adjacent graft muscle tissue. In double labelling experiments, single myogenic cells have been observed that seem on their way from one muscle to the other. In the reverse experiments, most fibres with the host label are found near the centre of the cross section of the graft where the highest level of ischemia causes the most intense degeneration and regeneration (Carlson, 1986).

The migration of myogenic cells from intact host muscle into the regenerating graft may be stimulated by 'wound hormones', that is. chemoattractants diffusing from the graft into the neighbourhood (Watt et al., 1994; Grounds and Davies, 1996; Bischoff, 1997). On the other hand, the degeneration/regeneration processes within a muscle graft disconnected from its original blood and nervous supply lead to a massive activation of satellite cell proliferation (Carlson, 1986) and may also stimulate a long distance emigration of muscle precursor cells. At a low level, migration of juvenile and adult myogenic cells may take place even in the absence of a wound stimulus. This would constitute a potentially continuous repair mechanism. In GFP \leftrightarrow 0 chimeras, patches of GFP-free skeletal muscle fibres are extremely rare and only observed in special cases such as extraocular muscles and in chimeras with very low GFP-positive contributions (D. Eberhard and H.J., unpublished). This suggests an extensive migration and mixing of myogenic cells, at least during organogenesis.

The observation of a long-term exchange of myogenic cells across borders of individual muscles may have a bearing on the possibility of cell therapy of diseased muscle. Solid transplanted muscle tissue (Füchtbauer et al., 1988; Fan et al., 1996; Smythe and Grounds, 2001) may act as a source of muscle precursor cells for an extended period of time and may thus circumvent the low yields in myoblast and stem cell colonization of muscle (Hodgetts et al., 2000; Ferrari et al., 2001; Partridge, 2002) as a route for therapeutical improvement.

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