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Stem Cell Function, Self-Renewal, and Behavioral Heterogeneity of Cells from the Adult Muscle Satellite Cell Niche

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Summary

Satellite cells are situated beneath the basal lamina that surrounds each myofiber and function as myogenic precursors for muscle growth and repair. The source of satellite cell renewal is controversial and has been suggested to be a separate circulating or interstitial stem cell population. Here, we transplant single intact myofibers into radiation-ablated muscles and demonstrate that satellite cells are self-sufficient as a source of regeneration. As few as seven satellite cells associated with one transplanted myofiber can generate over 100 new myofibers containing thousands of myonuclei. Moreover, the transplanted satellite cells vigorously self-renew, expanding in number and repopulating the host muscle with new satellite cells. Following experimental injury, these cells proliferate extensively and regenerate large compact clusters of myofibers. Thus, within a normally stable tissue, the satellite cell exhibits archetypal stem cell properties and is competent to form the basal origin of adult muscle regeneration.

Introduction

The ability of so complex a tissue as adult skeletal muscle to regenerate, even after total disruption (Studitsky, 1964; Carlson, 1986), has conventionally been attributed to the satellite cell: a quiescent muscle precursor forming a small population beneath the basal lamina of each myofiber (Mauro, 1961). Myofibers, the contractile units of skeletal muscle, are giant syncytial cells, each containing several hundred myonuclei within a continuous cytoplasm. Mammalian myonuclei are terminally differentiated and cannot replace themselves. Muscle is normally a stable tissue but, after injury, regeneration occurs via the activation and proliferation of satellite cells to form a pool of myoblasts, which differentiate and fuse to provide the myonuclei required to repair or replace damaged myofibers (Moss and Leblond, 1971; Snow, 1978). Paradoxically, when satellite cells are enzymatically dissociated and transplanted into a new host, their subsequent contribution to muscle regeneration is at best inefficient, generating a quantity of new differentiated myonuclei that rarely exceeds the original number of undifferentiated satellite cells (reviewed in Partridge, 2003). This discrepancy, combined with the demonstrated myogenic contribution of nonsatellite cell phenotypes such as bone marrow-derived cells (Ferrari et al., 1998; LaBarge and Blau, 2002) and muscle interstitial cells (Asakura et al., 2002; Polesskaya et al., 2003), has called into question the validity of the original satellite cell-centered paradigm. Importantly, though a satellite cell population is maintained throughout the life span of an organism, the origin of postnatally renewed satellite cells remains controversial (reviewed in Zammit and Beauchamp, 2001). Transplanted bone marrow cells can enter the myofiber sublaminal niche and express satellite cell markers (LaBarge and Blau, 2002), but such cells do not appear to make a significant contribution to injury-induced regeneration or form substantial myogenic colonies upon reisolation from the muscle (Sherwood et al., 2004). Satellite cells are also formed from transplanted muscle-derived cells (Blaveri et al., 1999; Heslop et al., 2001), but since donor muscle does not increase over time (Morgan et al., 1993), their ability to function as myogenic progenitors is also equivocal. Therefore, although the ability of satellite cells to differentiate into muscle is beyond doubt, it has not been established whether the satellite cell population is self-sufficient as a source of myogenic cells or, alternatively, is a mere progenitor population renewed by a different interstitial (Asakura et al., 2002; Polesskaya et al., 2003) or circulating (LaBarge and Blau, 2002) stem cell. Determining the source of postnatal satellite cell renewal is key to understanding the mechanism by which the regenerative function of adult muscles is sustained.

Satellite cells are defined anatomically, by their position beneath the basal lamina of myofibers (Mauro, 1961), but their function has previously been studied using preparations of dissociated cells, containing both satellite cells derived from the sublaminal niche and other progenitors derived from the interstitium. Since such dissociated cells have poor myogenic potential, previous transplantation studies have been limited to using very large populations, typically 10^4–10^6 per engrafted host muscle. Here, we graft individual, freshly isolated myofibers into the radiation-ablated muscles of dystrophic *mdx*-nude mice, as a method of investigating the potential of pure and small populations of anatomically defined satellite cells. We show that, in

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vivo, as few as seven satellite cells associated with one myofiber can give rise to sufficient differentiation-competent progeny to generate thousands of myonuclei. Unexpectedly, we find that satellite cells from different hindlimb muscles display different behavioral characteristics after grafting into the same host environment. We show that satellite cells are capable of extensive self-renewal, repopulating the irradiation-depleted host muscle with functional myogenic cells that generate large clusters of new myofibers during subsequent rounds of injury-induced regeneration. The satellite cell therefore has potent myogenic potential and archetypal stem cell properties that suit it to form a self-sufficient source of regeneration in adult skeletal muscles.

Results

Myogenic Potential of Satellite Cells in Single Myofiber Grafts

To investigate the behavior of small and pure populations of satellite cells in vivo, individual myofibers were grafted into the tibialis anterior (TA) muscles of dystrophin (dys)-deficient *mdx*-nude mice (Partridge et al., 1989). Three days prior to grafting, the hindlimbs of host mice were irradiated to ablate host satellite cell function (Gross et al., 1999; Heslop et al., 2000). Donor myofibers were isolated from the extensor digitorum longus (EDL), soleus, and TA muscles of adult 3F-*nLacZ*-2E mice, in which β-galactosidase (β-gal) reports expression of the myosin light chain 3F gene in the nuclei of fast myofibers (Kelly et al., 1995), but not satellite cells (Beauchamp et al., 2000). Immunocytochemistry for the satellite cell marker Pax7 (Seale et al., 2000) showed that the average number of associated satellite cells was 7.23 (SE 0.43) for EDL myofibers and 22.08 (SE 1.52) for soleus myofibers, in accordance with our previous observations (Zammit et al., 2002), and 9.66 (SE 0.72) for TA myofibers. Coimmunostaining for the basal lamina protein laminin, identified an occasional Pax7$^-$ nucleus outside the basal lamina of 3.5% of myofibers, but no Pax7$^+$ extralaminal nuclei were seen (Figures 1A and 1B). Therefore, in accordance with our previous observations (Rosenblatt et al., 1995), the vast majority of myofibers were not contaminated by other cell types. Each host muscle was grafted with a single freshly isolated myofiber with its associated population of sublaminal satellite cells. Engrafted muscles were removed 3 weeks later and frozen for sectioning. Alternate sections were stained in X-gal to localize β-gal activity reporting myofiber-specific expression of the 3F transgene, or stained with an antibody against dystrophin (dys). While only a proportion of grafts generated muscle identifiable by these two markers, some individual single myofiber grafts generated clusters of muscle containing more than 100 new myofibers. Thus, in a 3 week period the small retinue of satellite cells associated with a single grafted myofiber underwent rapid and sustained proliferation to generate sufficient differentiation-competent progeny to regenerate large numbers of new myofibers in the host muscle (Figures 1C–1H).

For example, an individual EDL myofiber graft containing an average of 7 (and certainly fewer than 20) satellite cells generated 189 dys$^+$ myofibers, of which 100 were β-gal$^+$ (Figure 1C). Transplanted myoblasts both generate myofibers de novo and contribute myonuclei to repair existing host myofibers, forming mosaics for which dys expression in an *mdx* myofiber requires a minimum donor contribution of about 25% of myonuclei (Blaveri et al., 1999). Since each TA myofiber contains about 600 myonuclei (our unpublished data), at a conservative estimate, the observed absolute numbers of dys$^+$ myofibers would contain at least 25,000–30,000 differentiated donor myonuclei. Previously, grafts of dissociated myoblasts have been shown to generate a net yield of, at best, one myonucleus per grafted cell (reviewed in Partridge, 2003). Here, in some grafts the net yield per grafted satellite cell was several thousand myonuclei.

As a control, we isolated donor myofibers from 3F-*nLacZ*-2E muscles that had been exposed to 18 Gy γ-irradiation 72 hr previously. 18 Gy is sufficient to completely ablate replication-competent satellite cells, though the muscle still retains some regenerative potential and there is no overt damage to the myofibers themselves (Heslop et al., 2000). We found that grafts of freshly isolated irradiated soleus (n = 10) or EDL (n = 10) single myofibers did not generate any β-gal$^+$ nuclei in the host muscle (see Table S1 in the Supplemental Data available with this article online).

Functional Heterogeneity of Satellite Cells from Different Hindlimb Muscles

The frequency with which grafts successfully generated muscle appeared to be related to the average number of satellite cells originally present within the graft: soleus myofibers, with an average of 22 satellite cells, formed muscle in 75% (15/20) of grafts, whereas EDL myofibers, with an average of 7 satellite cells, formed muscle in 32% (11/34) of grafts, and TA myofibers, with an average of 10 satellite cells, formed muscle in 33% (14/42) of grafts. The quantity of muscle formed by each successful graft varied substantially, but not in proportion to satellite cell number. While pathological differences between individual graft sites are likely to have been a partial determinant of myogenic performance, we nevertheless observed a consistent pattern of variability between grafts derived from the three different muscle groups (Figures 1C–1H). The numbers of β-gal$^+$ myofibers generated by EDL, soleus, and TA myofiber grafts were compared using a zero-inflated negative binomial regression model, which takes into account the high proportion of zero values and the overdispersion associated with a Poisson distribution (Cheung, 2002). While there was no significant difference between the amounts of muscle formed from EDL and soleus myofiber grafts ($p = 0.22$), the amounts of muscle formed from TA myofiber grafts were found to be significantly smaller than those of the other two groups ($p < 0.001$ in both cases). The satellite cell in its myofiber sublaminal niche therefore exhibits functional properties distinct to its muscle of origin.

Satellite Cells Self-Renew and Repopulate Irradiation-Depleted Muscles

To investigate the ability of satellite cells to give rise to new satellite cells, we used myofiber grafts derived from

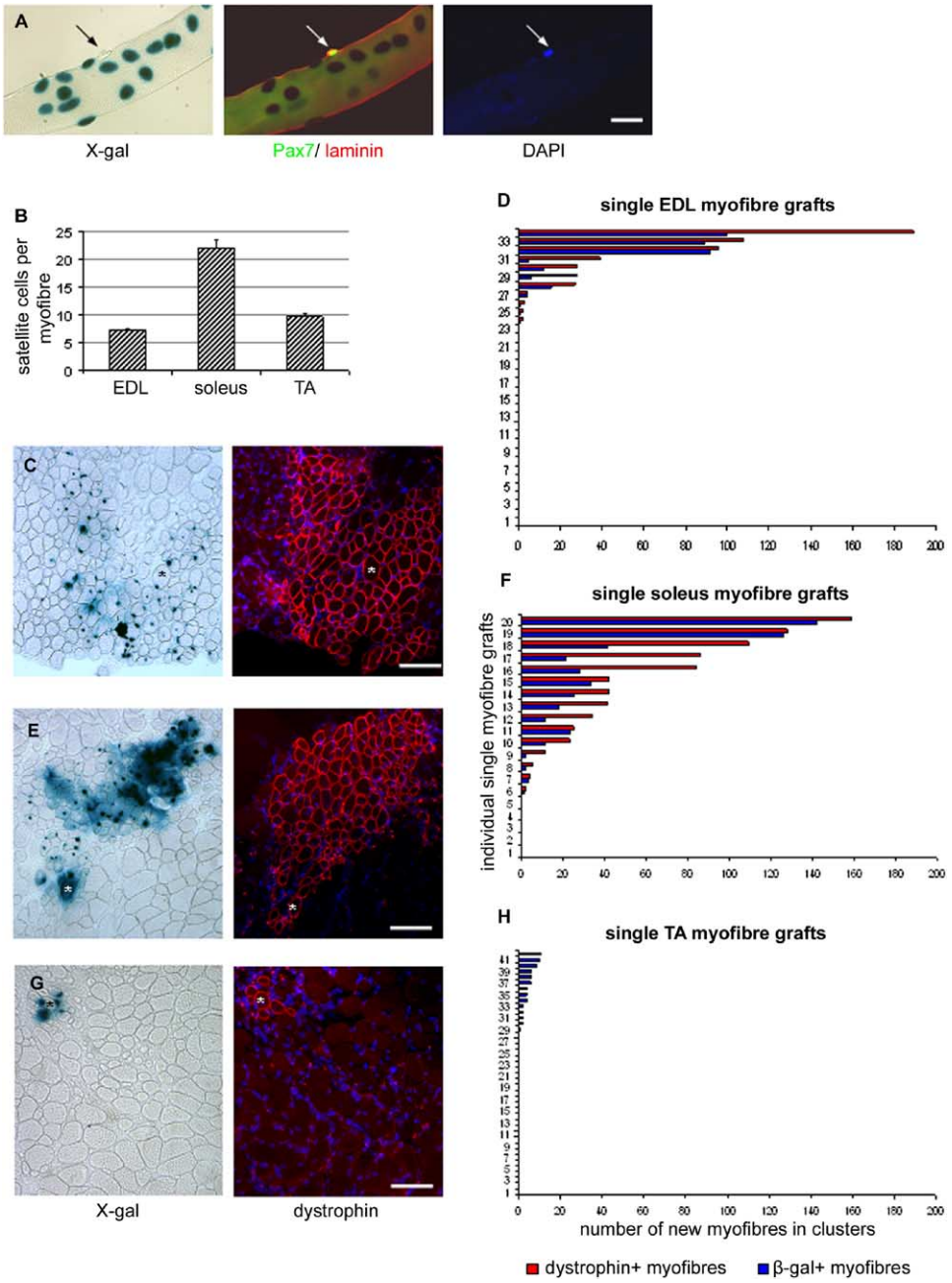

Figure 1. Myogenic Potential of Satellite Cells in Single Myofiber Grafts

(A) Single myofiber isolated from a donor 3F-*nLacZ*-2E mouse, stained in X-gal and immunostained for Pax7 and laminin. Myonuclei contain the blue reaction product of X-gal staining, which quenches DAPI immunofluorescence. A DAPI$^+$ nucleus (arrow) is identifiable as that of a satellite cell by its expression of Pax7 and its location beneath the basal lamina as visualized by laminin staining. Scale bar, 25 μm.

(B) Bar chart showing the mean numbers of Pax7$^+$ satellite cells on single myofibers isolated from EDL (n = 77 from five mice), soleus (n = 40 from three mice), and TA (n = 50 from three mice) muscles of adult 3F-*nLacZ*-2E mice. Each error bar represents a standard error.

(C and D) Grafts of single myofibers isolated from the EDL muscles of donor adult 3F-*nLacZ*-2E mice and inserted into the irradiated TA muscles of host *mdx*-nude mice. Three week time point. (C) Serial transverse sections of an engrafted muscle stained in X-gal solution or for dys. The average of seven satellite cells on the EDL myofiber underwent extensive proliferation to regenerate a cluster of 189 dys$^+$ myofibers, of which 100 were β-gal$^+$. (D) Bar chart depicting muscle generated by 34 individual EDL myofiber grafts derived from four donor animals. Ranked in order of the numbers of dys$^+$ myofibers.

(E and F) Muscle generated from grafts of 3F-*nLacZ*-2E single soleus myofibers into irradiated *mdx*-nude TA muscles. (E) Donor-derived muscle identified in serial sections by detection of β-gal activity and of dys. (F) Bar chart depicting muscle generated by 20 individual single myofiber grafts from three donor animals. Ranked in order of the number of dys$^+$ myofibers.

(G and H) Muscle generated from grafts of 3F-*nLacZ*-2E single TA myofibers into irradiated *mdx*-nude TA muscles. (G) Donor-derived muscle identified in serial sections by detection of β-gal activity and dys. (H) Bar chart depicting muscle formation from 42 individual myofiber grafts from three donor animals (β-gal$^+$ myofibers only are shown; dys staining was not carried out on most muscles). Ranked in order of the number of β-gal$^+$ myofibers. Scale bars, 100 μm. Asterisks mark the same myofiber in each series.

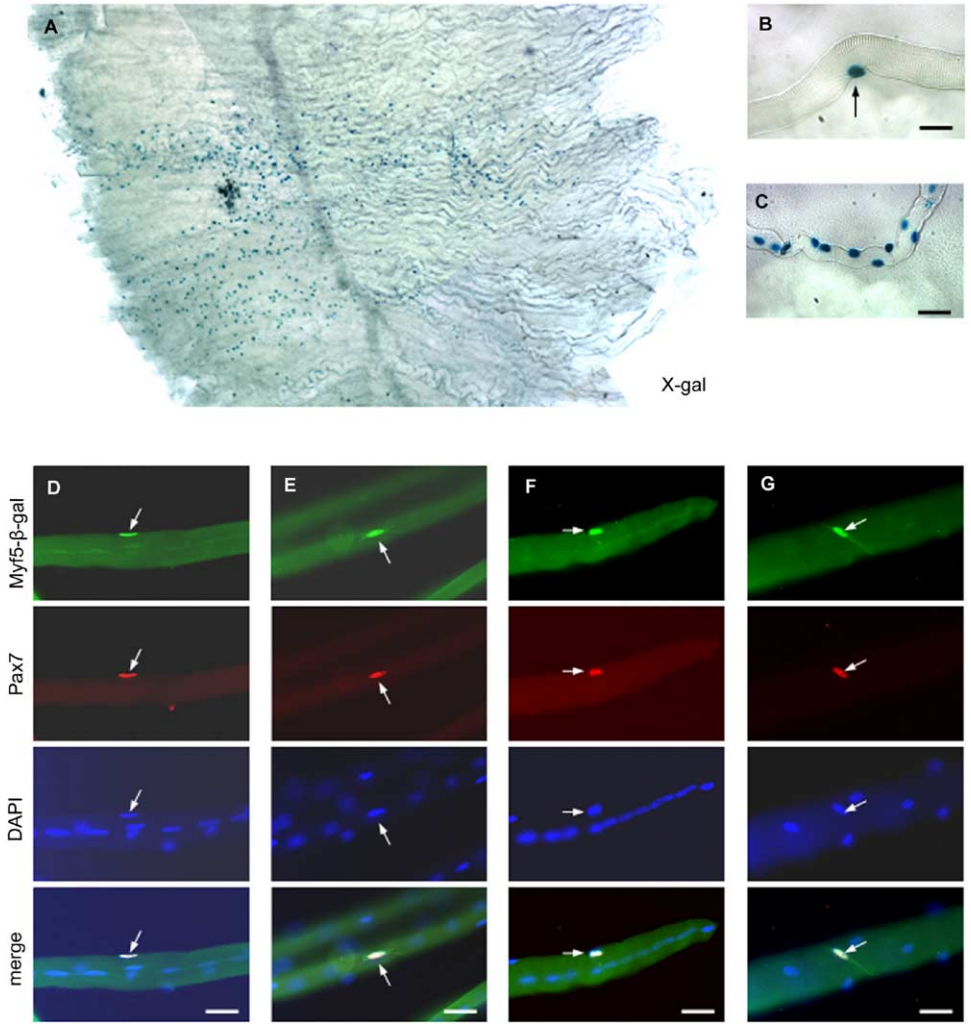

Figure 2. Satellite Cells Self-Renew and Repopulate Irradiation-Depleted Muscles

(A) X-gal-stained segment of an irradiated *mdx*-nude TA muscle that had been grafted with a single *Myf5*$^{nLacZ/+}$ soleus myofiber (with average 22 satellite cells) 3 weeks previously. Numerous Myf5-β-gal$^+$ mononucleate cells have repopulated a large area of muscle.

(B and C) X-gal-stained myofibers isolated from an irradiated *mdx*-nude TA muscle engrafted with a single *Myf5*$^{nLacZ/+}$ EDL myofiber 3 weeks previously. (B) Myofiber bearing a Myf5-β-gal$^+$ satellite cell (arrow). Scale bars, 25 μm. (C) A chain of newly formed donor β-gal$^+$ myonuclei in a newly formed donor myofiber. Scale bars, 50 μm.

(D–G) Single myofibers isolated from irradiated *mdx*-nude TA muscles, each engrafted with a *Myf5*$^{nLacZ/+}$ single soleus myofiber. Coexpression of Pax7 and Myf5-β-gal unequivocally identifies graft-derived satellite cells (arrows). (D and F) Weak β-gal staining of centrally located chains of Pax7$^-$ nuclei indicates a recent graft contribution to myonuclei. Scale bars, 25 μm.

the EDL, soleus, and TA muscles of adult *Myf5*$^{nLacZ/+}$ mice, in which β-gal reports expression of *Myf5* in the nuclei of satellite cells and also in newly formed, but not mature myofibers (Tajbakhsh et al., 1996; Beauchamp et al., 2000). Three weeks after grafting of a single myofiber, we isolated new single myofibers from each engrafted muscle and stained them, together with the undigested portion of muscle, in X-gal. Two out of ten muscles engrafted with a single EDL myofiber, two out of six muscles engrafted with a single soleus myofiber, and five out of ten muscles engrafted with a single TA myofiber contained several hundred Myf5-β-gal$^+$ mononucleate cells, often distributed over a considerable region of muscle (Figure 2A). Analysis of isolated myofibers confirmed that β-gal activity was commonly localized to the discrete nuclei of satellite cells (Figure 2B). In addition, chains of centrally located β-gal$^+$ myonuclei were seen in a few newly regenerated myofibers (Figure 2C). Since myonuclear β-gal translocates to adjacent myonuclei, the satellite cell nuclei could be distinguished from myonuclei by the absence of such translocation, implying lack of cytoplasmic continuity with the myofiber (Yang et al., 1997; Heslop et al., 2001).

In order to quantify the numbers of satellite cells formed, myofibers isolated from six further soleus myofiber-engrafted muscles were analyzed by immunostaining for Myf5-β-gal and Pax7. Graft-derived satellite cells were unequivocally identified by coexpression of these canonical markers in nuclei that were closely associated with myofibers (Figures 2D–2G). Pax7$^+$ β-gal$^-$

Table 1. Counts of Satellite Cells Generated from Individual Grafts of *Myf5*$^{nLacZ/+}$ Soleus Single Myofibers

	Mouse 1 (Right)	Mouse 1 (Left)	Mouse 2 (Right)	Mouse 2 (Left)	Mouse 3 (Right)	Mouse 3 (Left)	Average
Total sampled myofibers	27	40	32	28	25	29	—
Counted Pax7$^+$ satellite cells	31	55	9	10	50	70	—
Pax7$^+$ satellite cells per myofiber	1.15	1.38	0.28	0.36	2.00	2.41	1.26
[a]Calculated TOTAL (all Pax7$^+$) satellite cells in whole muscle	1193	1431	290	373	2074	2499	1310
Counted Pax7$^+$ β-gal$^-$ satellite cells	26	35	9	10	49	50	—
Pax7$^+$ β-gal$^-$ satellite cells per myofiber	0.96	0.88	0.28	0.36	1.96	1.72	1.03
[a]Calculated HOST (Pax7$^+$ β-gal$^-$) satellite cells in whole muscle	996	913	290	373	2033	1784	1065
Counted Pax7$^+$ β-gal$^+$ satellite cells	5	20	0	0	1	20	—
Pax7$^+$ β-gal$^+$ satellite cells per myofiber	0.19	0.50	0	0	0.04	0.69	0.24
[a]Calculated DONOR (Pax7$^+$ β-gal$^+$) satellite cells in whole muscle	197	519	0	0	41	716	246

A sample of myofibers was isolated from each engrafted muscle, and the number of host (Pax7$^+$ β-gal$^-$) and donor (Pax7$^+$ β-gal$^+$) satellite cells was counted. The total number of host and donor satellite cells per engrafted muscle was then calculated.
[a] Based on a mean of 1037 total myofibers per muscle (Table S2).

cells were assumed to be persistent host satellite cells; although radiation completely ablates replication-competent satellite cells (Heslop et al., 2000), apoptosis does not occur until cells commence mitosis. A total of 225 satellite cells associated with 186 myofibers isolated from six engrafted muscles were analyzed (Table 1). This sample represents about 3% of total myofibers, since sections of equivalent muscles engrafted with soleus myofibers had a mean of 1037 (SE 46) total myofibers per muscle (Table S2). We calculated that, on average, a total of 246 Pax7$^+$ β-gal$^+$ satellite cells were generated from each single myofiber graft (Table 1). Since the original number of satellite cells per graft averaged 22, formation of 246 new satellite cells represents a 10-fold population expansion. These data confirm that most β-gal$^+$ nuclei identified by X-gal staining of whole muscles were indeed those of satellite cells. The frequent association of graft-derived satellite cells with small, centrally nucleated myofibers implies that they were generated concomitantly with myonuclei (Figures 2D and 2F). These results demonstrate that the satellite cell population self-renews by an active physiological process. After proliferation, satellite cell progeny adopt divergent fates: while the majority population differentiates, some cells persist as an undifferentiated reserve population and occupy the sublaminal satellite cell niche. Moreover, because the observed output of satellite cells was 10-fold larger than the original input, these data show that a small satellite cell population can expand itself to repopulate a depleted muscle.

Myogenic Cells Derived from Single Myofiber Grafts Are Robustly Regenerative in Response to Subsequent Injury of the Engrafted Muscle

Satellite cells can be formed from bone marrow grafts (LaBarge and Blau, 2002), but their poor contribution to myotoxin-induced regeneration (Sherwood et al., 2004) argues that residency within the myofiber sublaminal niche does not necessarily predict myogenic potential. To evaluate the myogenic competence of the satellite cells derived from single myofiber grafts, we injured engrafted muscles with notexin, a myotoxin which destroys myofibers but spares satellite cells and leaves the basal laminae and microcirculation intact (reviewed in Harris, 2003). In control studies, notexin injected into donor strain *Myf5*$^{nLacZ/+}$ TA muscles destroyed 75%–80% of myofibers within 24 hr, leading 1 week later to the formation of newly regenerated myofibers that could be distinguished from the minority of surviving myofibers by their expression of myonuclear Myf5-β-gal and cytoplasmic neonatal myosin, and by their small size and central nucleation (Gross and Morgan, 1999) (Figure S1). In transplantation experiments, the TA muscles of *mdx*-nude hosts were each grafted with a single *Myf5*$^{nLacZ/+}$ myofiber, and some were injured 4 weeks later by notexin injection. A week after injury, muscles were removed to assess the regenerative response. Many engrafted muscles contained compact clusters of dys$^+$ muscle, some comprising over 100 myofibers. Graft-derived myofibers were small and centrally nucleated and robustly expressed the regenerative markers Myf5-β-gal and neonatal myosin (Figures 3 and 4). Satellite cell progeny derived from each single myofiber graft therefore underwent rapid proliferation in the week following notexin injury and had robust myogenic potential. This sustains the idea that satellite cells have sufficient proliferative and myogenic potential to constitute an exclusive origin of new myonuclei in regenerating muscles.

The Frequency of Muscle Formation Increases Both Spontaneously over Time and Further in Response to Experimental Injury

In the absence of experimental injury, we found that the proportion of grafts generating identifiable muscle was higher at the 5 week time point than in experiments analyzed at 3 weeks, rising from 32% to 40% for EDL grafts, 33% to 63% for TA grafts, and 75% to 100% for soleus grafts (Figure 5A). This indicates spontaneous recruitment of previously inactive cells into the myogenic program over time. The average number of donor myofibers per successfully engrafted muscle was also greater at 5 weeks than at 3 weeks, rising from 48 to 114 for EDL myofiber grafts, from 54 to 75 for soleus

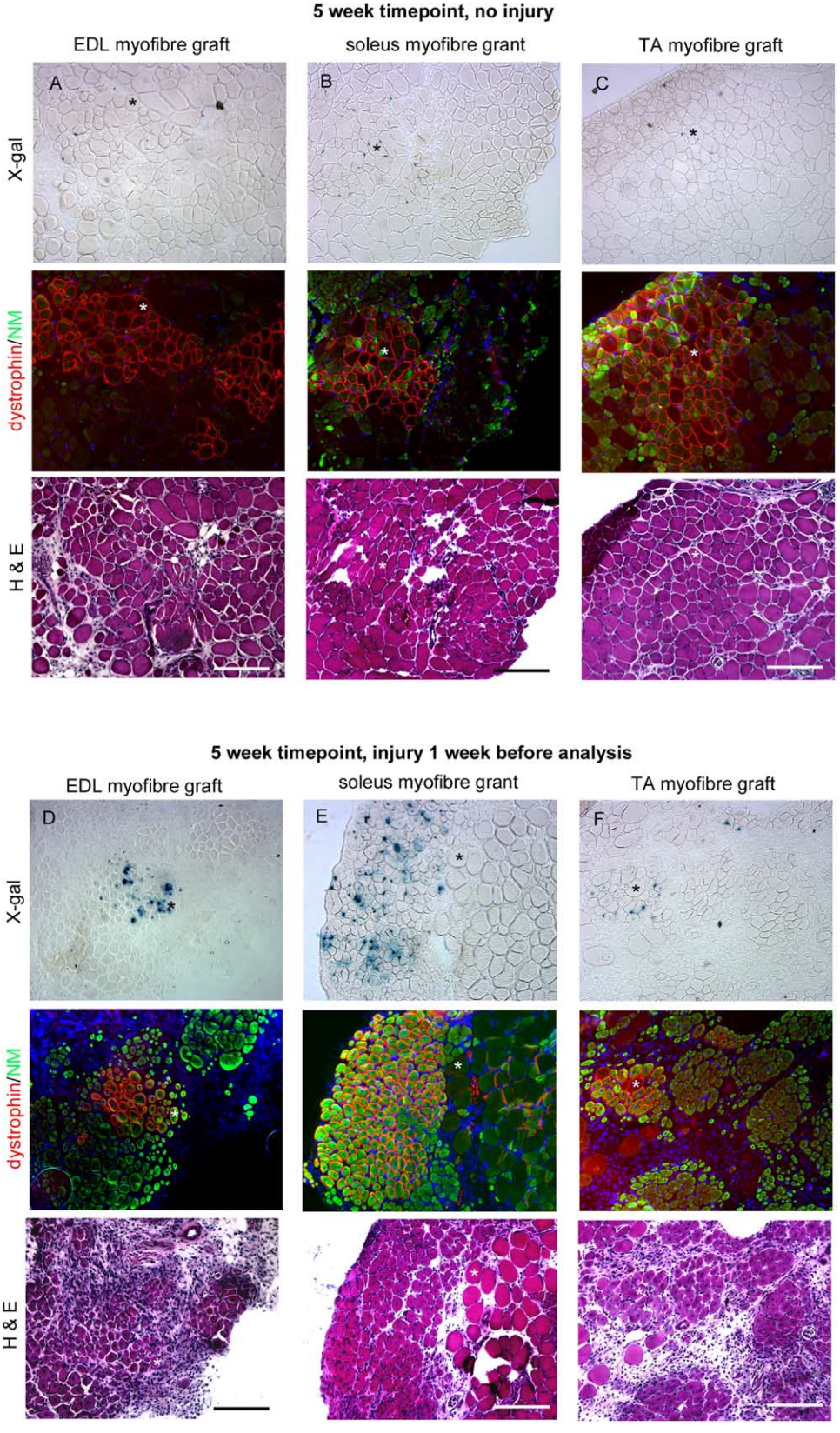

Figure 3. Muscle Regeneration by *Myf5*$^{nLacZ/+}$ Single Myofiber Graft-Derived Cells after Notexin Injury of the Engrafted Muscle

(A–C) Five week time point without injury. Serial transverse sections of irradiated *mdx*-nude TA muscles, each engrafted with a single *Myf5*$^{nLacZ/+}$ myofiber derived from an EDL, soleus, or TA muscle. (A) An EDL myofiber-engrafted muscle contains a large cluster of dys^{+}

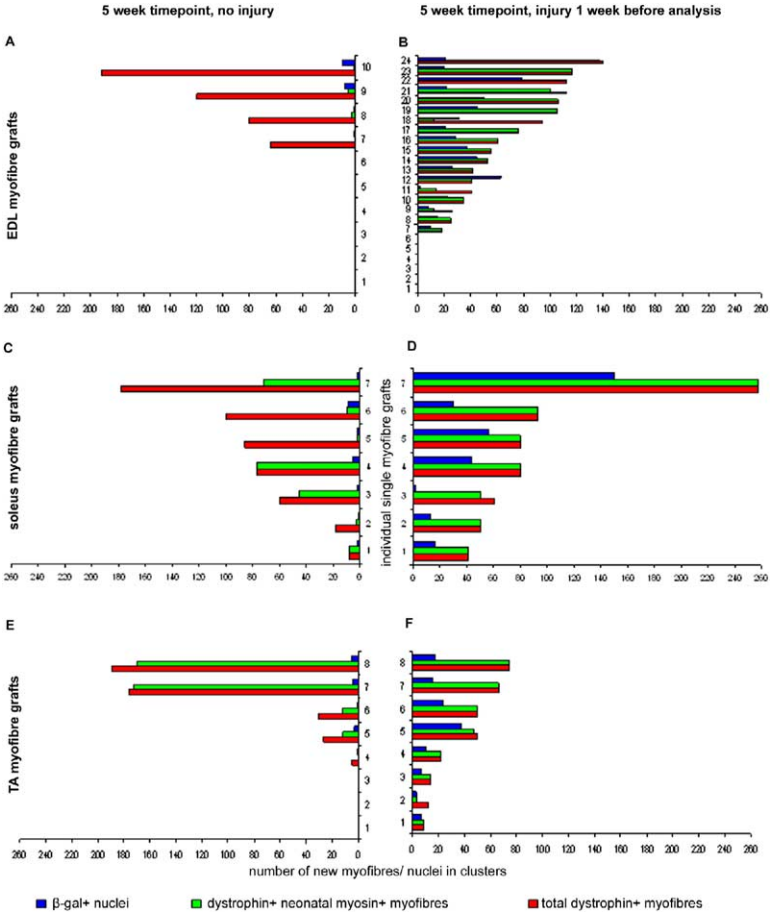

Figure 4. Bar Charts Depicting Counts of Myofibers Regenerated by *Myf5*$^{nLacZ/+}$ Single Myofiber Graft-Derived Cells after Notexin Injury of the Engrafted Muscle

Data ranked in order of the number of dys$^+$ myofibers. Engrafted muscles were injured 4 weeks after grafting and then removed 1 week later. (A and B) Bar charts showing muscle generated from EDL myofiber grafts. Injured muscles contained significantly higher numbers of dys$^+$ neonatal myosin$^+$ myofibers (Mann-Whitney, $p = 0.002$) and β-gal$^+$ nuclei (Mann-Whitney, $p = 0.005$) than uninjured muscles. (C and D) Bar charts showing muscle generated from soleus myofiber grafts. Injured muscles contained significantly higher numbers of dys$^+$ neonatal myosin$^+$ myofibers (Mann-Whitney, $p = 0.026$) and β-gal$^+$ nuclei (Mann-Whitney, $p = 0.007$) than uninjured muscles. (E and F) Bar charts showing muscle generated from TA myofiber grafts. Injured muscles contained significantly higher numbers of β-gal$^+$ nuclei (Mann-Whitney, $p < 0.001$), but not dys$^+$ neonatal myosin$^+$ myofibers (Mann-Whitney, $p = 0.235$), than uninjured muscles.

myofiber grafts, and most strikingly, from 5 to 86 for TA myofiber grafts (Figure 5A). In the absence of experimental injury, 2.0% of total dys$^+$ myofibers derived from EDL myofiber grafts and 41.2% of dys$^+$ myofibers formed from soleus myofiber grafts expressed neonatal myosin, indicating that they had been recently generated. However, neonatal myosin was seen in a much larger proportion, 85.5%, of myofibers derived from TA myofiber grafts, indicating the recent occurrence of regenerative events that would account for the substantial catch-up between weeks 3 and 5. Thus EDL, soleus, and TA satellite cells are all capable of generating large clusters of muscle, but TA satellite cells do so with a comparative delay, perhaps reflecting a slower proliferative rate.

Further evidence of a vigorous and persistent myogenic reserve derived from grafted satellite cells was the finding that injury of the engrafted muscles further augmented the proportion of grafts generating muscle (Figures 3, 4, and 5A). Thus, only 40% of EDL myofiber grafts generated muscle at the 5 week time point in the absence of experimental injury, but at the same time point, 75% of grafts (derived from the same donor) formed clusters of newly regenerated muscle where injury was inflicted 1 week prior to removal of the muscles. Similarly, TA myofiber grafts had generated myofibers in 64% of undamaged muscles by 5 weeks but had contributed to 100% of the contralateral muscles injured 1 week previously (Figures 4 and 5A). We infer from this that the lack of muscle formation by

myofibers, which are relatively mature as shown by their large size, mainly peripheral nucleation, and lack of neonatal myosin and Myf5-β-gal. Occasional peripheral β-gal$^+$ nuclei are candidate satellite cells. (B) A cluster of dys$^+$ muscle derived from a soleus myofiber graft contains mainly mature myofibers, but also a few that express neonatal myosin and Myf5-β-gal. (C) A large cluster of dys$^+$ muscle derived from a TA myofiber graft contains numerous neonatal myosin$^+$ myofibers and some β-gal$^+$ nuclei. The high proportion of recently regenerated myofibers is consistent with the large increase in cluster size observed here at 5 weeks relative to other experiments analyzed at 3 weeks (see Figure 1). (D–F) Five week time point with notexin injury 1 week before analysis. Serial sections of irradiated *mdx*-nude TA muscles, each engrafted with a single *Myf5*$^{nLacZ/+}$ myofiber, injected with notexin 4 weeks after grafting, and removed 1 week later. The formation of compact clusters of small, centrally nucleated dys$^+$ myofibers, which strongly express neonatal myosin and myonuclear Myf5-β-gal fusion protein, demonstrates that graft-derived cells responded to the injury by rapidly generating numerous progeny that differentiated to form new myofibers. Usually one, but occasionally two or three compact clusters were formed. Neonatal myosin$^+$ dys$^-$ myofibers are derived from host radiation-resistant progenitors that are activated in response to notexin injury (Heslop et al., 2000). Scale bars, 200 μm. Asterisks mark the same myofiber in each series.

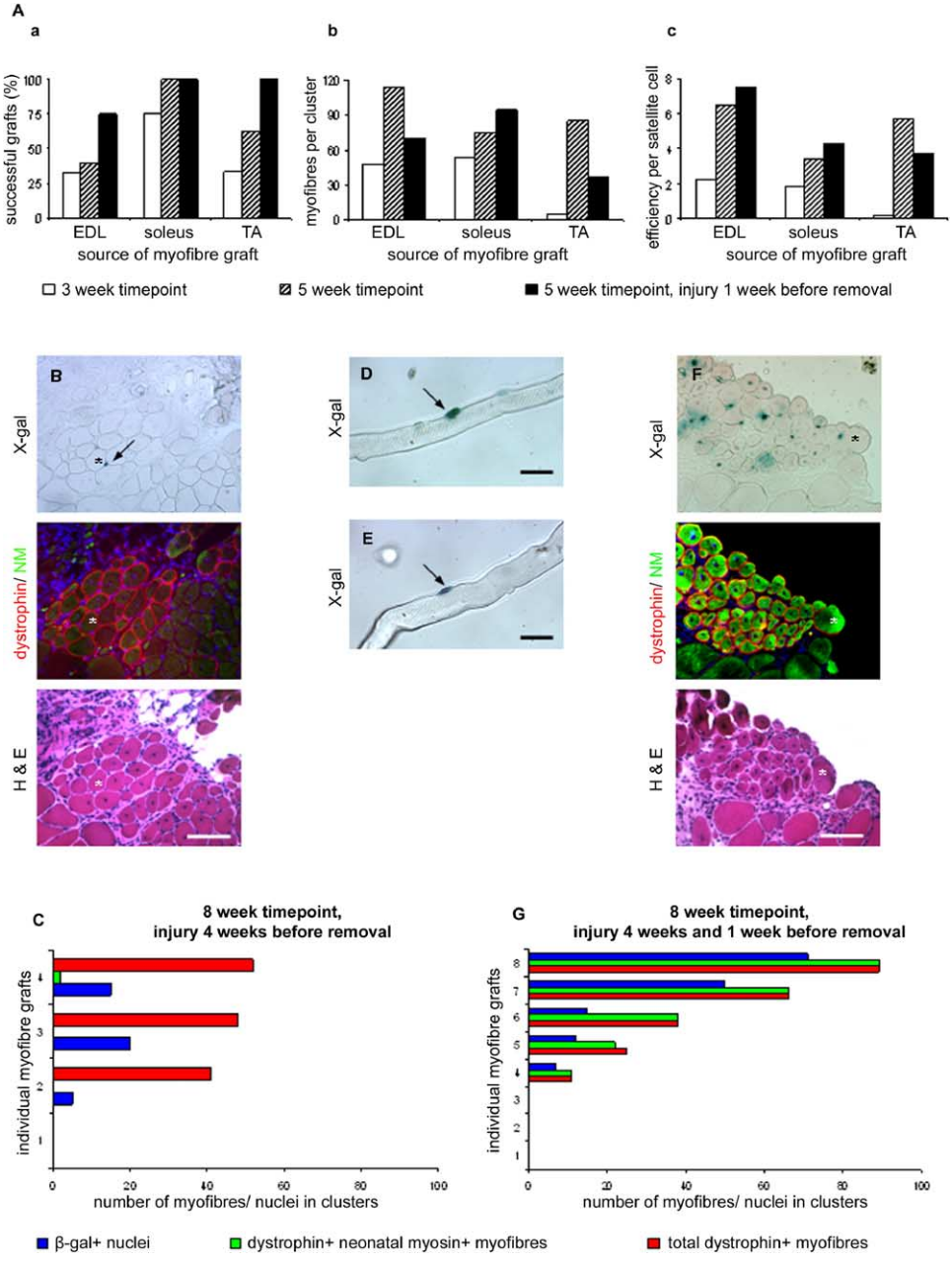

Figure 5. Persistence of Satellite Cells and Myogenic Stem Cell Function over Time and after Injury-Induced Regeneration

(A) Bar graphs showing, at different time points and in response to injury, the following: (Aa) The proportion of successful grafts, where a successful graft generates one or more β-gal$^+$ nuclei. (Ab) The average cluster size where data describe numbers of dys$^+$ myofibers (except for TA myofiber grafts at the 3 week time point, where data describe numbers of β-gal$^+$ myofibers). (Ac) The overall efficiency of muscle formation, where efficiency = the total number of myofibers formed by all grafts divided by the total number of satellite cells grafted (estimated using data derived from Pax7 counts; Figures 1A and 1B).

(B–E) Analysis of irradiated *mdx*-nude TA muscles engrafted with a single *Myf5*$^{nLacZ/+}$ soleus myofiber, injured 4 weeks after grafting and then left to regenerate for 4 weeks. (B) The left engrafted muscles were analyzed immunohistochemically. By this time point, dys$^+$ myofibers have downregulated neonatal myosin and myonuclear Myf5-β-gal. A single β-gal$^+$ nucleus (arrow) on the periphery of one myofiber (asterisk) is a candidate satellite cell. Scale bar, 100 μm. (C) Bar chart depicting muscle formation from four grafts. (D and E) The contralateral right engrafted muscles were analyzed by X-gal staining of isolated myofibers. Myf5-β-gal$^+$ satellite cells (arrows) were identified 4 weeks postinjury. Scale bars, 25 μm.

(F and G) Analysis of irradiated *mdx*-nude TA muscles engrafted with single *Myf5*$^{nLacZ/+}$ EDL myofibers, subjected to two rounds of injury at 4 weeks and 7 weeks after grafting and removed 1 week after the second injury. (F) The formation of a cluster of newly regenerated dys$^+$ myofibers demonstrates that the average seven grafted satellite cells generated progeny capable of participating in at least two rounds of injury-induced regeneration. Scale bar, 100 μm. (G) Bar chart depicting muscle formation by eight individual grafts. Asterisks mark the same myofiber in each series.

some grafts in the absence of experimental injury is largely attributable to physio-pathological factors rather than technical failure. Some grafted satellite cells therefore persisted in host muscles for several weeks without manifesting myogenic potential but were recruited into an active myogenic program in response to the increased regenerative demands induced by notexin injury.

Persistence of Satellite Cells and Myogenic Stem Cell Function over Time and after Injury-Induced Regeneration

To investigate whether the donor-derived satellite cell population persists after an acute regenerative event, we grafted single *Myf5*$^{nLacZ/+}$ soleus myofibers into the TA muscles of four *mdx*-nude recipient mice and injected notexin into the engrafted muscle 4 weeks later. Muscles were removed for analysis after a further 4 weeks to allow regeneration (i.e., 8 weeks after grafting). Histological analysis of the right engrafted muscles revealed clusters of donor dys$^+$ myofibers in three out of four cases. Neonatal myosin was absent from the vast majority of dys$^+$ myofibers, indicating that the regenerative response was largely complete by the time of analysis. Small numbers of β-gal$^+$ nuclei associated with dys$^+$ clusters were positioned mainly on the periphery of myofibers, and thus candidate satellite cells. The persistence of graft-derived satellite cells was confirmed by X-gal staining of myofibers isolated from the contralateral engrafted muscles, where three out of four preparations contained β-gal$^+$ satellite cells (Figures 5B–5E).

In another experiment, *Myf5*$^{nLacZ/+}$ EDL myofiber-engrafted muscles were subjected to two sequential notexin injuries at 4 and 7 weeks after grafting. One week after the second injury, five of out eight engrafted muscles contained substantial clusters of dys$^+$ myofibers. Almost all dys$^+$ myofibers strongly coexpressed neonatal myosin, and there were also numerous β-gal$^+$ nuclei, confirming that the clusters had been newly regenerated in the week following the second injury (Figures 5F and 5G).

Together, these findings show that the preinjury population of functional satellite cells is reestablished following regeneration, portraying the satellite cell as a vigorous and self-renewing myogenic stem cell capable of maintaining its own population throughout acute regenerative events.

Satellite Cell Myogenic Activity Does Not Require the Parental Myofiber

To investigate whether a viable parental myofiber is required for satellite cell function, we inserted grafts of 3F-*nLacZ*-2E EDL myofibers into host muscle sites that had been injected with notexin a few seconds previously, such that the viability of the myofiber was limited to a few hours. Engrafted muscles were examined after 3 weeks. Three out of six contained clusters of 30.3 (SE 10.1) dys$^+$ myofibers, of which 14.0 (SE 7.9) were β-gal$^+$. Thus, long-term persistence of the myofiber is not required for myogenic function of the associated satellite cells.

To investigate the ability of satellite cells to generate muscle after dissociation from their parental myofiber, we released satellite cells from beneath the basal lamina of 3F-*nLacZ*-2E EDL myofibers using physical trituration (see Experimental Procedures). The myofibers were then sieved out, leaving a population of small, mononucleate cells. Coimmunostaining for Pax7 and desmin showed that 95.3% of isolated cells expressed one or both markers, showing them to be a relatively homogeneous population of satellite cells (Figure 6A). Irradiated *mdx*-nude TA muscles (n = 8) were grafted with suspensions of 150 freshly isolated satellite cells and removed for analysis after 3 weeks. Grafts were robustly myogenic, several generating clusters of more than 100 β-gal$^+$ dys$^+$ myofibers (Figures 6B and 6C), showing that the myogenic potential of satellite cells was not substantially impaired by physical removal from the myofiber. We failed to isolate vigorously myogenic cells from myofibers enzymatically (data not shown), in accord with previous reports, for example, that grafts of 10^4 cells dissociated from myofibers by enzymatic digestion contributed to only 0.37% of host myofibers (Sherwood et al., 2004). This is some thousand-fold less efficient than the 8%–10% of host myofibers produced here from 150 physically separated satellite cells. The difference may be that, in our assays, satellite cells are not directly exposed to proteolytic enzymes that could cause damage to cell surface ligands or receptors. The method of satellite cell isolation therefore critically affects posttransplantation stem cell function.

Discussion

As a concept, the stem cell has been allocated a set of simple defining criteria: the ability to self-renew and to maintain at least one differentiated tissue type throughout the life span of an organism. The hematopoietic archetype also introduces multipotency as a common, though not required, feature of the stem cell (Raff, 2003; Wagers and Weissman, 2004). However, multipotency has featured most prominently in support of putative muscle stem cells, while rigorous tests of the more definitive benchmarks have been lacking. Thus, while the remarkable regenerative ability of skeletal muscle has long been recognized (Studitsky, 1964; Carlson, 1986) and is strongly resonant of stem cell activity, cells isolated from muscle and grafted into sites of muscle regeneration have shown disappointingly feeble myogenic activity, certainly nothing that would merit their designation as a stem cell by comparison with the hematopoietic stem cell (HSC) (reviewed in Partridge, 2003). The ability of satellite cells to generate differentiated myonuclei is indubitable both in vivo (Moss and Leblond, 1971; Snow, 1978) and in vitro (Rosenblatt et al., 1995), but without establishing the issue of autonomous self-renewal, this behavior is not incompatible with that of a progenitor population. Whereas a stem cell is defined by its functional attributes (Wagers and Weissman, 2004), the definition of a satellite cell is purely anatomical (Mauro, 1961). There is evidence that both muscle interstitium (Asakura et al., 2002; Polesskaya et al., 2003) and circulating bone marrow-derived (LaBarge and Blau, 2002) cell types can give rise to

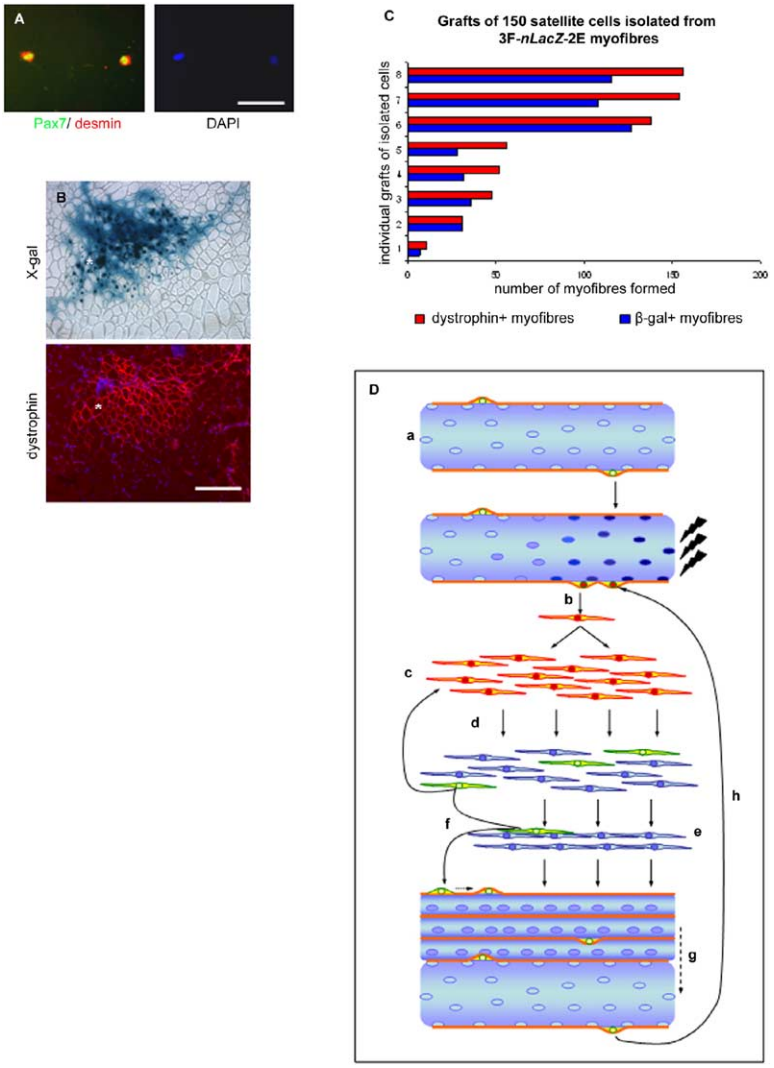

Figure 6. Self-Sufficient Stem Cell Function of Satellite Cells in Skeletal Muscles

(A) Immunocytochemistry for the satellite cell markers Pax7 and desmin in cells isolated from myofibers by physical trituration. 95.3% (82/86 cells examined) expressed one or both markers. Scale bar, 25 μm.

(B and C) Muscle generated by grafts of 150 satellite cells isolated from 3F-*nLacZ*-2E EDL myofibers. (B) Serial sections of a muscle grafted with 150 satellite cells and removed for analysis 3 weeks later, showing a large cluster of β-gal$^+$ dys$^+$ myofibers. Scale bar, 200 μm. Asterisks mark the same myofiber. (C) Bar graph showing the numbers of β-gal$^+$ and dys$^+$ myofibers formed from eight individual grafts of 150 cells. Ranked in order of the number of dys$^+$ myofibers.

(D) Model of satellite cell dynamics. (Da) Quiescent satellite cells beneath the basal lamina (orange) of a myofiber. (Db) Satellite cell activation after injury. Migration of daughter cells from beneath the basal lamina. (Dc) Satellite cell progeny proliferate extensively. (Dd) The progeny adopt alternative fates and either commit to differentiation (blue) or remain undifferentiated (yellow). (De) Differentiation-committed cells fuse to repair or replace damaged myofibers. (Df) Undifferentiated progenitors continue proliferating to derive the next cohort of myoblasts or are captured beneath the basal lamina of newly formed myofibers and renew the satellite cell population. (Dg) Myofiber maturation. (Dh) The new population of satellite cells is functionally competent.

satellite cells, but demonstration of such satellite cells having functional potential has been lacking, and the results of a recent study of cells extracted from regenerating skeletal muscle argues strongly that bone marrow is not a significant source of myogenic progenitors (Sherwood et al., 2004).

Here, we have shown that the satellite cell is a potent myogenic progenitor and is able to maintain its own functional population by self-renewal. We previously showed that, in suspension culture, the progeny of myofiber-associated satellite cells adopt divergent fates, with the majority population committing to differentiation while a minority remain undifferentiated and retain expression of Pax7 (Zammit et al., 2004). The prediction that satellite cells may be able to self-renew via the return to quiescence of their cycling progeny has been formally validated by the present findings. We also show that transplanted satellite cells are capable of expanding their own population to repopulate an irradiation-depleted muscle. Substantial graft-derived myogenic activity persists in host muscles for at least 8 weeks and, moreover, throughout recurrent injury, indicating that at least some of the new satellite cells have functional potential comparable to the original parental satellite cells from which they are derived. Therefore, at least some Pax7$^+$ satellite cells are true stem cells, constituting a self-sufficient source of regeneration in adult muscles (Figure 6D).

It is possible that only a proportion of satellite cells function as stem cells in our assay system. EDL myofibers have fewer satellite cells than those of the other two muscles, and after notexin injury, 100% of soleus and TA myofiber grafts, but only 75% of EDL myofiber grafts, generated new muscle. Hypothetically, the satellite cell population might consist of both true stem cells and other less proliferative progenitor cells. We plan further work to define muscle stem cells at the clonal level. In addition, our data do not exclude a possible role for interstitial myoblasts, which have previously been postulated as the earlier precursors of satellite cells (Asakura et al., 2002; Polesskaya et al., 2003). However, we suggest that the reverse hypothesis, whereby interstitial myoblasts are themselves the progeny of satellite cells, is equally plausible.

As with muscle satellite cells, epithelial stem cells can be identified by anatomical criteria, being found predominantly in the bulge region of hair follicles (Wagers and Weissman, 2004). A recent study showed that epithelial stem cells are normally quiescent within their niche but can be stimulated to self-renew extensively in vitro and are multipotent in vivo after grafting (Blanpain et al., 2004). Since 5 × 10^6 cells were used per graft, it is unclear whether they were able to undergo population expansion in vivo, as shown with grafted HSC, and as we have shown, satellite cells. Epithelial stem cells can be sorted from skin cell suspensions on the basis of specific cell-surface markers, and this has allowed functional potential to be characterized in phenotypically defined subsets of cells (Blanpain et al., 2004). Although several phenotypic markers of satellite cells have been identified, including Pax7 (Seale et al., 2000) and Myf5 and CD34 (Beauchamp et al., 2000), their relationship to stem cell function has not yet been characterized.

An unexpected adjunct to this work was the finding that the satellite cells of different adult muscles exhibit distinct patterns of behavior when transplanted into a common host muscle, the TA. Whereas EDL and soleus satellite cells were capable of generating large clusters of muscle by the 3 week time point, TA satellite cells did so only at the later 5 week time point. This difference in regenerative rate does not correlate with the starting number of satellite cells but might reflect differences between the parental myofibers themselves. For instance, EDL and soleus myofibers have very similar average surface areas, at 0.63 and 0.64 mm^2, respectively (Zammit et al., 2002), but the average surface area of TA myofibers is greater at 1.23 mm^2 (our unpublished data). Alternatively, the evident variability between satellite cells from different muscles could result from cell-intrinsic factors.

Although we do not exclude the possibility that myofibers have a role in regulating satellite cell function, separation of satellite cells from their parental myofibers by physical trituration did not result in substantial impairment of their myogenic potential. We were, however, unable to isolate comparably myogenic cells from myofibers by enzymatic digestion, and the results of other studies similarly suggest that cells separated from myofibers enzymatically generate muscle inefficiently in vivo. Although isolation of single myofibers involves collagenase digestion, satellite cells are shielded by the intact basal lamina, especially since we routinely select batches of enzyme for their low proteolytic contamination. Previous studies of posttransplantation myogenic potential have employed cells isolated by direct enzymatic disaggregation and have demonstrated almost uniformly inefficient muscle regeneration (reviewed in Partridge, 2003). Historical studies of minced muscle grafts by Studitsky (1964) and Carlson (1986) are the exception; these experiments did not involve the use of enzymes and indeed demonstrated remarkably efficient regeneration. It may also be significant that the archetypal HSC is routinely isolated using nonenzymatic techniques.

Our findings show that stem cells can be isolated from a normally stable, low-turnover adult tissue and predict that other adult organs might harbor tissue-specific stem cells that are incapacitated by inappropriate isolation or handling. Identification of the environmental components necessary for isolation of functional stem cells from adult tissues could lead to new sources of stem cells for regenerative therapies and, ultimately, treatments for both degenerative muscle disorders and other human diseases.

Experimental Procedures

Single Myofiber Isolation

Single myofibers were prepared from the EDL, TA, and soleus muscles of 6- to 8-week-old 3F-*nlacZ*-2E mice (Kelly et al., 1995) and *Myf5*$^{nLacZ/+}$ mice (Tajbakhsh et al., 1996), and from the engrafted TA muscles of *mdx*-nude mice, as described by Rosenblatt et al. (1995). Muscles were dissected and digested in 2% (w/v) collagenase type 1 (Sigma) in Dulbecco's modified Eagle's medium (DMEM) (Gibco) for 1.5–2 hr in a 35°C water bath. Myofibers were isolated by gentle trituration of the muscle using a customized heat-polished Pasteur pipette (~7 mm diameter). Individual unblemished myofibers were washed by using a fine heat-polished Pasteur pipette (~1 mm diameter) to serially transfer them through three dishes of warmed DMEM supplemented with 4 mM L-glutamine (Sigma) and 1% penicillin and streptomycin solution (Sigma). Dishes and pipettes were prerinsed with horse serum to prevent myofiber adherence. Prepared myofibers were incubated at 37°C/5% CO_2 for up to 1 hr before grafting.

Isolation of Satellite Cells from Myofibers by Physical Trituration

Satellite cells were separated from myofibers using a method adapted from Shefer et al. (2004). Two thousand intact myofibers were isolated from the EDL muscles of 3F-*nLacZ*-2E mice, washed, and suspended in 8 ml plating medium consisting of DMEM supplemented with 10% horse serum (PAA Laboratories), 0.5% chick embryo extract, 4 mM L-glutamine (Sigma), and 1% penicillin and streptomycin solution (Sigma). Myofibers were triturated for 5 min with a 19G needle mounted on a 1 ml syringe. The suspension was passed through a 40 μm cell sieve to remove the myofibers. The remaining satellite cell suspension was centrifuged for 15 min at 450 RCF, and the resultant pellet was resuspended in medium. A viable (trypan blue-excluding) count was performed. Cells were kept on ice until grafting within 1 hr of isolation. Aliquots of 10^3 cells were seeded onto Matrigel (1 mg/ml) (BD Biosciences) and cultured overnight in plating medium before immunocytochemistry of adherent cells.

Grafting of Myofibers or Isolated Satellite Cells into Mouse Muscles

Mice were bred and experimental procedures were carried out in the Biological Services Unit, Imperial College Faculty of Medicine, Hammersmith Hospital, in accordance with the Animals (Scientific Procedures) Act 1986. Donor myofibers or satellite cell suspensions were grafted into the TA muscles of 24-day-old *mdx*-nude mice (Partridge et al., 1989). Three days before grafting, the satellite cell populations of recipient hindlimb muscles were ablated by exposure to 18 Gy γ-irradiation (Gross et al., 1999). Individual myofibers or suspensions of 150 satellite cells were drawn into fine glass needles in 3–4 μl medium and inserted into recipient muscles under microscopic observation.

Model of Muscle Injury

To injure muscles, 10 μl *Notechis scutatus scutatus* notexin (Latoxan, Valence, France) (10 μg/ml) was injected using a Hamilton syringe (Gross and Morgan, 1999).

Immunohistochemistry of Tissue Sections

Engrafted muscles were frozen in isopentane cooled in liquid nitrogen. Serial 7 μm cryosections were collected at 100 μm intervals throughout the entire muscle. For immunofluorescence of unfixed cryosections, we used rabbit anti-dystrophin (P7) and mouse anti-neonatal myosin (BF34) antibodies. For secondary detection, we

used TRITC-conjugated swine anti-rabbit Ig (DakoCytomation) and Alexa Fluor 488-conjugated goat anti-mouse Ig (Molecular Probes). 4′,6-diamidino-2-phenylindole (DAPI) was used as a nuclear counterstain. Staining was preceded by blocking with 10% goat serum and 10% swine serum. X-gal and H&E staining were performed as described (Gross and Morgan, 1999).

Immunocytochemistry of Isolated Myofibers and Satellite Cells

3F-*nLacZ*-2E myofibers were fixed and stained in X-gal as described in Beauchamp et al. (2000), permeabilized with 0.5% Triton X-100 (Sigma) and blocked with 10% goat serum and 10% fetal calf serum. Myofibers were incubated overnight with mouse anti-Pax7 (Developmental Studies Hybridoma Bank) and rabbit anti-laminin (Sigma) antibodies. For secondary detection, TRITC-conjugated swine anti-rabbit Ig and Alexa Fluor 488-conjugated goat anti-mouse Ig were used. Myofibers isolated from *Myf5*$^{nLacZ/+}$ myofiber-engrafted *mdx*-nude TA muscles were fixed, permeabilized, and blocked as above, and then incubated overnight with mouse anti-Pax7 and rabbit anti-β-gal (Molecular Probes) antibodies. Alexa Fluor 488-conjugated goat anti-rabbit Ig (Molecular Probes) and Alexa Fluor 594-conjugated goat anti-mouse Ig (Molecular Probes) were used for secondary detection. Satellite cell cultures were fixed, permeabilized, and blocked as above, and then incubated overnight with mouse anti-Pax7 and rabbit anti-desmin (Sigma) antibodies. TRITC-conjugated swine anti-rabbit Ig and Alexa Fluor 488-conjugated goat anti-mouse Ig were used for secondary detection. DAPI was used as a nuclear counterstain.

Microscopy

Fluorescence and bright-field microscopy and image capture were performed using a Zeiss Axiophot microscope and Metamorph software. Minor adjustments to brightness and contrast were made using Adobe Photoshop version 5.

Data Analysis

Counts were made of the maximum number of donor myofibers in single serial sections of host muscles. Data were pooled from multiple donor animals as indicated in figure legends. Stata version 8.00 (Stata Corporation) was used for the negative binomial regression model (Cheung, 2002). INSTAT was used for Mann-Whitney tests. p values of < 0.05 were taken as significant.

Supplemental Data

The Supplemental Data include two tables and one figure and can be found with this article online at http://www.cell.com/cgi/content/full/122/2/289/DC1/.

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