

Direct Isolation of Satellite Cells for Skeletal Muscle Regeneration

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Muscle satellite cells contribute to muscle regeneration. We have used a *Pax3*^{GFP/+} mouse line to directly isolate (Pax3)(green fluorescent protein)-expressing muscle satellite cells, by flow cytometry from adult skeletal muscles, as a homogeneous population of small, nongranular, Pax7⁺, CD34⁺, CD45⁻, Sca1⁻ cells. The flow cytometry parameters thus established enabled us to isolate satellite cells from wild-type muscles. Such cells, grafted into muscles of *mdx nu/nu* mice, contributed both to fiber repair and to the muscle satellite cell compartment. Expansion of these cells in culture before engraftment reduced their regenerative capacity.

Satellite cells are skeletal muscle progenitor cells responsible for postnatal growth and repair (1). The difficulty of isolating pure populations of satellite cells in sufficient number

has precluded their use in cell-based tissue repair assays. These assays have, therefore, employed either muscle precursor cells that correspond to the progeny of muscle satellite cells, obtained after activation and proliferation in culture (2–4), or mixtures of cells obtained after enzymatic dissociation of skeletal muscles (5, 6). In vivo, quiescent muscle satellite cells are characterized by the expression of surface markers such as M-cadherin (7, 8), syndecan 3 and 4 (9), and CD34 (10); however, none of these permit unequivocal isolation because of the lack of specificity or availability of suitable reagents. Satellite cells also express transcription factors, notably Pax7, a member of the homeodomain/paired box family of Pax

proteins (11). Recently, we have shown that, Pax3, the paralog of Pax7, is also expressed in quiescent muscle satellite cells in a subset of muscles (12, 13). The generation of a green fluorescent protein (GFP)-tagged Pax3 mouse line (*Pax3*^{GFP/+}) (14) permitted us to isolate (Pax3)GFP-expressing cells from adult skeletal muscles by flow cytometry.

Previous observations on *Pax3*^{nlacZ/+} mice indicated that satellite cells expressing the transcriptional regulator Pax3 were limited to a subset of adult skeletal muscles, including the diaphragm, most trunk muscles, and some limb muscles (13). The *Pax3*^{GFP/+} mouse line shows similar expression. As illustrated in the diaphragm (Fig. 1A), (Pax3)GFP⁺ cells are found in a typical satellite cell position beneath the layer of laminin that surrounds muscle fibers. Most of these cells also express the transcriptional regulator Pax7 (Fig. 1B), which marks muscle satellite cells (11).

Flow cytometric analysis of the cells prepared from diaphragm muscle of adult *Pax3*^{GFP/+} mice (Fig. 1C) indicated that (Pax3)GFP⁺ cells constitute a population that is negative for CD45 and Sca1 and positive for CD34. These cells were also negative for the endothelial markers CD31 and Flk1 (fig. S1). Forward and side scatter gating (reflecting the size and granularity of the cells, respectively) (Fig. 1C, left) indicated that (Pax3)GFP⁺ cells constitute a homogeneous population of small, nongranular, mononucleated cells. Immediately after sorting, immunodetection showed the presence of 93% Pax7⁺ cells and 8% MyoD⁺

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cells. Pax7 marks both quiescent and activated satellite cells (15), whereas MyoD marks activated satellite cells only (16, 17). This indicates that the majority of the cells did not undergo activation during the few (4 to 6) hours that were required for dissociation and sorting. Colony assays further established the identity of these cells as muscle progenitors, giving rise to 100% Pax7- and MyoD- expressing cells after 3 days in culture (fig. S2). The (Pax3)GFP+ fraction that we isolated thus constitutes a pure population of myogenic cells.

We functionally characterized (Pax3)GFP+ cells, isolated by flow cytometry from adult diaphragm muscle, by grafting them into irradiated tibialis anterior (TA) muscles of immunodeficient *nude mdx* mice (*mdx nu/nu*). These mice lack dystrophin, a structural protein that is mutated in Duchenne muscular dystrophy patients (18). Satellite cells of the TA, like those of other lower hindlimb muscles, do not normally express Pax3. The contribution of (Pax3)GFP+ cells to fiber repair was measured by the restoration of dystrophin expression in muscle fibers of host mice, 3 weeks after grafting. Numerous dystrophin-positive fibers were readily detected in the grafted muscles (Fig. 1D, top). Only occasional dystrophin-positive fibers, probably revertant fibers (19), were found in the control contralateral, non-grafted TA muscles (Fig. 1D, bottom). Grafting of 2×10^4 cells led to dystrophin expression in an average number of 587 fibers, and grafts of as few as 10^3 cells still resulted in dystrophin expression in an average of 160 fibers (Fig. 1E, left). These yields are comparable to those obtained after grafting 5×10^5 cells isolated by enzymatic dissociation of whole adult muscles (5, 6).

Most grafting experiments have employed muscle precursor cells obtained after a phase of amplification in culture (2, 3). To determine whether such culturing procedures could alter the capacity of cells to contribute to tissue reconstitution, we grafted cultured and noncultured (Pax3)GFP+ cells. Grafting 10^4 noncultured cells led to restoration of dystrophin expression in an average number of 300 fibers, whereas grafting the same number of cultured cells resulted in significantly fewer dystrophin-positive fibers (mean = 88 fibers, $P < 0.02$) (Fig. 1E, right). We also grafted 10^5 cells, corresponding to the progeny after 3 days in culture of 10^4 (Pax3)GFP+ cells. These cells led to restoration of dystrophin expression in an average number of 265 fibers, a figure that is similar to that obtained when grafting 10^4 noncultured cells (Fig. 1E, right). These results show that culturing muscle satellite cells for a few days before grafting reduces their efficiency in fiber repair, suggesting that in vitro expansion is disadvantageous. Clonal assays indicated that cultured cells display a lower proliferation potential than freshly isolated cells and a tendency to differentiate more

rapidly (table S1). These features may account for their reduced regenerative capacity.

(Pax3)GFP+ CD34+ donor cells could be recovered by flow cytometry from grafted muscles (Fig. 2A). These cells displayed a myo-

genic phenotype in culture, expressing MyoD and Pax7 and differentiating into TroponinT-expressing myotubes (Fig. 2B). Single fibers prepared from grafted muscles (Fig. 2C) carried cells of donor origin in a muscle sat-

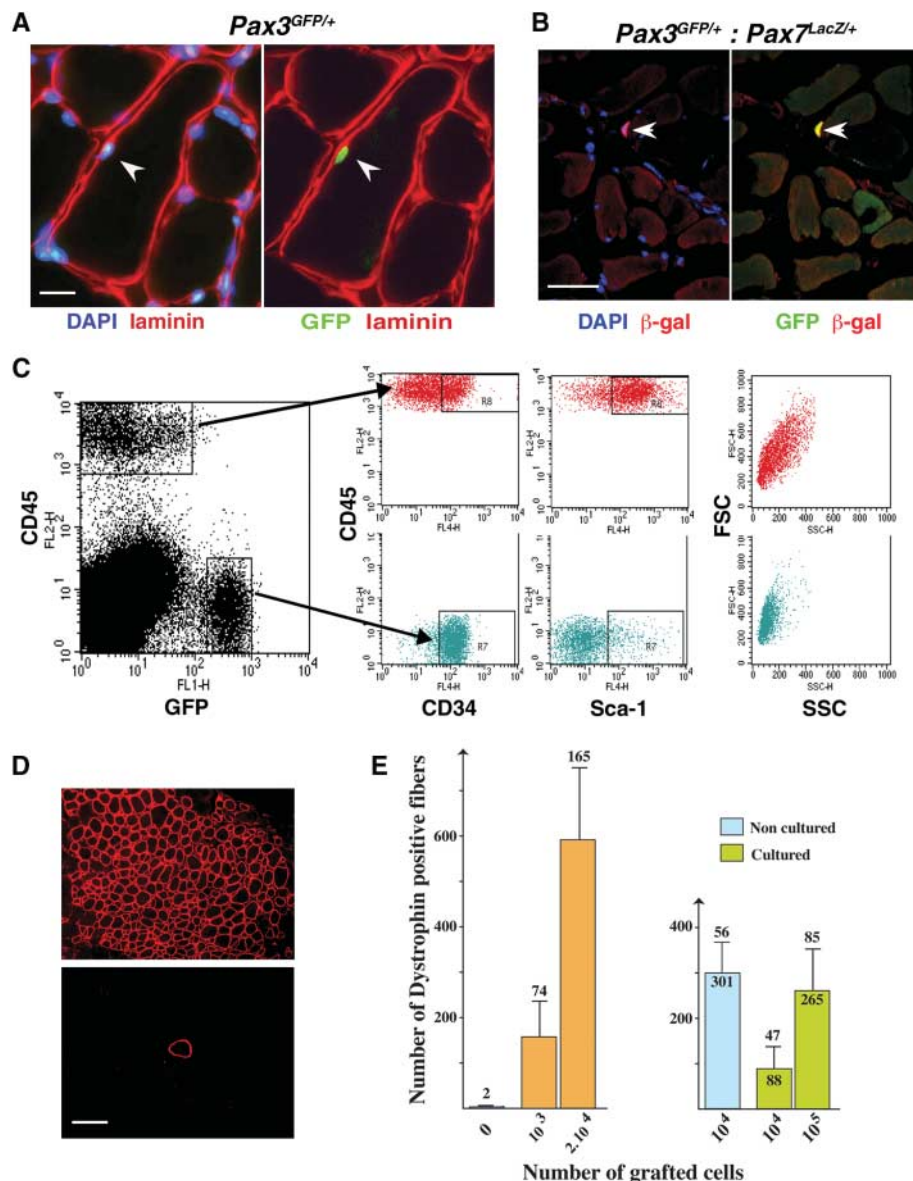


Fig. 1. Characterization of (Pax3)GFP-expressing cells from the diaphragm muscle of adult mice. (A) Transverse section of a *Pax3*^{GFP/+} mouse. Left: Immunodetection of laminin (red staining), with 4',6-diamidino-2-phenylindole (DAPI) coloration. Right: Direct fluorescent detection of GFP+ cells (green staining), together with immunodetection of laminin. Scale bar, 20 μ m. (B) Transverse section from a *Pax3*^{GFP/+}; *Pax7*^{LacZ/+} mouse. Left: Immunodetection of β -galactosidase (β -gal, red staining) from the *Pax7* allele, together with DAPI staining. Right: Detection of GFP fluorescence (green staining) from the *Pax3* allele and co-immunodetection of β -galactosidase (red staining) from the *Pax7* allele, resulting in yellow staining. Scale bar, 100 μ m. Arrowheads indicate candidate muscle cells. (C) Flow cytometry analysis of cells from *Pax3*^{GFP/+} mice. CD34 and Sca1 expression on CD45+ cells (red, top panels) and on GFP+ cells (blue, bottom panels). Top and bottom right panels correspond to forward scatter (FSC) and side scatter (SSC) gating of CD45+ and GFP+ cells, respectively. (D) Detection of dystrophin-positive fibers in grafted muscles. Three weeks after grafting with (Pax3)GFP+ cells, TA muscles of *mdx nu/nu* mice were processed for detection of dystrophin. Top: Transverse section of grafted muscle. Bottom: Control contralateral nongrafted TA. Scale bar, 200 μ m. (E) Quantitative analysis of experiments as shown in (D). Cells were grafted immediately after sorting (left). The effect of cell culture was examined by injecting cultured or noncultured cells (right). The numbers of injected mice were, from left to right: 4, 5, 4, 6, 5, and 4. Labeled error bars represent SD.

ellite cell position, co-expressing (Pax3)GFP and Pax7. Of 569 cells detected on the surface of 120 single fibers from grafted TA muscles, 17% were satellite cells of donor origin co-expressing Pax7 and (Pax3)GFP. These results show that grafted muscle satellite cells contribute not only to muscle fiber repair but also to the muscle satellite cell compartment. They also show that (Pax3)GFP+ cells retain their Pax3+ identity in the environment of the TA muscle, where endogenous satellite cells do not express Pax3. Injured, as well as intact, TA muscle from *Pax3^{GFP/+}* mice does not normally contain (Pax3)GFP+ cells (fig. S3).

Flow cytometric analysis indicated that (Pax3)GFP+ cells express the surface marker CD34. We used this surface marker and the parameters defined by forward and side scatter gating for (Pax3)GFP+ cells (Fig. 1C) to determine whether muscle progenitor cells that

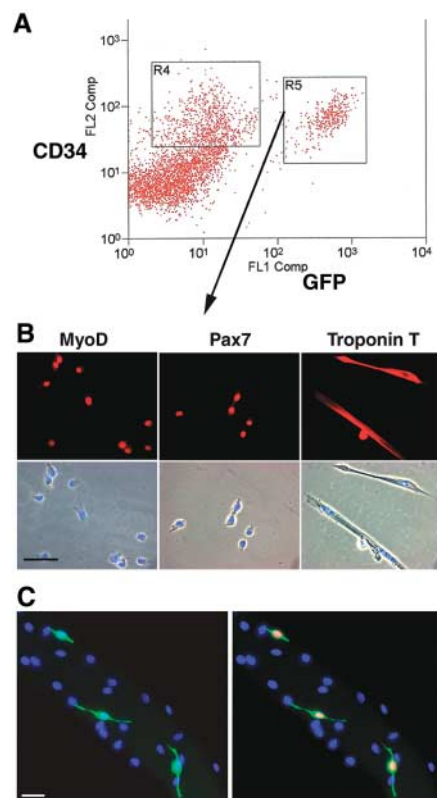


Fig. 2. Recovery of (Pax3)GFP+ cells from grafted TA muscles. (A) Three weeks after grafting, TA muscles were enzymatically dissociated and (Pax3)GFP+ CD34+ cells were isolated by flow cytometry. (B) Immediately after sorting, cells were plated and their myogenic identity determined by immunodetection of MyoD, Pax7, and Troponin T, after 3, 3, and 5 days of culture, respectively (top panels). Bottom panels show DAPI nuclear stain and phase contrast. Scale bar, 20 μ m. (C) Detection of (Pax3)GFP+ satellite cells on single fibers of grafted TA muscles. Left: Immunodetection of GFP with DAPI staining. Right: Co-immunodetection of Pax7 and GFP with DAPI staining on the same section. Scale bar, 15 μ m. GFP marks both the cytoplasm and the nucleus of satellite cells, whereas Pax7 marks only the nucleus.

do not express (Pax3)GFP could also be isolated from adult muscles. The GFP+ CD34+ cells isolated from the diaphragm of adult mice (Fig. 3A) represented 47% of the cells analyzed by flow cytometry. Clonal analysis of the cells from each fraction showed that all of the clones formed (78 out of 192 single cells) were myogenic, as monitored by immunodetection of MyoD and Pax7 and by myotube formation. In contrast, the GFP- CD34+ cells (Fig. 3A) displayed a cloning efficiency of 6% and gave rise to only 2 myogenic clones out of 192 plated cells. The same cell fractions from the lower hind leg muscles (Fig. 3B) gave markedly different results. GFP+ CD34+ cells, representing only 0.25% of the cells, gave rise to 33 clones (out of 96 single cells), all of which were myogenic. The GFP- CD34+ cells, which now represented 52% of the population, gave rise only to myogenic clones, with a cloning efficiency of 39% (76 out of 192 single cells). These results confirm that adult muscle progenitor cells belong to the (Pax3)GFP+ CD34+ cell fraction in the diaphragm, whereas, in lower hind leg muscles, they are in the (Pax3)GFP- CD34+ fraction. Both cell fractions express Pax7 (fig. S4). Thus, the parameters of size and granularity defined for (Pax3)GFP+ cells permit an equally efficient isolation of muscle satellite cells by sorting on the basis of CD34 expression. Skeletal muscle repair assays confirm and extend these observations. Grafting of GFP+ CD34+ cells from the diaphragm of adult mice or GFP- CD34+ cells from lower hind leg muscles of the same mice into TA muscles of *mdx nu/nu* recipients produced comparable restoration of dystrophin expression (Fig. 3, C and D). Thus both preparations participate similarly in muscle fiber repair.

Flow cytometric analysis and characterization of (Pax3)GFP+ cells present in skeletal muscles of adult *Pax3^{GFP/+}* mice have per-

mitted us to define parameters for isolating adult muscle progenitor cells. These cells comprise a population of small, nongranular, CD34+ CD45- Sca1- cells expressing Pax7. In accordance with this, a CD34+ cell fraction from skeletal muscles has been shown to be enriched in myogenic cells (20). In a recent study (21), adult muscle-associated progenitor cells were also shown to belong to a fraction of CD45- Sca1- CD34+ cells. We have shown recently that the progenitor cells of skeletal muscle during late embryonic and fetal development depend on both Pax3 and Pax7 (14). In the adult, not all muscle satellite cells express both genes. As shown here, although those in the diaphragm are Pax3+ Pax7+, satellite cells in lower hindlimb muscles express only Pax7. This distinction is maintained in regenerating muscles as seen in the TA after injury, where satellite cells remain (Pax3)GFP-negative. This is in contrast to a previous report on cells cultured from injured muscle (22). When we extended this flow cytometric analysis to a much larger gating window, we still did not detect any (Pax3)GFP+ cells after TA muscle injury (23). In muscles such as the diaphragm, Pax3 expression is cell-autonomous because (Pax3)GFP+ cells engrafted into the Pax3-negative TA muscle retain their initial phenotype. The myogenic potential of Pax3-expressing or -nonexpressing satellite cells is indistinguishable in both in vitro and in vivo assays. Distinguishing the potential role of Pax3 in subpopulations of adult muscle satellite cells awaits a conditional mutant, because embryos do not survive in the absence of Pax3.

Assays for muscle repair that have been developed to date are based on the injection of 5×10^5 to 10^6 cells into the muscles of *mdx* mice. Most have been performed with cells either directly obtained by enzymatic dissociation of muscles (5, 6) or after a phase of selection and amplification in culture (2, 3). When 5×10^5 cells from freshly disaggregated

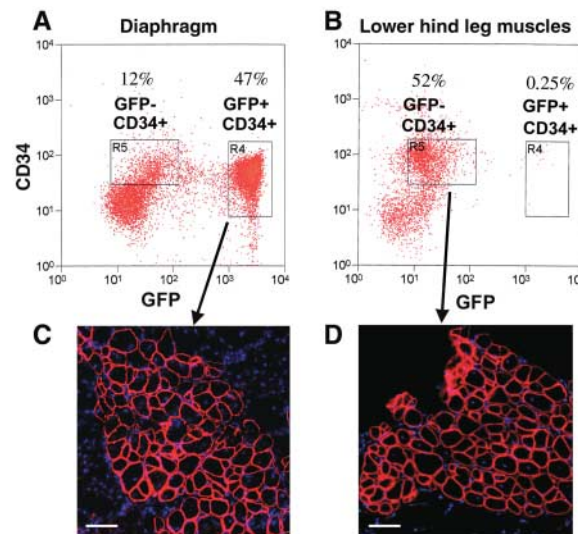


Fig. 3. Isolation of muscle satellite cells in the absence of (Pax3)GFP expression. Flow cytometric analysis of cells from the diaphragm and lower hind leg muscles of adult *Pax3^{GFP/+}* mice. (A and B) Cells from the diaphragm and from lower hind leg muscles were analyzed for both GFP and CD34 expression as indicated in each panel. The percentages shown correspond to the fraction of positive cells within a FSC/SSC gate as shown in Fig. 1C. (C and D) Immunodetection of dystrophin in TA muscles of *mdx nu/nu* mice 3 weeks after grafting of 2×10^4 cells from the fractions indicated by the arrows. Scale bar, 50 μ m.

muscle were implanted into the TA of irradiated *mdx nu/nu* mice, they formed a mean of 328 dystrophin-positive fibers (5). Similar results were obtained after injecting one to two million muscle-derived cultured cells into limb muscle (2, 3). Our results now show that purified satellite cells are much more efficient than these crude or cultured cell populations in contributing to muscle repair.

The culture of muscle progenitor cells before grafting markedly reduces their regenerative efficiency such that the culture expansion itself is an “empty” process, yielding the same amount of muscle as the number of cells from which the culture was initiated. Culture-induced modifications may affect survival or engraftment capacity of the cells (24, 25). However, we did not detect a difference in survival between cultured and freshly isolated cells 1 day after grafting (23). The activated state of the grafted cells may diminish their regenerative potential, because freshly isolated progenitor cells are not activated at the time of grafting, unlike their cultured progeny that express MyoD. Clonal assays suggest that the lower regenerative capacity of cultured cells reflects their more rapid differentiation. A similar situation is encountered with hematopoietic stem cells, which begin to differentiate and to lose their tissue reconstitution capacity when cultured (26).

Not only do purified muscle satellite cells contribute to muscle repair when engrafted into regenerating *mdx* muscles but some also persist as progenitor cells, adopting a satellite cell position and expressing Pax7. These re-

sults, therefore, point to muscle satellite cell self-renewal. The fact that (Pax3)GFP+ cells can be recovered from the muscles into which they were originally transplanted and shown to differentiate into muscle cells in culture also argues in favor of self-renewal. We therefore conclude that the satellite cell selection procedure described here results in cells that can both repair and contribute to the progenitor cell population of damaged muscles. There may be other stem cell types that can be mobilized to contribute to this process (27), but the muscle satellite cell population isolated by the flow cytometry parameters that we have defined is clearly a major contributor to muscle regeneration and a potential therapeutic agent.

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Supporting Online Material

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Materials and Methods

Figs. S1 to S4

Table S1

References and Notes

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