The role of CD34 expression and cellular fusion in the regeneration capacity of myogenic progenitor cells

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Summary

Characterization of myogenic subpopulations has traditionally been performed independently of their functional performance following transplantation. Using the preplate technique, which separates cells based on their variable adhesion characteristics, we investigated the use of cell surface proteins to potentially identify progenitors with enhanced regeneration capabilities. Based on previous studies, we used cell sorting to investigate stem cell antigen-1 (Sca-1) and CD34 expression on myogenic populations with late adhesion characteristics. We compared the regeneration efficiency of these sorted progenitors, as well as those displaying early adhesion characteristics, by quantifying their ability to regenerate skeletal muscle and restore dystrophin following transplantation into allogenic dystrophic host muscle.
    Identification and utilization of late adhering populations based on CD34 expression led to differential regeneration, with CD34-positive populations exhibiting significant improvements in dystrophin restoration compared with both their CD34-negative counterparts and early adhering cell populations. Regenerative capacity was found to correspond to the level of myogenic commitment, defined by myogenic regulatory factor expression, and the rate and degree of induced cell differentiation and fusion. These results demonstrate the ability to separate definable subpopulations of myogenic progenitors based on CD34 expression and reveal the potential implications of defining myogenic cell behavioral and phenotypic characteristics in relation to their regenerative capacity in vivo.

Key words: Skeletal muscle, CD34, Sca-1, Muscular dystrophy, Transplantation

Introduction

Cell biologists interested in the development and maintenance of the skeletal muscle compartment have characterized and identified proteins instrumental in the regulation of myogenic differentiation. Observed differential expression patterns of these proteins, specifically a family of transcription factors termed myogenic regulatory factors (MRF), by mononucleated cells has led to proposed organizational models of hierarchy among myogenic progenitor cells (Cornelison and Wold, 1997; Miller et al., 1999; Seale and Rudnicki, 2000). Level of myogenic commitment defined by expression of early or late stage MRF proteins indicates cell progression toward terminal differentiation and fusion into multinucleated myotubes and myofibers (Weintraub, 1993; Smith et al., 1994; Megeney and Rudnicki, 1995; Rudnicki and Jaenisch, 1995; Yoshida et al., 1998; Sabourin et al., 1999).

Aside from these well-defined and characterized intracellular proteins, recent interest in the isolation of myogenic populations with potential stem cell-like abilities has led to enhanced interest in the characterization of myogenic cells in terms of surface protein expression. Of interest are those that have been previously used to define murine bone marrow-derived hematopoietic stem cells, primarily: CD34, stem cell antigen-1 (Sca-1), and c-kit (Okada et al., 1992; Osawa et al., 1996; Goodell, 1999). Of these proteins, Sca-1 and CD34 are increasingly becoming associated with myogenic progenitor cells (Gussoni et al., 1999; Jackson et al., 1999; Lee et al., 2000; Torrente et al., 2001; Qu-Petersen et al., 2002). In addition, CD34, a transmembrane glycophosphoprotein known to be expressed by human hematopoietic progenitor cells as well as endothelial and certain fibroblastic cells (Yamazaki and Eyden, 1995; Krause et al., 1996; Vanderwinden et al., 1999), has recently been associated with both the quiescent and activated states of myogenic satellite cells (Beauchamp et al., 2000). Researchers are now performing these types of characterizations on donor populations prior to transplantation while focusing on defining populations with enhanced regeneration and dystrophin restoring abilities for use in clinical cellular therapies for muscular dystrophies.

Despite these recent efforts, an uncomfortable gap between muscle cell precursor biology and cell transplantation efficiency currently exists. The lack of information regarding the isolation and utilization of defined populations of primary myogenic cells, for comparison of regeneration characteristics, has led to presumptions of desirable donor cell characteristics for transplantation purposes that have yet to be challenged.

A preplate cell isolation procedure has been previously used to separate myogenic cells based upon variable adhesion characteristics and has demonstrated higher degrees of muscle

regeneration following transplantation through the utilization of late adhering (or late preplate) populations (Qu et al., 1998). It has also been previously shown that this technique allows for the enrichment of distinct, but heterogeneous, populations of myogenic progenitors within these late adhering cultures in terms of their phenotypic expression of surface markers Sca-1 and CD34 (Jankowski et al., 2001; Torrente et al., 2001). Using this information we were able to separate two phenotypically-distinct progenitor populations from primary cultures based on CD34 expression which displayed variable and definable regeneration capabilities in terms of restoration of dystrophin within dystrophic host skeletal muscle. With these known differential regeneration abilities in hand, we investigated in vitro cellular characteristics to elucidate potential mechanisms behind this in vivo behavior. Our results suggest that in vitro cell characteristics regarding differentiation status and cell fusion kinetics are important indicators of in vivo regenerative capacity.

Materials and Methods

Cell isolation (preplate technique) and culture

The preplate technique was utilized to obtain various myogenic cultures from dissociated skeletal muscle (Richler and Yaffe, 1970; Rando and Blau, 1994; Qu et al., 1998). Using a previously described protocol (Jankowski et al., 2001), a single cell suspension was obtained by enzymatic dissociation of gastrocnemius muscles from normal male mice (C57 BL/10J; Jackson Laboratories, Bar Harbor, ME) between 6 and 9 weeks of age. Enzymatic dissociation was performed by serial digestion of minced muscles in 0.2% collagenase-type XI solution (Sigma) for 1 hour, 0.3% dispase (Gibco-BRL) for 45 minutes, and 0.1% trypsin (Life Technologies) for 30 minutes. The cell suspension was added to a collagen-coated flask (collagen Type I, Sigma, St Louis, MO) in Dulbecco's modified Eagle's medium (DMEM, containing 10% fetal bovine serum, 10% horse serum, 1% penicillin/streptomyocin, and 0.5% chick embryo extract; Gibco-BRL). After 1 hour, non-adherent cells contained within the supernatant were removed and transferred to a second flask for a period of 2 hours. Fresh medium was added to the first set of adherent cells (termed preplate 1, or PP1) and this procedure was continued for PP3 through PP6 at subsequent 24 hour periods. This process resulted in six to seven cultures of adherent cells with different adhesion characteristics. For simplification of nomenclature, myogenic cells derived from preplates 1 through 3 ($\leq$27 hour initial adhesion) are referred herein as EP, for early preplate. Likewise, cells obtained from preplates 4 and beyond (>27 hour initial adhesion) are referred to as LP, for late preplate. An overview of this process and the cell populations used in the experimental investigations is shown in Fig. 1.

Adhesion of non-myogenic cells to the collagen-coated culture flasks occurs at a faster rate compared to myogenic cells. Continued passage of the supernatant into fresh flasks during the preplating process results in the purification of myogenic cells. Thus, EP cultures initially contained a higher percentage of non-myogenic cells. Prior to their use, the myogenic cells found within the EP cultures were purified by re-plating, as previously described (Qu and Huard, 2000; Qu-Petersen et al., 2002). Using this technique, EP cultures were purified to 80-90% desmin-positive. Although this additional step was performed to obtain the EP cells, the time from initial cell isolation to their use in the experiments was the same for both EP and LP populations.

Sorting of cultured myogenic cells and transplantation into dystrophic hosts (mdx)

A general overview of the transplantation protocol and the sorting procedure used in this study is shown in Fig. 1. Details of this procedure are described below.

Primary LP myogenic cells, isolated via the preplate technique, were trypsinized and sorted for the presence or absence of the cell surface proteins Sca-1 and CD34, in separate experiments, using a magnetic antibody cell sorting separation system as follows (MACS; Miltenyi Biotec, Auburn, CA). Following trypsinization, cells were maintained on ice throughout the procedure. For CD34 separation, cells were labeled with biotinylated rat anti-mouse CD34 monoclonal antibody (RAM34; Pharmingen, San Diego, CA) followed by anti-biotin microbeads (Miltenyi Biotec). This CD34 antibody recognizes both truncated and full-length isoforms. Purity of separation and expression of other proteins was assessed by labeling portions of both positively-selected and negative fractions with streptavidin (SA)-allophycocyanin conjugate, R-phycoerythrin (R-PE)-conjugated Sca-1 (Ly-6A/E) and FITC-conjugated CD45 rat anti-mouse antibodies (Pharmingen).

For Sca-1 separation, cells were labeled with microbead-conjugated rat anti-mouse Sca-1 monoclonal antibody (Miltenyi Biotec). Purity of separation and expression of other proteins was assessed by labeling portions of positive and negative fractions with R-PE-conjugated Sca-1, biotinylated CD34 and FITC-conjugated CD45 rat anti-mouse monoclonal antibodies (Pharmingen), followed by SA-allophycocyanin.

Following either separation, separate cell portions were labeled with appropriately conjugated isotype-matched control antibodies. 7-amino-actinomycin D (7-AAD; Pharmingen) was added to all tubes for exclusion of nonviable cells in the analysis. Separation purity was assessed using a FACSCalibur flow cytometer and analyzed with CellQuest software (both Becton Dickinson).

Portions of sorted populations were also placed back into culture and their phenotype re-evaluated at 2 and 5 days following sorting. Other sorted portions were used for comparison of cell characteristics such as cell division and fusion behavior, MHC-I expression, and expression of other myogenic proteins. MHC-I expression was assessed using similar labeling and gating techniques as those described here, and previously (Qu-Petersen et al., 2002), utilizing an R-PE-conjugated anti-mouse H-2K^b monoclonal antibody (Pharmingen).

The transplantation results provided represent data collected for experiments performed utilizing cells from over 20 separate isolation procedures. For injection into dystrophic mice, both EP and LP-sorted populations were washed twice in Hank's Balanced Salt Solution and injected into the gastrocnemius muscle of 7-9 week old female dystrophic mice (C57BL/10ScSn-DMD^{mdx}, Jackson Laboratory). For LP-sorted populations, single paired injections with the negative-sorted population in one limb and the positive-sorted population in the other were performed. Some additional injections were also performed in a non-paired fashion. Seven days following injection, the number of dystrophin-positive fibers for each cell injection was evaluated. An area containing the maximum number of dystrophin-positive fibers for each injection was used to determine the regenerative efficiency index, defined as: (number of dystrophin-positive myofibers at the injection site/number of viable desmin-positive cells injected) multiplied by 1×10^5, for ease of comparison and graphical display. Dystrophin-positive myofibers were counted manually from acquired digital images using ImageView software (Automated Cell Technologies, Oakmont, PA).

Dystrophin immunohistochemistry

Injected gastrocnemius muscles were flash frozen in liquid-nitrogen-cooled 2-methylbutane, serial-sectioned and evaluated for the number of dystrophin-positive myofibers. Dystrophin staining of cryopreserved tissue was performed on acetone-fixed, horse serum blocked sections using a rabbit anti-dystrophin antibody (1:1000; gift from Terry Partridge). Sections were then washed in PBS and incubated with biotinylated anti-rabbit IgG antibody, followed by additional washing and incubation with SA-Cy3 or SA-FITC (Sigma).
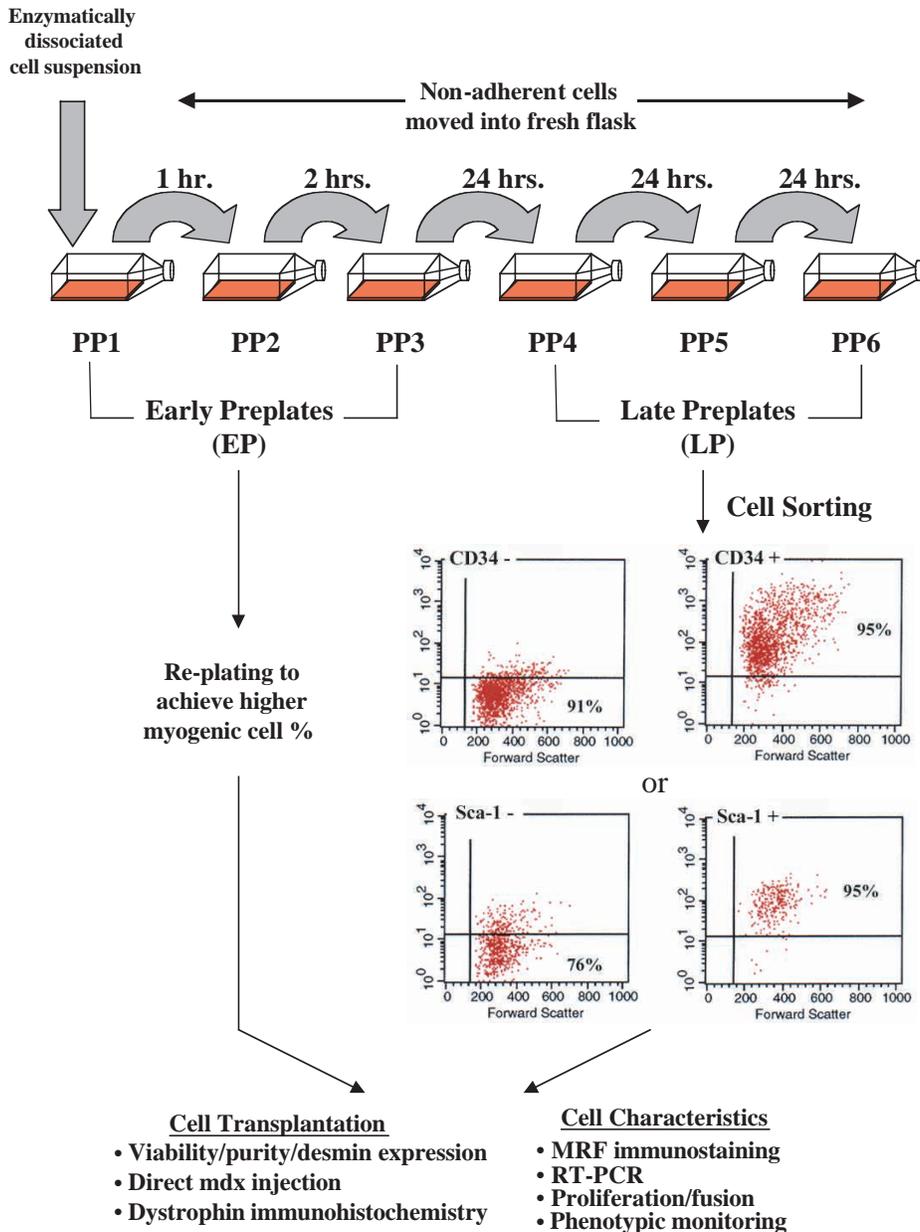
Fig. 1. Overview of experimental procedure. The preplate isolation technique was used to obtain two cell populations used for experimentation, found within early adhering preplates (EP) and late adhering preplates (LP). Myogenic (desmin-expressing) cells are enriched with each passage, thus the EP population was re-plated prior to their utilization, in order to further purify the myogenic cells. LP cells were sorted based on either Sca-1 or CD34 expression, in separate experiments, prior to their utilization. Representative dot plots of sorted cell populations, analyzed by flow cytometry, following magnetic antibody cell sorting are shown. Following their isolation, each cell population was injected into mdx skeletal muscle and monitored for their ability to restore dystrophin. They were also examined in terms of various in vitro cell characteristics.

in PBS. The cells were incubated with primary antibodies at room temperature for 2 hours at the following dilutions in PBS containing 5% goat serum: mouse IgG anti-desmin (1:250; Sigma), mouse IgG anti-MyoD (1:250; Pharmingen), mouse IgG anti-myogenin (1:250; Pharmingen), rabbit IgG anti-Myf5 (1:300; Santa Cruz Biotechnology), rabbit IgG anti-M-cadherin (1:50; gift of Anthony Wernig). Following thorough rinsing with PBS, cells were incubated with either biotinylated goat anti-mouse IgG or biotinylated goat anti-rabbit IgG secondary antibodies (both 1:250; Vector) for 30 minutes. Finally, cells were washed and incubated with SA-Cy3 (1:500) for 10 minutes. Negative control staining was performed using an identical procedure, with omission of the primary antibody.

RT-PCR

Total RNA was isolated using TRIzol reagent (Life Technologies) and 1 μg of RNA for each cell type was used for reverse transcription (RT). RT was performed using the SuperScript™ First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions. PCR primer sequences and reaction parameters were utilized as previously described: myogenin and MyoD (Rohwedel et al., 1995), M-cadherin and Myf5 (Cornelison and Wold, 1997). PCR products were obtained following 35 cycles and separated on agarose gels. Expected product sizes were observed for all reactions: myogenin, 86 bp; MyoD, 147 bp; M-cadherin, 446 bp; and Myf5, 352 bp. Genomic DNA contamination was excluded by the fact that all primers spanned an intron, as well as parallel RT controls that were performed without reverse transcriptase. C2C12 cells, used as a control, were obtained from the American Type Culture Collection and PP1 cells were obtained through the preplate technique, as described previously within the Cell Isolation section.

Cell-cycle analysis

Sorted cell suspensions were centrifuged, washed in PBS and permeabilized by addition of 0.05% saponin in PBS. 7-AAD, a

Myofiber size analysis

Dimensional analysis of dystrophin-positive fibers generated by the injection of the various donor myogenic cell populations was performed on digital images using Northern Eclipse software (v6.0, Epix Imaging). Using a threshold to distinguish the immunofluorescence signal from background, the software determines the boundary of each fiber and uses this determination to provide dimensional data for each fiber separately. Fiber size data was generated from the same images used in calculating the regenerative efficiency index, and the reported diameter represents maximum fiber thickness. For comparison, analysis of fiber sizes taken from non-injected areas of the mdx muscle was also performed.

In vitro immunocytochemistry

A fraction of each myogenic population was evaluated by immunofluorescent staining for expression of the myogenic proteins desmin, Myf5, MyoD, myogenin, and M-cadherin. Analysis was performed on methanol-fixed cells pre-incubated with 5% goat serum

nucleic acid dye, was added for 20 minutes before washing with PBS and analyzing on a flow cytometer using a linear scale. Analysis was performed by gating singlets in the forward/side scatter profile, and the peak channel fluorescence for cells in the G_2/M region was confirmed to be twice that of the G_0/G_1 region.

Cell division and fusion characteristics

A novel microscopic imaging system was used to acquire data pertaining to both cell division and fusion characteristics, in separate studies, through time-lapsed lightfield imaging (Greenberger et al., 2000; Deasy et al., 2002). This system uses a biobox incubator mounted to the stage of an inverted microscope linked to a CCD camera (Automated Cell Technologies). Using preselected coordinate positions, view fields were recorded by the CytoWorks software program that subsequently controlled the time and position of the microscope stage movement. For both cell division and fusion experiments, images were acquired at 10-minute intervals using a 20× objective over a period of 4 days.

For cell division analysis, cells were plated at an initial density of 1,000 cells/well of a collagen Type-I coated 12-well plate. In terms of proliferation kinetics the total cell number is dependent upon two parameters, the division time and the mitotic fraction. Division time was measured directly through individual observations of cytokinesis. The mitotic fraction (α) was calculated using a previously described non-exponential growth model, which utilizes the experimentally determined cell number and division time for each population (Sherley et al., 1995; Deasy et al., 2002):

$$N = N_0 \left[0.5 + \frac{1 - (2\alpha)^{(t/DT)+1}}{(2 - 4\alpha)} \right],$$

where N is the number of cells at any time t, N_0 is the initial number of cells, and both division time (DT) and cell number at each time point are experimentally determined through image analysis. Non-linear least squares regression, used to determine the best fit to the growth model equation, was performed using a statistical software package (SigmaStat v2.0; Jandel Corp.).

Both the rate and degree of fusion was monitored for each cell population. Cells were plated at an initial density of 1,250 cells/cm² in multi-well plates containing high serum DMEM medium, described previously. Following cell adhesion, the culture medium was replaced with DMEM containing 2% serum (1% fetal bovine, 1% horse), and cell fusion into multinucleated and elongated myotubes was monitored over a 96 hour period, along with total cell number. Immediately following this 96 hour observation period, immunofluorescent staining (as described previously) was performed for evaluation of desmin and myosin heavy chain (1:250; clone MY-32, Sigma) in one protocol, and desmin and CD34 (biotin conjugated, RAM34, 1:250) in another protocol. Cells were also counterstained with Hoechst (33328; Sigma) dye to reveal nuclei. Random fields in each well were evaluated for the total number of myogenic nuclei, the number of mononuclear desmin-positive cells, the number of myosin heavy chain-positive myotubes (containing ≥2 nuclei), and the number of nuclei within these myotubes. The fusion index is defined as: (nuclei within myosin heavy chain-expressing myotubes/total number of myogenic nuclei) × 100 (Sabourin et al., 1999).

Animals

All animals were housed in the Rangos Research Center Animal Facility of the Children's Hospital of Pittsburgh. The policies and procedures of the animal laboratory are in accordance with those detailed in the guide for the 'Care and Use of Laboratory Animals' published by the US Department of Health and Human Services. Protocols used for these experiments were approved by the Animal Research and Care Committee of the Children's Hospital of Pittsburgh and the University of Pittsburgh (Protocol #7/00).

Statistical analysis

For all analyses, results demonstrating $P<0.05$ were considered statistically significant. Multiple group comparisons were made using one-way ANOVA, using Student-Newman-Keuls pairwise comparisons to determine significance level. Non-parametric distributions were also detected and comparisons made using Kruskal-Wallis one-way ANOVA on ranks, with Dunn's test for pairwise comparisons. Direct comparisons between LP phenotypic subfractions were also made by Student's t-test or Mann-Whitney Rank Sum test where appropriate. All comparisons were made using SigmaStat (v2.0, Jandel Scientific) statistical software. Data are presented as mean±standard error of the mean, unless otherwise noted.

Results

Determination of myogenic commitment

Immunofluorescence staining with regard to Myf-5, MyoD, myogenin, and M-cadherin was performed on early adhering (EP) and late adhering (LP) myogenic populations to assess their level of expression of various proteins relating to myogenic differentiation status. All comparisons were made using cells evaluated at identical time points, from initial adhesion to time of fixation, in order to control for differences in expression that may arise with time in culture. As shown in Fig. 2, differences in the myogenic phenotype between EP and LP cells are apparent. EP myogenic cells, shown in Fig. 2 (top rows), are characterized in our cultures as containing the following percentages of cells expressing the protein of interest: M-cadherin, 70-80%; Myf5, >90%; MyoD, >85%; and myogenin, 45-65%. In contrast, LP myogenic cells, shown in Fig. 2 (bottom rows), are characterized as displaying the following phenotype: M-cadherin, >90%; Myf-5, >90%; MyoD, 45-70%; and myogenin, <25%. Taken in context with the patterns of expression of these proteins described in the literature, these data suggest that the majority of the EP myogenic cultures contain cells that display a higher degree of myogenic commitment, as determined by coexpression of multiple MRF proteins and a higher percentage of cells that have entered the terminal differentiation program associated with cell fusion as determined by myogenin expression (Smith et al., 1994; Yoshida et al., 1998; Miller et al., 1999). In contrast, the LP myogenic cultures contain a larger percentage of cells displaying a less differentiated myogenic phenotype, consistent with that used to describe satellite cells, and a smaller percentage of cells entering the terminal differentiation stage of the myogenic lineage.

Cell transplantation in mdx mice

LP cell sorting based on CD34 or Sca-1 expression

Following MACS separation, both negative and positive fractions were assessed to determine cell viability, purity of separation, desmin expression, and in some cases phenotype, prior to transplantation. Representative flow cytometry dot plots are displayed in Fig. 1, and collective results of the separation characteristics are described in Table 1. Relevant EP population characteristics are also provided within this table.

The purity of the separated populations can be assessed by describing the percentage of each population that emits fluorescence above or below that of the isotype control-labeled fraction. It was seen that separation of the Sca-1 fractions often resulted in the elution of cells expressing low levels of Sca-1 within the negative fraction (Fig. 1). Indeed, larger separation between positive and negative fractions, and potential elimination of cell populations that are expressing a lesser degree of the protein or are in the process of upregulating or downregulating the protein of interest, may be accomplished through the use of FACS separation. However, we also observed many advantages in using the MACS technique, such as increased cell viability and yield following separation. In addition, when examining the fluorescence intensity for each sorted population, it was observed that the separation occurred such that the Sca-1-positive fraction contained cells with mean fluorescence intensity ten times greater than that of the negative fraction. Separation percentages based on CD34 expression were cleaner, possibly due to differences in the expression of the proteins or differential antibody binding characteristics. Comparison of mean channel fluorescence between negative and positive fractions for both Sca-1 and CD34 separations was significant ($P<0.001$).

For several sorts, phenotypic characterization of expression of both sorted and non-sorted proteins was performed. For Sca-1 sorted cells, the negative fraction contained an average of $26.7\pm1.1\%$ CD34-positive cells, while the Sca-1-positive fraction contained $29.6\pm5.2\%$ CD34-positive cells. For CD34 sorted cells, the negative fraction contained $49.3\pm3.1\%$ Sca-1-positive cells, while the CD34-positive fraction contained $69.9\pm2.9\%$ Sca-1-positive cells (data not presented in Table 1).

As described previously (Qu et al., 1998; Jankowski et al., 2001), the preplate technique allows the enrichment of cultures containing myogenic cells, as assessed by desmin expression, from whole dissociated muscle. As shown in Table 1, slight differences in the percentage of desmin-expressing cells following sorting were consistently observed. Prior to sorting, all primary cell cultures remained in culture for only a few days while maintaining an undifferentiated morphology of small, round cells. These small and round cells expressed desmin, while the desmin-negative cells were usually much larger and generally exhibited a fibroblast-like morphology. Overall, there was no difference in either morphology or size between the negative and positive sorted LP fractions, as seen in the forward scatter profiles in Fig. 1. The number of cells in the positive and negative fractions that could be obtained from sorting was variable for all isolations,

however, on a consistent basis only a low number of the Sca-1-positive population was able to be obtained.

Dystrophin restoration

For LP-sorted populations, head-to-head injections with similar numbers of transplanted cells were performed. However, in many experiments the number of cells in each fraction available for injection varied, as well as the percentage of desmin-positive cells in each fraction. In order to more accurately assess the ability of each population to contribute to the myofiber regeneration process and the expression of dystrophin, the number of desmin-positive cells injected, rather than the total number injected, was used to define each transplanted population. A regenerative efficiency index was utilized for comparison of isolated populations to account for possible variations in the number of myogenic and viable cells injected following sorting of LP cells or re-plating of EP cells.

Comparison of LP subpopulations identified by the presence or absence of the surface proteins Sca-1 or CD34 demonstrated a variable regenerative efficiency. On a per cell basis, those expressing the CD34 protein were significantly more efficient in generating dystrophin-expressing myofibers at 7 days post-injection compared to their CD34-negative counterparts (Fig. 3A; 199.0 ± 33.2 vs. 87.0 ± 10.3, t-test $P=0.006$ and $P<0.05$
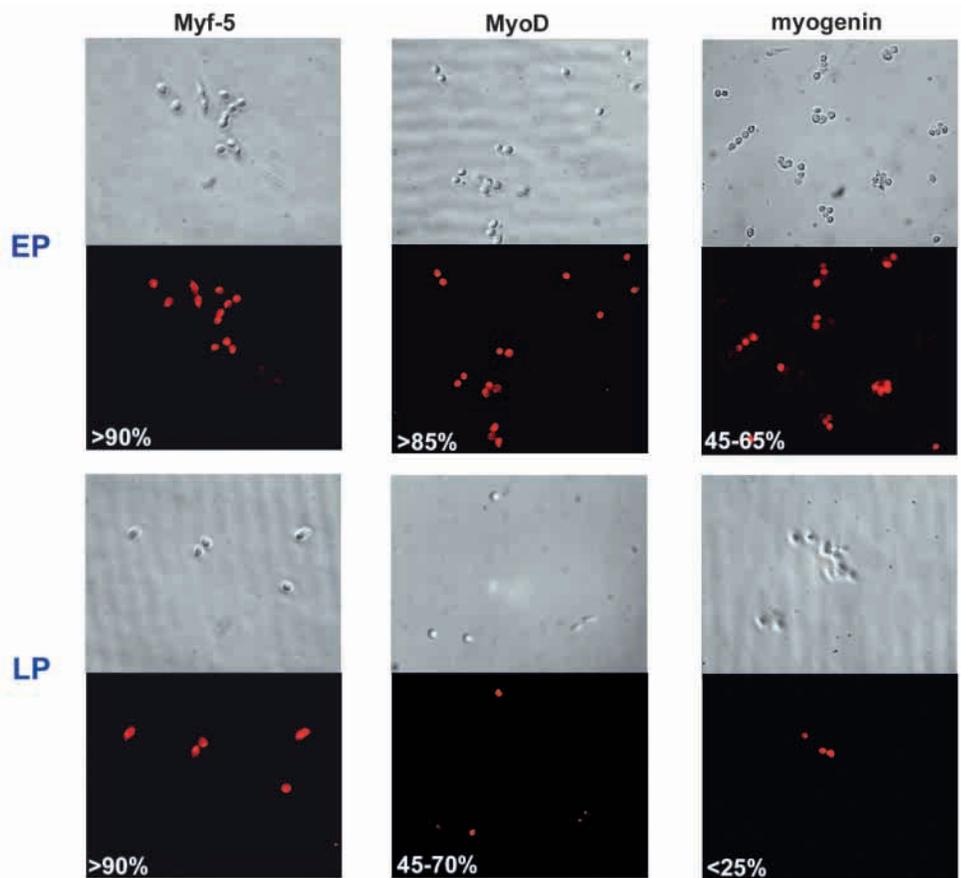

Fig. 2. Immunofluorescent characterization of MRF protein expression by EP and LP myogenic populations. According to current proposed models of myogenic precursor hierarchy, these patterns of MRF expression indicate that the EP cells are more myogenically committed, and thus closer to terminal differentiation, compared with the LP cells.

Table 1. Characteristics of transplanted cell populations, including sorting characteristics of LP myogenic cells separated based on either Sca-1 or CD34 expression

	EP Non-sorted	LP Sca-1 sorted cells		LP CD34 sorted cells	
		Negative fraction (Sca-1$^-$)	Positive fraction (Sca-1$^+$)	Negative fraction (CD34$^-$)	Positive fraction (CD34$^+$)
% Viability prior to transplantation	94.9±1.3	93.0±1.1	98.7±0.8	92.1±2.2	94.2±2.1
% Purity*	n/a	72.7±2.0	93.3±1.6	91.1±1.7	88.3±1.3
Mean fluorescence	n/a	16.9±1.0	171.8±16.5	12.3±2.4	217.4±25.6
% Desmin positive	84.1±4.2	82.1±2.4	90.6±1.5	82.2±1.5	78.4±2.4

*Determined by comparison to isotype control-labeled fractions.

using ANOVA comparison including EP). When comparing the Sca-1-negative and -positive fractions at the same time point there was a non-significant trend toward a higher regenerative index for Sca-1-positive cells (Fig. 3A; 157.1±71.5 vs. 99.2±26.6, P=0.382). Evaluation at longer time points post-injection did not alter these observations (data not shown).

The EP myogenic population demonstrated the least regenerative ability when compared to all subfractions of LP cells (Regenerative Index=63.3±14.0). A significant difference between EP cells and the CD34-positive LP fraction was observed (Fig. 3A; P<0.05), but not in comparison to the CD34-negative fraction.

Sizes and distribution of dystrophin-positive fibers generated by each cell population have also been monitored and are presented in Table 2 and Fig. 4. Overall, it was observed that myofibers generated by the EP population were larger in both cross-sectional area and diameter. In particular, a larger

percentage of myofibers within the 10-20 μm diameter and 0-100 μm^2 area ranges, representative of newly-formed myofibers, are observed with the LP populations. Higher percentages of EP-restored myofibers are observed with larger dimensions, falling within the range corresponding to the average size of non-dystrophin expressing mdx myofibers. Taken together, these in vivo observations are
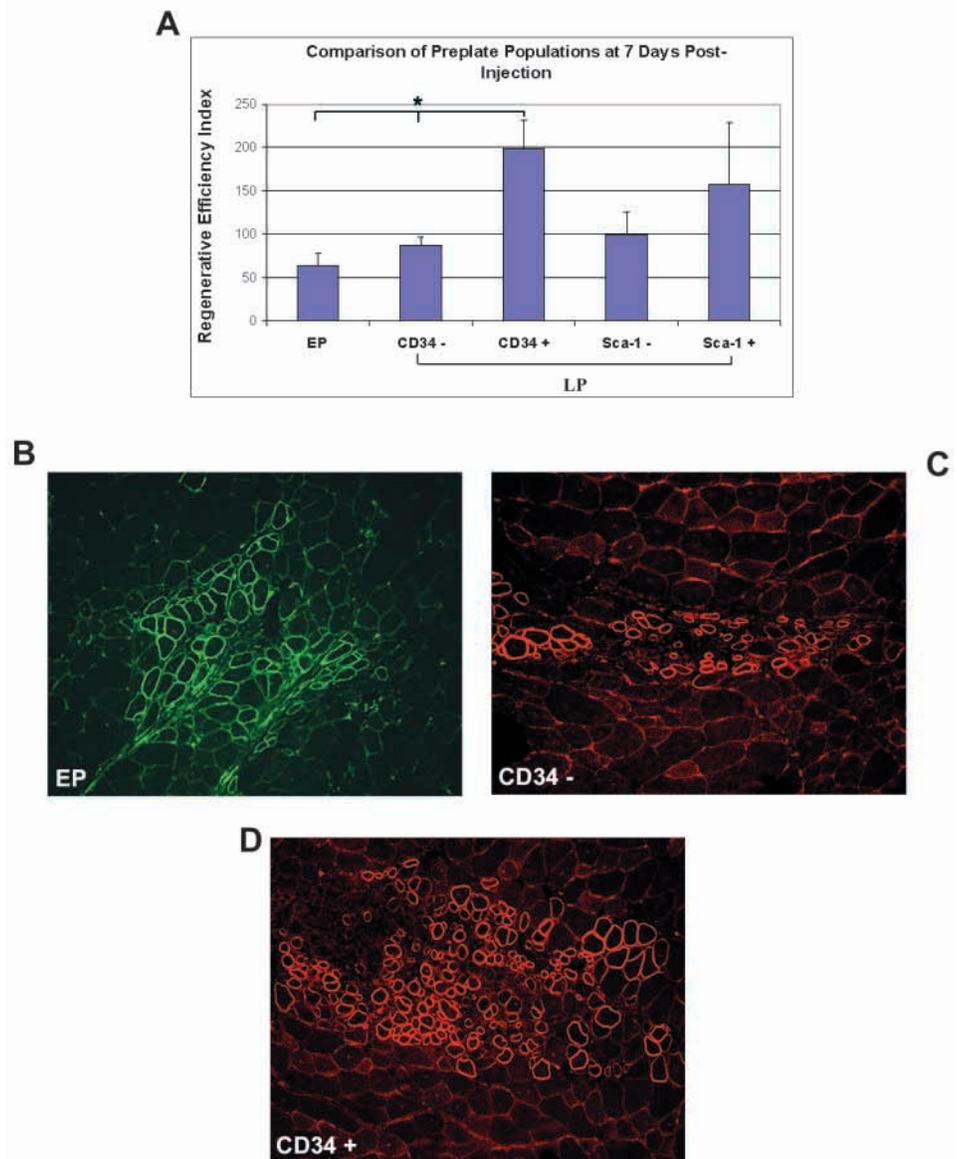

Fig. 3. (A) Efficiency of dystrophin restoration in mdx gastrocnemius muscle by myogenic precursor populations at 7 days following injection. CD34 and Sca-1 sorted LP subpopulations were obtained from separate isolations. A regenerative index, comparing restoration of dystrophin on a per cell basis (desmin-positive cells), was used for a more effective comparison of the various populations. CD34-positive LP cells demonstrated the highest dystrophin restoration (*P<0.05, CD34-positive vs. EP and CD34-negative). Cryosections taken from mdx muscle following transplantation and immunochemically stained to reveal dystrophin expression following injection of EP (B; 220,000 myogenic cells) and CD34-negative and -positive cells (C,D; approximately 95,000 myogenic cells each) are shown (20× objective).

Table 2. Dimensional characteristics of dystrophin-expressing myofibers generated by each injected myogenic donor cell population

	Myofiber diameter (μm)			Myofiber area (μm^2)		
	Mean±s.d.	Median	Percentiles 25-75%	Mean±s.d.	Median	Percentiles 25-75%
mdx	49.3±21.9	46.3	32.9-63.1	1135.7±25.9	842.0	413.6-1661.8
EP	26.9±14.2	22.7	16.5-35.0	316.9±11.0	184.7	89.4-450.9
LP CD34$^-$	18.4±10.4	16.5	10.4-22.7	176.3±6.3	108.2	43.4-220.3
LP CD34$^+$	20.3±11.1	18.1	11.4-26.7	198.3±6.0	121.7	47.6-273.0

suggestive of a more rapid donor cell fusion or a higher degree of fusion with pre-existing host fibers, or both, with the EP population. They also suggest that differential routes or mechanisms by which the various cell populations restore dystrophin expression may exist, and may correspond to differentiation status at the time of injection.

To examine the predictability of the regeneration responses observed with the phenotypically-purified LP populations, and in order to ensure that the number of cells injected relative to the area of injury created by the syringe needle did not limit the degree of regeneration observed, we examined the relationship between injected cell number and number of dystrophin-positive fibers generated. As demonstrated in Fig. 5, the number of myofibers expressing dystrophin increased in relation to the number of transplanted cells in a relatively linear fashion for all sorted LP populations. In addition, even though the number of data points collected was not sufficiently extensive to generate high correlation curve-fits, the responses observed suggest that selection of specific precursor populations using identifiable surface markers may enable a relatively accurate prediction of the myofiber regeneration response in relation to the number of transplanted myogenic cells.

Identifying potential sources of differential regeneration capacity: comparison of CD34-sorted LP cell in vitro characteristics

Using the established differential regeneration between purified LP CD34-negative and -positive populations as a basis, we investigated a number of in vitro cellular characteristics that we believed had the potential to influence their in vivo behavior. Characteristics that were examined included: reassessment of level of myogenic differentiation status via MRF expression, cell division time and mitotic fraction, fusion behavior, position within the cell cycle at the time of transplantation, and expression of MHC-1.

Expression of myogenic proteins

Both immunofluorescent staining and RT-PCR were performed on LP subpopulations following sorting. Both CD34-negative and -positive fractions were found to express similar levels of all myogenic proteins examined by immunofluorescence staining, falling within the range of expression previously defined for unsorted LP cells within the Results (Determination of myogenic commitment) section. In order to confirm these results RT-PCR was also performed on both CD34-negative and -positive LP subpopulations, as well as C2C12 cells and PP1 cells as a positive and negative control, respectively. Although non-quantitative, both LP subpopulations expressed

similar levels of the satellite cell-associated M-cadherin, as well as the MRF proteins Myf5, MyoD and myogenin, thus confirming the immunofluorescence observations. C2C12 cells expressed similar levels of all MRFs. PP1 cells, used as a negative control, contained primarily fibroblasts (>90%) and did not express M-cadherin, but did display a low level of expression of the MRFs due to the presence of the small percentage of desmin-expressing cells.

Cell-cycle analysis

Results of the cell-cycle analysis show no statistical differences between the CD34-negative and positive populations in terms of the percentage of cells in any particular stage of the mitotic
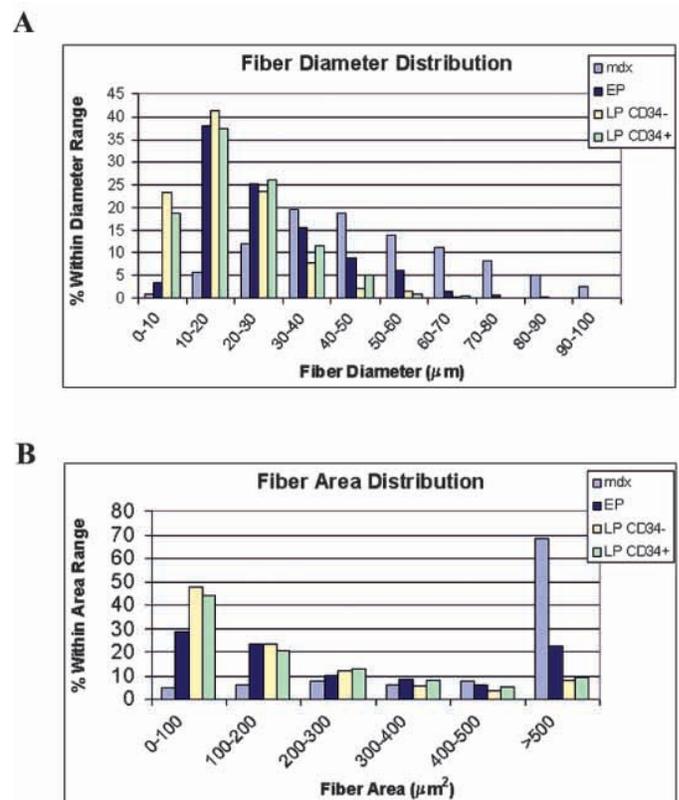

Fig. 4. Fiber diameter (A) and area (B) distributions of restored dystrophin-expressing myofibers generated by the various myogenic populations. A larger percentage of small, newly formed fibers is seen with the LP population, and the overall range of distribution of the EP-generated fibers suggests that they may be more likely to fuse with larger host myofibers. Mdx myofiber analysis of non-injected areas was used as a control.
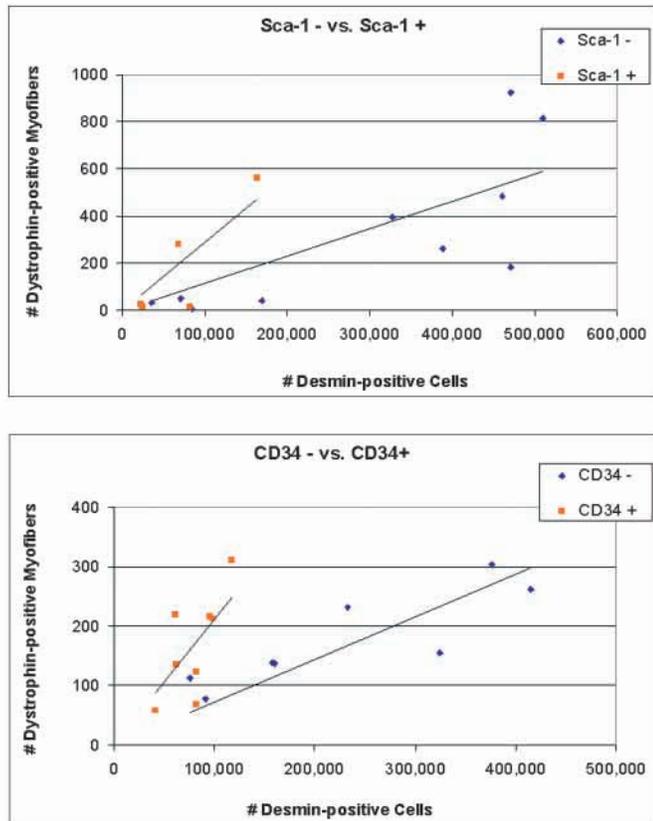
Fig. 5. Relationship between the number of LP cells injected and the number of dystrophin-positive myofibers observed at 7 days post-injection. The greater slope of the CD34$^+$ population's relationship re-emphasizes its higher level of regeneration efficiency. Relatively linear and predictable increases in number of dystrophin-positive myofibers generated with respect to injected cell number were observed for all populations (R^2: 0.676, 0.758, 0.736, and 0.497 for Sca-1$^-$, Sca-1$^+$, CD34$^-$, and CD34$^+$, respectively).

cycle. The percentage of cells in the G_0/G_1, S and G_2/M stages, respectively, for CD34-negative cells was found to be 75.9±1.4, 12.1±2.2 and 12.0±1.2. Similarly, for the CD34-positive population the percentage of cells in each stage was found to be 73.4±3.2, 11.8±1.4 and 14.8±3.0.

This similar level of cell-cycle progression and myogenic regulatory factor expression observed, as described, may be interrelated as it has been previously suggested that muscle differentiation signaling pathways may correlate to distinct patterns of cell-cycle status (Kitzmann et al., 1998; Lindon et al., 1998).

Immunogenicity

Statistical differences in MHC-I expression between the LP populations could not be detected by flow cytometric analysis, as the percentages of cells with detectable levels of expression were 13.0±6.3 and 19.1±8.3 for the CD34-negative and -positive sorted fractions, respectively (P=0.594). Although this LP intergroup comparison is similar, this expression is lower than that observed with EP populations (Qu-Petersen et al., 2002).

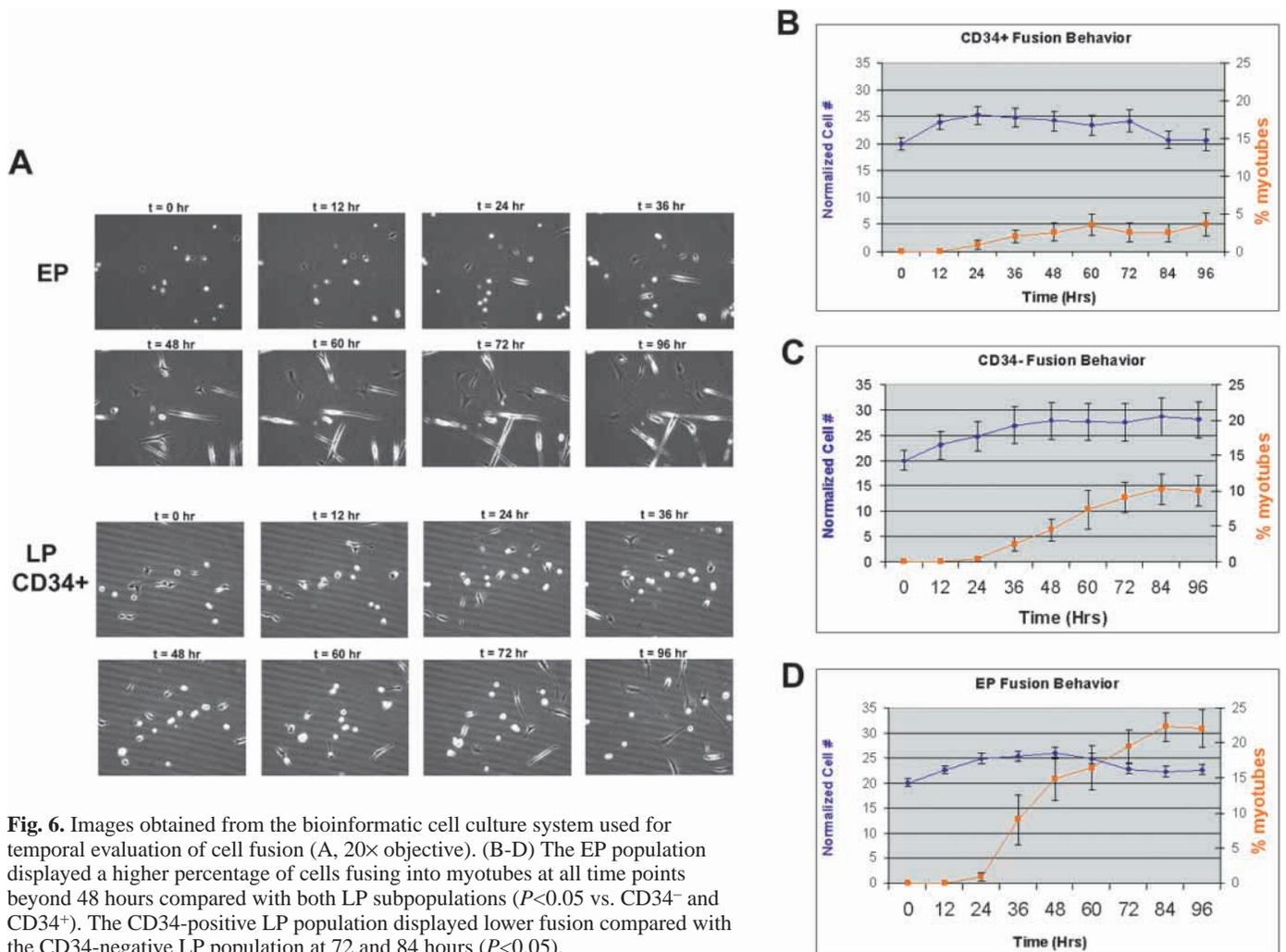### Cell division and fusion characteristics

Both cell division in culture (under high serum 'growth' conditions) and cell fusion into multinucleated myotubes (under low serum 'fusing' conditions) were monitored in vitro using a novel cell culture imaging system that allowed the cells to be closely monitored over extended culture periods. Information pertaining to cell division was collected and analyzed at 10 minute intervals and allowed for the determination of division times for both CD34-positive and -negative fractions in vitro. Average division times for dividing cells were determined to be 15.19±0.57 in the CD34-negative fraction and 14.76±0.49 in the positive fraction (P=0.988). In addition, from information gathered regarding the total cell number observed at specific time points, the mitotic fraction of each population was calculated using the non-exponential growth model described. No difference in the estimated mitotic fraction (α) could be determined between the two populations (P=0.259; α=0.398±0.081 and 0.358±0.094 for CD34-negative and -positive, respectively).

In separate experiments, under low serum conditions, lightfield observations (example provided in Fig. 6A) regarding the fusion and formation of multinucleated myotubes revealed differences between CD34-negative and -positive populations. From these observations, the CD34-negative population contained a higher percentage of cells fusing into myotubes at all time points beyond 72 hours when compared to the CD34-positive population (P<0.05 from 72 to 96 hours), as shown in Fig. 6B,C. Fixation and immunofluorescent staining at the 96 hour time point revealed that the fusion index (% of total myogenic nuclei contained within myotubes) was significantly higher for the CD34-negative population compared to its CD34-positive counterpart (Fig. 7C; P=0.034). Correspondingly, the ratio of mononuclear myogenic cells to the number of myotubes was significantly higher for the CD34-positive population (Fig. 7D; P=0.029).

Based on the regenerative efficiency of these LP subpopulations and their observed differences in fusion behavior, we hypothesized that the EP cells displaying the lowest regenerative capability would correspondingly display the highest degree of short-term fusion in the same assay. As shown in Figs 6 and 7, EP cells indeed displayed the highest rate and degree of cell fusion in comparison to both LP subpopulations. A significantly higher percentage of myotube formation was observed for EP cells at all time points beyond 48 hours (Fig. 6B-D, P<0.05 vs. both LP subpopulations). Also, the high degree of EP cell fusion is demonstrated by both the fusion index and ratio of mononuclear cells to myotubes (Fig. 7C,D; fusion index, P<0.05 vs. both CD34-negative and -positive; mononuclear ratio, P<0.05 vs. CD34-positive only). This difference can be further appreciated in the immunofluorescence staining shown in Fig. 7A,B.

Monitoring CD34 expression in vitro

Both CD34-negative and -positive sorted LP cells were placed back into culture, under high serum conditions, and re-evaluated for CD34 expression at various time points. As shown in Fig. 8A, a CD34-positive subpopulation is re-established in LP cultures initially depleted of CD34-expressing cells. In comparison, CD34 expression is

Fig. 6. Images obtained from the bioinformatic cell culture system used for temporal evaluation of cell fusion (A, 20× objective). (B-D) The EP population displayed a higher percentage of cells fusing into myotubes at all time points beyond 48 hours compared with both LP subpopulations ($P<0.05$ vs. CD34$^-$ and CD34$^+$). The CD34-positive LP population displayed lower fusion compared with the CD34-negative LP population at 72 and 84 hours ($P<0.05$).

maintained in cultures initially containing the CD34-purified fraction.

Based on this observation and the differential fusion characteristics displayed by the LP subpopulations, we examined the CD34 status of the non-fused cells remaining in culture at the conclusion of the fusion study. As expected, the non-fused myogenic cells within the CD34-positive LP cultures were found to express CD34 (>95%). Interestingly though, in cultures comprised initially of CD34-negative LP cells, we observed that the majority (>90%) of unfused myogenic cells that remained following 4 days of low serum conditions expressed CD34 (Fig. 8B,C). Thus, within CD34-depleted cultures, the CD34-positive subpopulation that is re-established represents the majority of the non-terminally differentiated, mononucleated fraction.

Discussion

We have investigated and compared the ability of myogenic progenitor cell populations, isolated via differential adhesion characteristics, to regenerate skeletal muscle and restore dystrophin expression within mdx host muscle. These populations displayed varying degrees of myogenic commitment and variable regeneration efficiencies. In an allogenic transplantation model, early adhering populations (EP) displayed lower levels of regeneration capacity. A slower adhering population (LP), expressing markers characteristic of satellite cells, was further identified and separated based on variable expression of the cell surface proteins Sca-1 and CD34. The expression of CD34 was associated with a statistically significant, and nearly three-fold, increase in cellular efficiency in the restoration of dystrophin-expressing myofibers. Utilization of a Sca-1-expressing population was associated only with a trend toward increased restoration.

Our observations with regard to the myogenic cell separation that occurs during the preplating process suggest that early adhesion is associated with a more progressive state of differentiation, as defined by MRF expression. We have confirmed through in vitro analysis of fusion characteristics that this higher level of myogenic commitment is associated with both increased rates and degrees of fusion, as expected, when compared to cells displaying a less-differentiated phenotype (as seen in LP cultures). However, within the LP cultures there appears to be subpopulations of cells identified by differential CD34 expression with seemingly similar levels of myogenic commitment, but with variable fusion characteristics. Similar observations have been reported previously in the literature. Through in vivo proliferation
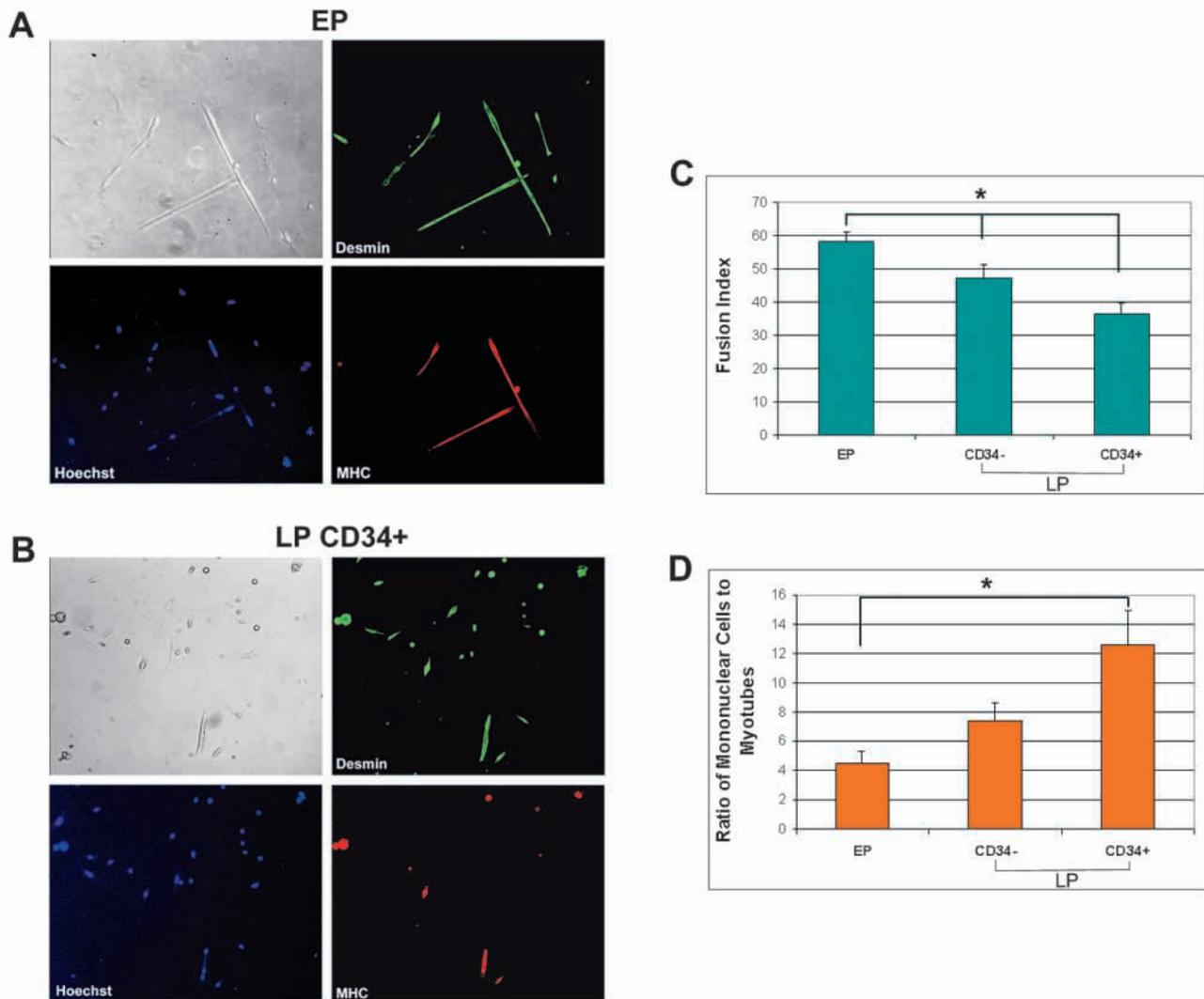
Fig. 7. Cultures exposed to low serum fusing conditions were evaluated by immunofluorescence to determine the number of myogenic nuclei associated with fused and myosin-heavy-chain-expressing (MHC) myotubes at 96 hours (representative images of EP and LP CD34-positive populations are shown in A and B). Significant differences in the degree of fusion, defined by the fusion index (C), and ratio of mononuclear cells to myotubes formed (D) were observed between EP and LP populations (fusion index, *$P<0.05$ for CD34-positive vs. EP and CD34-negative; ratio of mononuclear to myotubes, *$P<0.05$ for CD34-positive vs. EP only).

studies, Schultz described two different satellite cell progenitor populations: one termed the reserve population and the other the producer population (Schultz, 1996). The producer population was identified as providing nuclei directly to growing myofibers by fusion following a limited number of mitotic divisions. In contrast, the reserve population is believed to generate the producer population through asymmetric cell divisions. Additional evidence is presented by in vitro studies that have reported variations in the size of the colonies generated by individual satellite cells and behavioral heterogeneity upon induction of differentiation (Schultz and Lipton, 1982; Baroffio et al., 1996; Molnar et al., 1996; Yoshida et al., 1998). In this study, we demonstrate that CD34 expression, within LP cultures displaying satellite cell characteristics, may be useful in identifying these subpopulations and furthermore utilize this differential expression to demonstrate that these variations in fusion

characteristics appear to play a role in the regenerative abilities of these cells following transplantation into dystrophic hosts. Based on our results, decreased fusion is associated with increased dystrophin restoration and regeneration capacity in vivo. It is logical to envision a scenario in which transplanted populations demonstrating both rapid and high degrees of fusion would limit donor cells available to proliferate and further participate in the regeneration process, as myogenic cells that have participated in the formation of a myotube are considered terminally differentiated and withdrawn from the cell cycle (Bischoff, 1994). Correspondingly, cells that remain in an undifferentiated and unfused state retain the potential to proliferate and thus increase the yield of myogenic nuclei derived from an initial donor injection.

Recent results from our lab (not shown) lend further support to our hypothesis regarding delayed differentiation and fusion. We have been able to isolate a muscle-derived stem cell
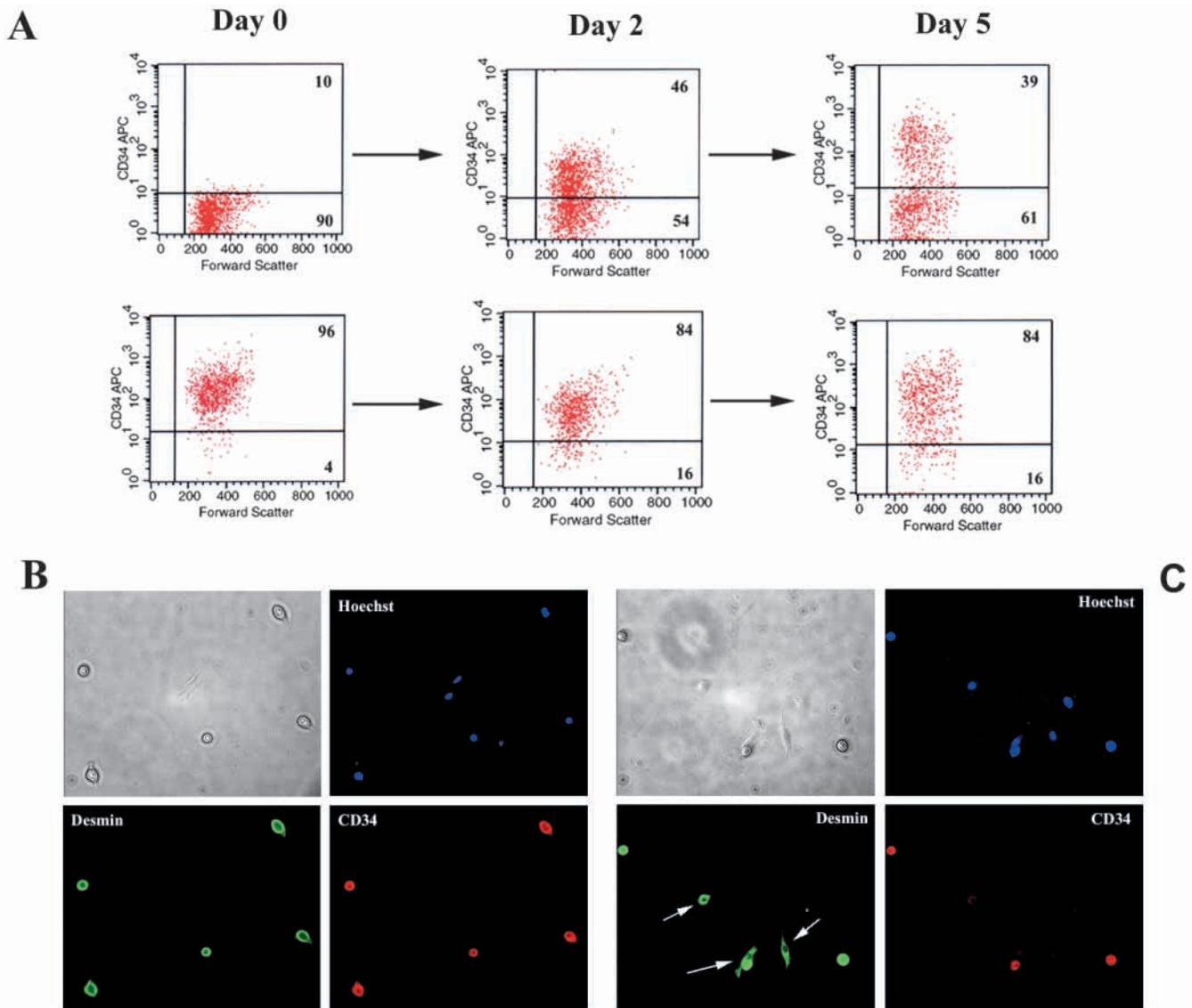
Fig. 8. (A) Establishment of a CD34-positive subpopulation is observed when culturing CD34-depleted cultures under high serum culturing conditions. Cells enriched for CD34 expression maintain their phenotype over the same time period (representative dot plots are shown along with averaged percentages of CD34-negative and -positive cells at each time point). (B,C) Immunofluorescence and brightfield images of mononuclear myogenic cells found within cultures initially depleted of a CD34-expressing population and exposed to low serum fusion medium. The majority of the undifferentiated myogenic cells were found to express CD34 (B), although some CD34-negative cells are present (C, arrows; 20× objective).

candidate, using the preplate technique, from the LP cultures. This cell population, termed LTP for long-term proliferating, has demonstrated multiple lineage differentiation capacity as well as an exceptional ability to regenerate muscle and restore dystrophin (Qu-Petersen et al., 2002). Within the same low serum fusion assay described, this cell population exhibits extremely low fusion (<6% fusion index) but also displays a large proliferative capacity, in contrast to the LP populations described herein. Such demonstrations again suggest that fusion characteristics, and proliferation prior to fusion in vivo, may play a key role in the regenerative efficiency of myogenic populations following transplantation. However, in contrast to all other published transplantation reports, this LTP cell population also displays prolonged dystrophin restoration even in the allogenic setting.

Investigation into other contributing mechanism(s) of the high level of dystrophin restoration with this particular population is ongoing.

While fusion characteristics seem to play a role in the outcome of dystrophin restoration, other important and related characteristics such as survival must not be overlooked or ignored. A rapid demise of donor cells has been observed (Huard et al., 1994; Kinoshita et al., 1994; Fan et al., 1996; Beauchamp et al., 1999) and has been implicated as one of the reasons for the lack of clinical successes in myoblast transfer therapy for the treatment of muscular dystrophies (Gussoni et al., 1992; Tremblay et al., 1993; Mendell et al., 1995; Neumeyer et al., 1998). Although significant improvements in cell survival have been achieved through delivery of anti-

inflammatory agents (Guerette et al., 1997; Qu et al., 1998; Merly et al., 1998), rapid donor cell fusion in combination with poor survival rates may indirectly worsen an already undesirable situation, by limiting or preventing surviving cells from expanding the donor population.

Deleterious effects of delayed fusion in the in vivo setting must also be considered. It has been previously suggested that the rate of donor cell fusion in vivo may play a role in the recognition of non-self antigens (Grounds, 1990). It is known that the MHC class I antigen, which inhibits the action of natural killer cells (Smythe et al., 2001), is down-regulated with the formation of mature myofibers in normal muscle (Ponder et al., 1983; Karpati, 1990; Pavlath et al., 1994). Even though regenerating myofibers (such as those observed within dystrophic muscle) display some increase in MHC-I expression (Emslie-Smith et al., 1989; McDouall et al., 1989), the fusion of donor cells could serve to protect them from host immune responses, but consequently limit the degree of dystrophin restoration. An ability to proliferate prior to fusion would enhance the degree of restoration, however, it could also lead to exposure to cytotoxic host cell responses, unless the proliferation was rapid enough. One such myogenic population, demonstrating highly prolific behavior upon engraftment, has been described previously through in vivo labeling techniques that allowed quantification and tracking of transplanted precursors (Beauchamp et al., 1999). Further investigation is required to determine if similar behavior is displayed by the CD34-positive population described here.

It is important to note that over long time periods the expression of dystrophin may serve as an adequate antigen for antibody production and eventual myofiber destruction (Ohtsuka et al., 1998; Ferrer et al., 2000), although conflicting evidence exists (Vilquin et al., 1995). Indeed, the eventual elimination of dystrophin-expressing myofibers in this allogenic study was evident within the immunocompetent host muscle of both EP and LP injected cell populations (results not shown). However, it is also worth mentioning that the muscle regeneration described herein was observed in a model in which there was no attempt to enhance the regenerative response or create host environmental conditions optimal for myogenic cell transplantation. Methods previously described to ensure optimal transplantation efficiency include attempts to block the host's regenerative response by pre-irradiation of the muscle to be injected (Morgan et al., 1990), prior injection of necrotic agents to stimulate the regenerative response, or both (Huard et al., 1994; Vilquin et al., 1995). Additionally, the myogenic donor cells used in this study were obtained from primary cultures, not clonal cell lines, which were derived from adult-aged skeletal muscle.

The CD34-positive phenotype discussed here has been recently described as representative of the majority of committed satellite cell precursors within skeletal muscle (Beauchamp et al., 2000). Our findings demonstrate some important similarities with those previously described, primarily the expression of both Myf5 and M-cadherin and the observed low level of fusion into myotubes in culture. As has been previously suggested with regard to the myogenic lineage, the timing of CD34 upregulation and downregulation may play a role in the prevention of differentiation (Zammit and Beauchamp, 2001). In a non-myogenic setting, CD34 expression has also been suggested to play a role in the inhibition of hematopoietic differentiation (Fackler et al., 1995). These observations agree with the results of our fusion studies and would support our hypothesis that the CD34-positive cells may be associated with delayed myogenic differentiation and fusion upon injection in vivo. Our findings associating the CD34-positive phenotype with undifferentiated myogenic cells, exposed to fusing conditions in vitro, have also been observed in differentiation studies utilizing cell lines (Beauchamp et al., 2000). However, our results further show that in a growth culture setting, a dominant CD34 phenotype seems to be relatively conserved. This does not appear to be the case with CD34-depleted cultures, which quickly re-establish a CD34-expressing subpopulation. This emerging CD34 subpopulation also seems to display delayed fusion characteristics when challenged in vitro, further suggesting a potential role of CD34 in the regulation of differentiation within myogenic cells. While clarification of such issues is necessary, it is becoming increasingly apparent that CD34 will play a significant role in identifying myogenic progenitor hierarchy and characterizing cell behavior both in vitro and in vivo.

In conclusion, using information regarding myogenic precursor cell heterogeneity we were able to isolate phenotypically-pure populations based on surface protein expression and demonstrate that the CD34-positive population of late adhering myogenic cells, displaying delayed fusion kinetics in vitro, displays an enhanced restoration of dystrophin expression within dystrophic skeletal muscle. Our results suggest that myogenic cell differentiation status plays an important role in the functional outcomes of cell transplantation, and continued understanding of myogenic cell biology is fundamental to the future utilization of precursor cells for therapeutic purposes. Although utilized within the context of dystrophin delivery, the results presented here may be applicable to other areas of research including muscle cell-mediated gene delivery or other muscle-related cell therapy applications.

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