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Cell heterogeneity upon myogenic differentiation: down-regulation of MyoD and Myf-5 generates 'reserve cells'

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SUMMARY

When a proliferating myoblast culture is induced to differentiate by deprivation of serum in the medium, a significant proportion of cells escape from terminal differentiation, while the rest of the cells differentiate. Using C2C12 mouse myoblast cells, this heterogeneity observed upon differentiation was investigated with an emphasis on the myogenic regulatory factors.

The differentiating part of the cell population followed a series of well-described events, including expression of myogenin, p21^{WAF1}, and contractile proteins, permanent withdrawal from the cell cycle and cell fusion, whereas the rest of the cells did not initiate any of these events. Interestingly, the latter cells showed an undetectable or greatly reduced level of MyoD and Myf-5 expression, which had been originally expressed in the undifferentiated proliferating myoblasts. When these undifferentiated cells were isolated and returned to the growth conditions, they progressed through the cell cycle and regained MyoD expression. These cells demonstrated identical features with the original culture on the deprivation of serum. They produced both MyoD-positive differentiating and MyoD-negative undifferentiated populations once again. Thus the undifferentiated cells in the serum-deprived culture were designated 'reserve cells'.

Upon serum deprivation, MyoD expression rapidly decreased as a result of down-regulation in approximately 50% of the cells. After this heterogenization, MyoD positive cells expressed myogenin, which is the earliest known event of terminal differentiation and marks irreversible commitment to this, while MyoD-negative cells did not differentiate and became the reserve cells. We also demonstrated that ectopic expression of MyoD converted the reserve cells to differentiating cells, indicating that down-regulation of MyoD is a causal event in the formation of reserve cells.

Key words: Myogenic regulatory factor, MyoD, Myf-5, Myogenin, Differentiation, Heterogeneity

INTRODUCTION

Many tissues contain not only differentiated cells but also undifferentiated cells that retain differentiation potential, i.e. stem cells. This heterogeneity is important for normal turnover, growth, or repair of tissues. Both types of cells play important roles in normal functioning of the tissue and have an intimate relationship with each other throughout life.

In the in vitro system, when a clonal cell population is induced for terminal differentiation, it is sometimes observed that not all the cells differentiate. Rather, a subpopulation of cells seem to escape from this process. For example, PC12 rat pheochromocytoma cells and mouse erythroleukemia (MEL) cells differentiate in response to induction signals such as nerve growth factor and dimethyl sulfoxide, respectively. In both cases, not all the cells terminally differentiate, but a subpopulation of cells remain undifferentiated (Gunning et al., 1981; Friend et al., 1971). Thus, it seems to be common among various cell types that differentiated and non-differentiated cells are generated from the original homogeneous cell population upon terminal differentiation. However, detailed observations explaining how this heterogeneity is produced have not been given.

We addressed this issue using an in vitro myogenic differentiation system in the present report. Discovery of the myogenic regulatory factors (MRFs) has made the myogenic cells one of the best systems to study cellular differentiation (Weintraub et al., 1991; Buckingham, 1992; Olson, 1992; Weintraub, 1993). MRFs are also called the MyoD family, consisting of MyoD (Davis et al., 1987), Myf-5 (Braun et al., 1989), myogenin (Wright et al., 1989), and MRF4/herculin/Myf-6 (Rhodes and Konieczny, 1989; Miner and Wold, 1990; Braun et al., 1990), which are transcription factors sharing the basic helix-loop-helix (bHLH) motif and are

able to activate skeletal muscle-specific genes through a consensus DNA element termed E box. Each member of this family is expressed and plays important roles at various stages of differentiation, as revealed by targeted gene knock out and other experiments. MyoD and Myf-5 are expressed in undifferentiated proliferating myoblasts and myotubes (Tapscott et al., 1988; Braun et al., 1989) and play crucial roles in the determination and/or maintenance of myogenic identity (Rudnicki et al., 1992, 1993; Braun et al., 1992; Weintraub, 1993). Therefore, they are often called the ‘determination class’ of MRFs. MyoD is also required for healthy self-renewing proliferation of the adult skeletal muscle stem cells, the satellite cells (Megeney et al., 1996). Myogenin is induced upon early steps of terminal differentiation and is essential for this process (Wright et al., 1989; Nabeshima et al., 1993; Hasty et al., 1993). MRF4 contributes to the later maturation steps (Rhodes and Konieczny, 1989; Braun and Arnold, 1995; Zhang et al., 1995; Patapoutian et al., 1995; Olson et al., 1996).

Mouse C2 myoblast (Yaffe and Saxel, 1977), and its subclone C2C12 (Blau et al., 1983) are the best characterized myogenic cell lines to date. In the presence of a high concentration of serum, they proliferate as an undifferentiated population each expressing MyoD and Myf-5. Terminal differentiation of C2C12 is most typically induced by serum deprivation, which causes a series of chronologically ordered events. Expression of myogenin is the earliest that has been reported to occur (Andres and Walsh, 1996). After that, cells withdraw from cell cycles permanently and express $p21^{WAF1}$, a cyclin-dependent kinase inhibitor, followed by the production of contractile proteins and cell fusion resulting in the formation of myotubes (Miller, 1990; Halevy et al., 1995; Parker et al., 1995; Guo et al., 1995; Andres and Walsh, 1996).

It has been observed that only a fraction of C2C12 myoblast cells undergo differentiation upon serum deprivation, while others do not follow the cellular events described above (Miller, 1990). However, not so much attention has been paid to these ‘escapees’ of differentiation. In this study, we investigated the characteristics of this undifferentiated cell population found in a ‘differentiated’ culture of C2C12 cells and analyzed how this heterogeneity is generated during the differentiation process, with an emphasis on the MyoD family of MRFs.

MATERIALS AND METHODS

Cells and cultivation

C2C12, a subclone of the C2 mouse myoblast cell line (Yaffe and Saxel, 1977; Blau et al., 1983), was obtained from the American Type Culture Collection. A subclone of C2C12, designated C2/4 (Yoshida et al., 1996), was used and referred to simply as C2C12 in this paper. The cells were routinely propagated in the growth medium, DMEM supplemented with 10% fetal calf serum (FCS) and 60 μg/ml of kanamycin. To induce differentiation, cells were seeded at a density of 2×10^4 cells/well (12-well plate) or equivalent in growth medium and then 24 hours later switched to ITS serum-free medium (DMEM supplemented with 10 μg/ml of insulin, 5 μg/ml of transferrin, 10 nmol of sodium selenite, 1 mg/ml of bovine serum albumin (BSA) and 60 μg/ml of kanamycin). The ITS medium was routinely changed every 24 hours. Insulin (human), transferrin (human) and BSA were purchased from Sigma Chemical Co. In every situation collagen type I-coated culture plates (Iwaki) were used.

Immunostaining

Cells were fixed with 2% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 10 minutes and permeabilized with 0.25% Triton X-100 in PBS for 20 minutes at room temperature. For the staining with anti-proliferating cell nuclear antigen (PCNA), cells were treated with 100% methanol at room temperature for 5 minutes after fixation instead of the detergent permeabilization. Non-specific binding sites were blocked with 5% skim milk in PBS for 1 hour, and the cells were incubated with the primary antibodies (4°C, overnight) and then with the appropriate fluorochrome-conjugated secondary antibodies (37°C, 30 minutes). Double staining was performed by subsequent incubations with another set of first and second antibodies. After DNA-staining with 4′,6-diamidino-2-phenylindole (DAPI, Molecular Probe), specimens were mounted and observed with an Axiophoto fluorescence microscope (Zeiss). In some experiments, biotinylated anti-mouse IgG (diluted at 1:2,000, Vector) and a Vectastain Elite ABC kit (Vector) was used.

The primary antibodies used in this study are as follows: anti-MyoD mouse mAb, 5.8A (used in dilution 1:50; Dias et al., 1992), affinity-purified anti-MyoD rabbit polyclonal antibody (pAb) (1:100; Koishi et al., 1995), anti-Myf-5 rabbit pAb (IgG fraction; Smith et al., 1993), anti-myogenin mAb, F5D (1:100; Wright et al., 1989), anti-$p21^{WAF1}$ rabbit pAb (1:2,000; PharMingen), anti-troponin T mouse mAb, NT302 (1:2,000; Abe et al., 1986), anti-skeletal myosin rabbit pAb (1:500; Sigma Chemical Co.) and anti-PCNA mouse mAb, PC10 (1:500; Novocastra Laboratories Ltd). Secondary antibodies were all purchased from Organon Teknika Co./Cappel and used in dilution at 1:200: FITC-conjugated anti-mouse IgG, FITC-conjugated anti-rabbit IgG ($F(ab')_2$ fragment), Texas Red-conjugated anti-mouse IgG ($F(ab')_2$ fragment), and Texas Red-conjugated anti-rabbit IgG.

Transient transfection experiments

All the transfections were performed using Lipofectamine reagent (Gibco BRL) according to the manufacturer’s instructions. The MyoD expression plasmid, pcDNA3/MMyoD was generated by insertion of an open reading frame sequence encoding wild-type mouse MyoD (Fujisawa-Sehara et al., 1990) into pcDNA3 (Invitrogen). Exponentially growing C2C12 cells grown on 12-well culture plates were transfected with 0.75 μg of pcDNA3 or pcDNA3/MMyoD and 0.25 μg of pcDNA3/NLSLacZ, which is also a pcDNA3 derivative encoding nuclear localization signal-tagged bacterial LacZ protein. The transfected cells were incubated in ITS medium for a further 3 days and stained chemically for β-galactosidase activity with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) and immunochemically for troponin T expression with a Vectastain Elite ABC kit (Vector).

Stable transformants

Stable transformants used in this study were generated using the LacSwitch inducible mammalian expression system (Stratagene). pOPRSVIMyoD was generated by replacement of the CAT gene in the operator vector, pOPRSVICAT, with the 1.2 kbp *Eco*RI-*Hin*dIII fragment containing the mouse MyoD coding sequence (Fujisawa-Sehara et al., 1990).

C2C12 cells were first transfected with the Lac repressor vector p3′SS and selected by hygromycin B (300 μg/ml, Calbiochem) to isolate a clone, C23′SS3, which constitutively expressed LacI. C23′SS3 cells were subsequently transfected with pOPRSVICAT (for control) or pOPRSVIMyoD, and transformants were selected in 400 μg/ml of geneticin (Gibco) and 300 μg/ml of hygromycin B. A clone, C2lacMyoD, which expresses exogenous MyoD conditionally in the presence of 5 mM of isopropyl-β-D-thiogalactoside (IPTG) was then isolated.

Northern blot hybridization was carried out as described (Yoshida et al., 1996) with a few modifications. Briefly, 15 μg each of total RNA was separated in a 1.2% agarose gel containing 7% formaldehyde and the electrophoresed gel was photographed. RNA

was capillary-transferred onto Hybond N^+ membrane (Amersham) and hybridized with a ^{32}P-labeled probe prepared from the MyoD coding fragment. Conditions for hybridization, washing and autoradiography were as described previously (Yoshida et al., 1996).

RESULTS

Cell heterogeneity in the differentiated C2C12 culture

Fig. 1 indicates the kinetics of myotube formation of C2C12 cells observed in the ITS serum-free differentiation condition. Approximately 50% of the total number of cells never fused into myotubes even after a long culture period. The differentiation status of individual cells in the differentiated culture was examined by immunostaining for muscle contractile proteins (Fig. 2). While multinucleated myotubes and some of the mononucleated cells (myocytes) were positive for troponin T or skeletal myosin, a significant proportion of mononucleated cells did not express these proteins (Fig. 2, arrowheads). This is consistent with the previous observation that only a portion of myoblast cells differentiate even in the serum-deprived condition, and the rest remain mononucleated and undifferentiated (Miller, 1990). The final percentage of differentiated cells was highly constant and was always between 40% and 60% in the case of our clonal subline of C2C12 cells (C2/4). Although many investigations have aimed at the mechanisms that drive differentiation, not so much attention has been paid to these escapees of differentiation.

To further characterize the heterogeneity of the cell population, the expression of MRFs was examined by immunofluorescence. MyoD and Myf-5 play roles in acquirement and/or maintenance of myogenic potential, and are expressed in undifferentiated C2 myoblasts in the growth medium (Tapscott et al., 1988; Braun et al., 1989). In the differentiated culture, MyoD nuclear immunoreactivity was restricted to the troponin T-positive myotubes or myocytes; no expression, or only trace amounts, was detected in the undifferentiated, mononucleated cells. Similarly, while Myf-5 was clearly detected in all the myotube and myocyte nuclei like MyoD, the undifferentiated mononucleated cells showed the apparently weaker staining of Myf-5 in their nuclei, although the intensities varied from cell to cell (Fig. 2B).

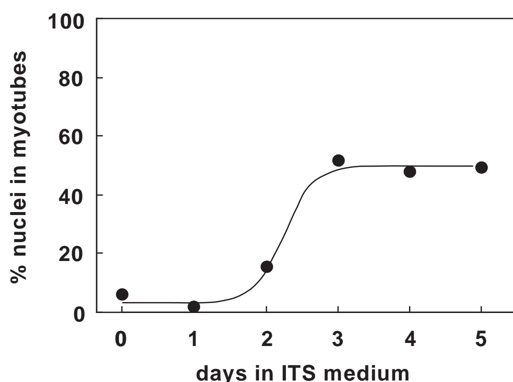

Fig. 1. Time course of myotube formation of the C2C12 cell culture induced to differentiate in ITS medium. The fusion index (percentage nuclei incorporated in myotubes) at each time point was determined as previously described (Yoshida et al., 1996).

Myogenin, which is expressed upon and important for the terminal differentiation step, was present in the nuclei of skeletal myosin-positive cells, but was not detected in the undifferentiated cells. MRF4 was not detected in this culture system.

The proliferation status of the cells in differentiated culture

Then, we examined the expression of proliferating cell nuclear antigen (PCNA) and $p21^{WAF1}$ to investigate the proliferation status of individual cells in the differentiated C2C12 culture. $p21^{WAF1}$ is a cyclin-dependent kinase inhibitor known to be expressed in parallel with the irreversible withdrawal from cell cycle during myogenic differentiation (Halevy et al., 1995; Parker et al., 1995; Guo et al., 1995). As described previously, myotubes and myocytes were positive for $p21^{WAF1}$ and negative for PCNA (Figs 2D, 3A). PCNA was never expressed in myotubes even after the re-addition of serum (Fig. 3B; Andres and Walsh, 1996). Thus, the differentiated cells are irreversibly exited from the cell cycle.

On the other hand, the undifferentiated mononucleated cells were negative for $p21^{WAF1}$ (Fig. 2D, arrowheads) and showed no or very weak staining of PCNA. The latter molecule was highly induced by the second addition of serum as reported (Fig. 3, arrowheads; Schneider et al., 1994; Andres and Walsh, 1996). BrdU(5-bromo-2′-deoxyuridine) incorporation experiments gave similar results; while 70% of the cells incorporated BrdU in growth condition, no myotubes and only 14% of the mononucleated cells incorporated it in differentiated culture. Thus, the undifferentiated mononucleated cells cycle slowly but are not permanently withdrawn from the cell cycle.

The undifferentiated cells are the reserve cells

Have the MyoD-negative undifferentiated cells lost their potentials to express MyoD or to differentiate? To test this, the mononucleated cells were isolated from the differentiated culture as described in Fig. 4. The isolated cells were almost mononucleated and negative for MyoD (Fig. 4A) and the calculated yield was 99%, indicating that the undifferentiated cells were efficiently isolated by this treatment. After two days of culture in growth medium, essentially all these mononucleated cells became MyoD positive and myogenin negative (Fig. 4B,C). They were also positive for Myf-5 (data not shown). They were in an active stage of the cell cycle as indicated by PCNA expression (Fig. 4D).

When these cells were transferred to the differentiation condition for the second time, they formed troponin T and MyoD positive myotubes again (Fig. 5A). MyoD-negative mononucleated cells also appeared (Fig. 5A, arrowheads). Interestingly, the proportions of MyoD-positive cells and differentiated cells (myogenin positive) were the same as for the first-time differentiation (Fig. 5B).

To further characterize the mononucleated cells, a clonal assay was performed to trace the fate of each single cell's progeny. Isolated mononucleated cells were grown in growth medium for 6 days to form colonies and then moved to ITS to test their differentiation property (Fig. 5C). Essentially all the colonies showed a mixed morphology consisting of both differentiated myotubes and mononucleated cells. Notably,

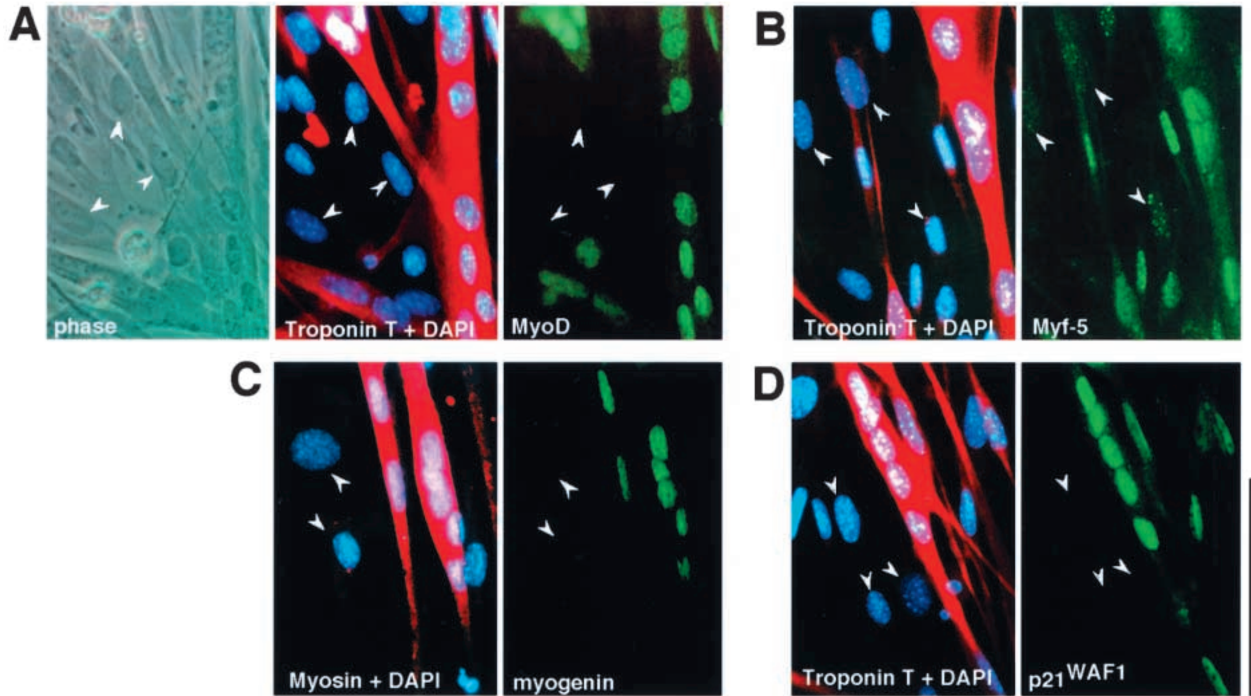

Fig. 2. Expression of myogenic regulatory factors, muscle contractile proteins and p21^{WAF1} in the differentiated state of C2C12 cells after 4 days in ITS. A fixed culture was subjected to double immunofluorescence with (A) anti-troponin T (red) and anti-MyoD pAb (green), (B) anti-troponin T (red) and anti-Myf-5 (green), (C) anti-skeletal myosin (red) and anti-myogenin (green), (D) anti-troponin T and (red) anti-p21^{WAF1} (green) antibodies and counterstained with DAPI. Each set of panels shows a single representative field. (A) Phase contrast image. Arrowheads indicate undifferentiated mononucleated reserve cells (see text for details). Bar, 100 μm.

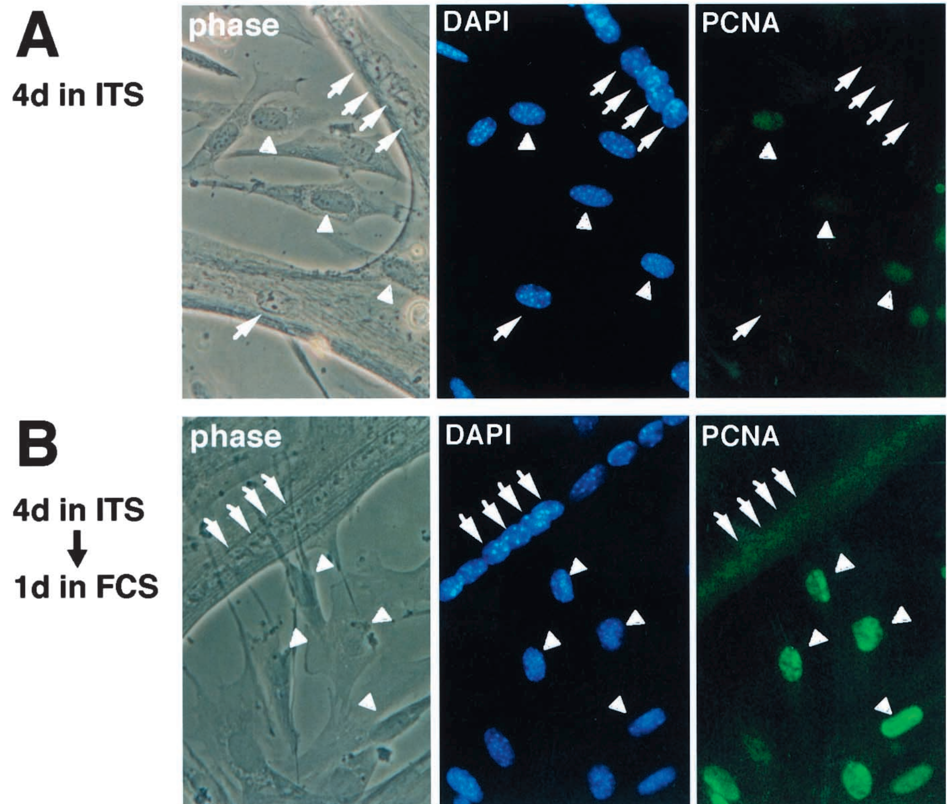

Fig. 3. Expression of PCNA in (A) differentiated (4 days in ITS) and (B) serum-restimulated (1 day in growth medium after 4 days in ITS) C2C12 culture. Cells were fixed and stained by anti-PCNA mAb and DAPI. Phase contrast image, DAPI and anti-PCNA stained views of each representative field are shown. Arrowheads and arrows indicate the nuclei of mononucleated cells and of myotubes, respectively. Bar, 100 μm.

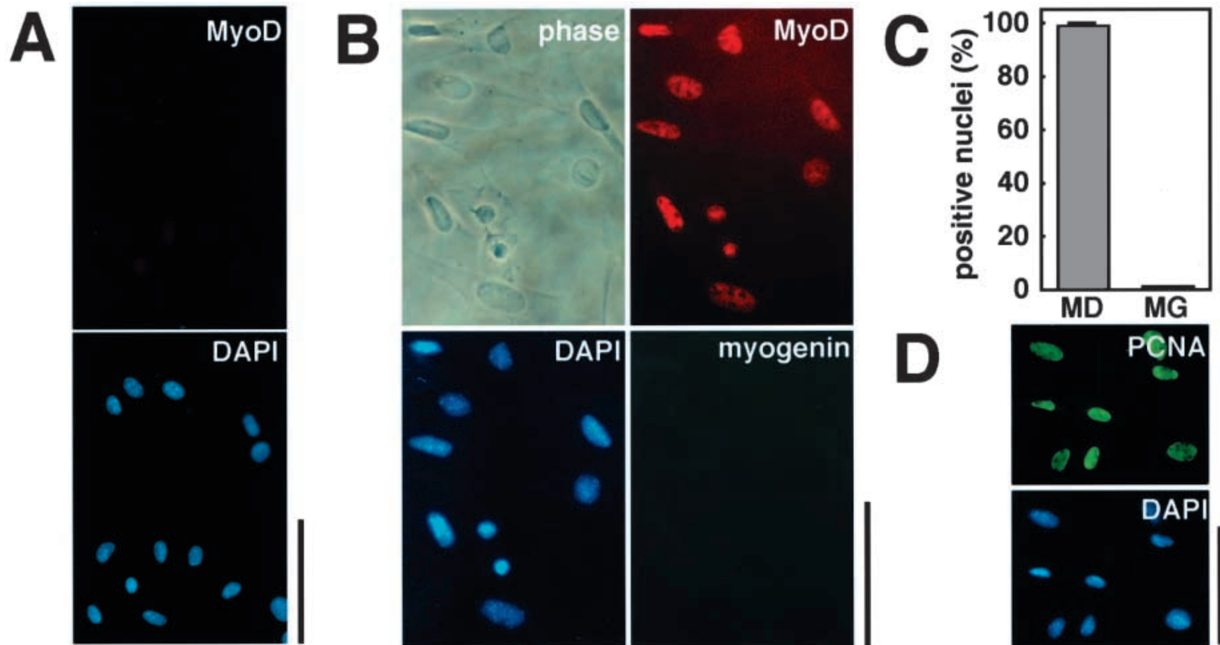

Fig. 4. Isolation of mononucleated cells. Mononucleated cells in a differentiated C2C12 culture (4 days in ITS) were collected by trypsinization and reseeded onto a new dish in growth medium. The fragments of floating myotubes were removed after 30 minutes when the mononucleated cells had attached to the culture dish. At this time, these cells were stained with anti-MyoD mAb and DAPI (A). After 48 hours of culture in growth medium, double immunofluorescence staining was performed (B). Phase contrast image, DAPI, anti-MyoD and anti-myogenin-stained views of a single representative field are shown. (C) Percentages of the nuclei positive for MyoD and myogenin were counted in parallel cultures to that in B. Three independent fields of 1 mm^2 each were counted. Bars, s.e.m. (D) Expression of PCNA. A parallel culture to that in B was stained with anti-PCNA mAb and DAPI. Bars, 100 μm.

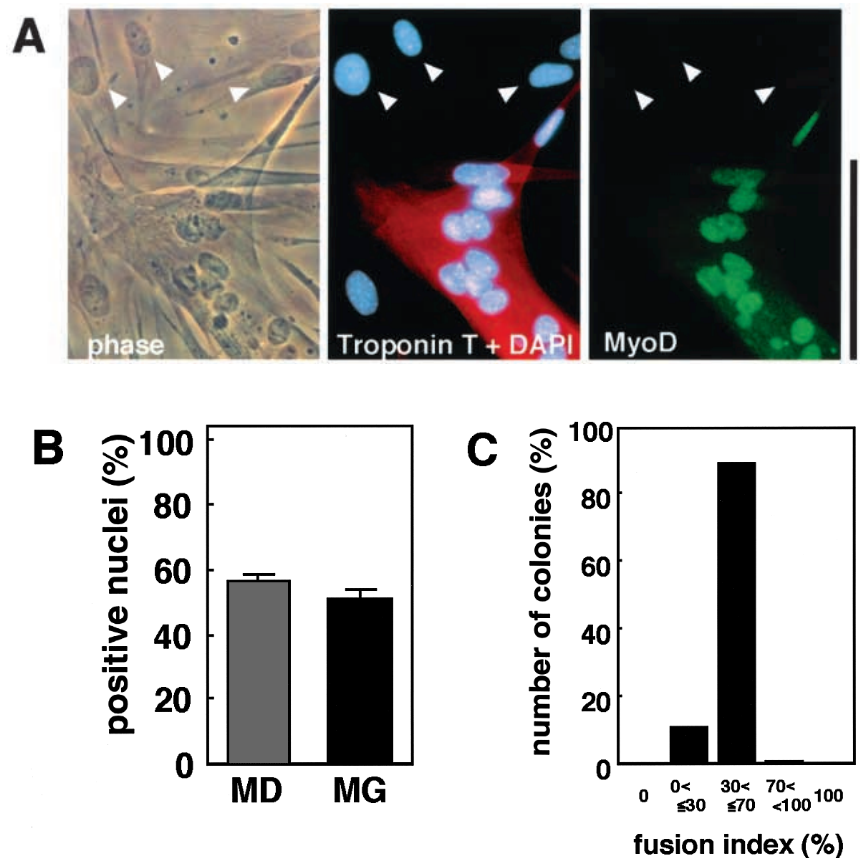

Fig. 5. Myogenic potential of serum-restimulated reserve cells. (A) Cells shown in Fig. 4B were incubated in ITS medium for another 4 days, then fixed and stained with anti-troponin T mAb (red) and anti-MyoD pAb (green). Phase contrast (left), troponin T/DAPI staining (middle) and MyoD staining (right) images of a single, representative field are shown. Arrowheads indicate the undifferentiated MyoD-negative cells. Bar, 100 μm. (B) Percentage positive nuclei for MyoD and myogenin were counted in parallel cultures to that in A. Three independent fields of 1 mm^2 each were counted. Bars, s.e.m. (C) A clonal assay. Freshly isolated reserve cells described in Fig. 4A were allowed to make colonies by incubating at a clonal density in growth medium. After 6 days, when each colony grew up to about 2 mm in diameter, colonies were induced to differentiate in ITS medium for another 3 days. Thereafter, colonies were classified by their morphology indexed by the percentage nuclei incorporated into myotubes (fusion index). Percentages of the colonies of indicated indexes are shown. Total colony number counted is 309.

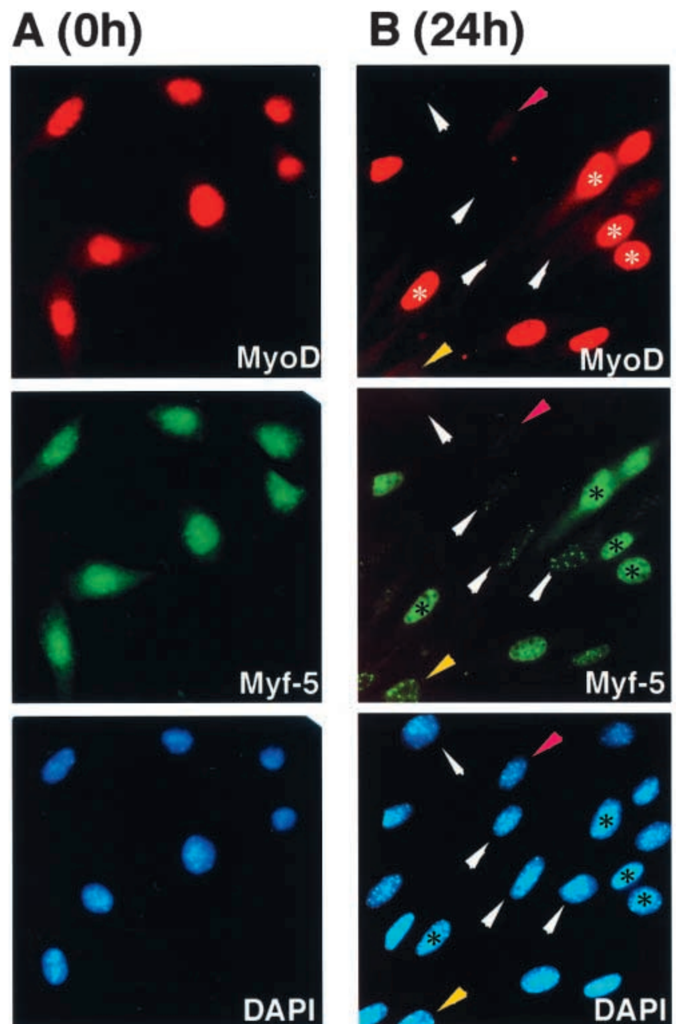

Fig. 6. Double staining of MyoD and Myf-5 in growing and differentiating C2C12 cells. Exponentially growing (A) and differentiating cells (24 hours in ITS) (B) were fixed, subjected to double immunostaining with anti-MyoD mAb (red) and anti-Myf-5 pAb (green), and counterstained with DAPI. Note that each cell expresses both MyoD and Myf-5 in A. In B, their expression is largely overlapped and a major part of the cells are either double positive (asterisk) or double negative (including MyoD negative and Myf-5 low, white arrowheads). However, MyoD weakly positive/Myf-5-negative cells (pink arrowhead) and MyoD-negative/Myf-5-positive cells (yellow arrowhead) are also observed. Bar, 100 µm.

about 90% of the colonies exhibited a comparable ratio of differentiated to undifferentiated cells with the original C2C12 cells. These results strongly suggest that each MyoD-negative undifferentiated cell that appeared in the differentiated culture can act as a source of the differentiated cells in response to appropriate stimuli. Therefore, these cells can be referred to as reserve cells.

It must be mentioned, however, that because the plating efficiency was about 20% (reasonable for this kind of experiment), we cannot exclude the possibility that only a subpopulation of the mononucleated cells was preferentially expanded in these experiments.

MyoD expression is rapidly down-regulated in a subset of cells

Given that the initial, undifferentiated proliferating myoblasts are essentially all positive for MyoD and Myf-5, and that a significant proportion (in our case, about 50%) of cells show negative or decreased expression for them in the differentiated culture, the amounts of MyoD and Myf-5 proteins in individual cells must change dynamically in the ITS medium.

We, therefore, performed a double staining experiment for MyoD and Myf-5 during the differentiation. Both MyoD and Myf-5 were clearly detected in essentially all the proliferating C2C12 cell nuclei, with relatively small variations in their intensities between the cells as were pointed out for MyoD (Fig. 6A; Tapscott et al., 1988). When the cells were transferred to ITS medium, a fraction of the cells began to down-regulate their expression. As shown in Fig. 6B, after 24 hours of culture in ITS, MyoD expression was repressed in a subset of cells to an almost undetectable level. Myf-5 expression exhibited a relationship with MyoD; most MyoD-positive nuclei showed clear staining for Myf-5 (asterisk) and MyoD-negative nuclei tended to show a weak or undetectable level of Myf-5 (white arrowheads). However, a small portion of cells did not exhibit parallel expression (colored arrowheads), suggesting that MyoD and Myf-5 are not down-regulated by identical mechanisms in the same population of cells.

A detailed time course of MyoD expression indicated that down-regulation of MyoD became apparent as early as 6 hours after transfer to ITS medium and reached a final level by 12 hours after the shift (Fig. 7A). Fig. 7B shows the actual numbers of MyoD-positive and -negative cells, indicating that the initial cell numbers doubled in the first 18 to 20 hours, almost the same as the population doubling time in the growth condition. Therefore, decrease of the cell number caused by apoptotic cell death, which is associated with myogenic differentiation (Wang and Walsh, 1996), was not apparent within the experimental period of Fig. 7A,B. We believe that the increase in the MyoD-negative cell portion is not simply due to the selective elimination of MyoD-positive cells, rather, it is caused by down-regulation of MyoD in some of the MyoD-expressing cells. Down-regulation of Myf-5 seemed to occur more slowly than that of MyoD.

MyoD divergence precedes the myogenin expression and irreversible commitment

As the above experiment identified the time course of MyoD divergence, we investigated its chronological relationship to the two marked events associated with terminal differentiation; the myogenin expression and the irreversible commitment. The myogenin expression is the earliest reported event related to the terminal differentiation (Andres and Walsh, 1996).

A double staining experiment (Fig. 7C) showed that myogenin expression became obvious after the MyoD expression was diverged (12 hours in ITS) and occurred only in the MyoD-positive cells. Towards the end of the incubation time, eventually all the MyoD-positive cells became myogenin positive and the MyoD-negative cells did not express myogenin. Fig. 7D shows the detailed kinetics of myogenin expression and the appearance of the cells irreversibly committed to terminal differentiation, defined by their continuous expression of myogenin even after the shift back into the growth condition. The accumulation of committed

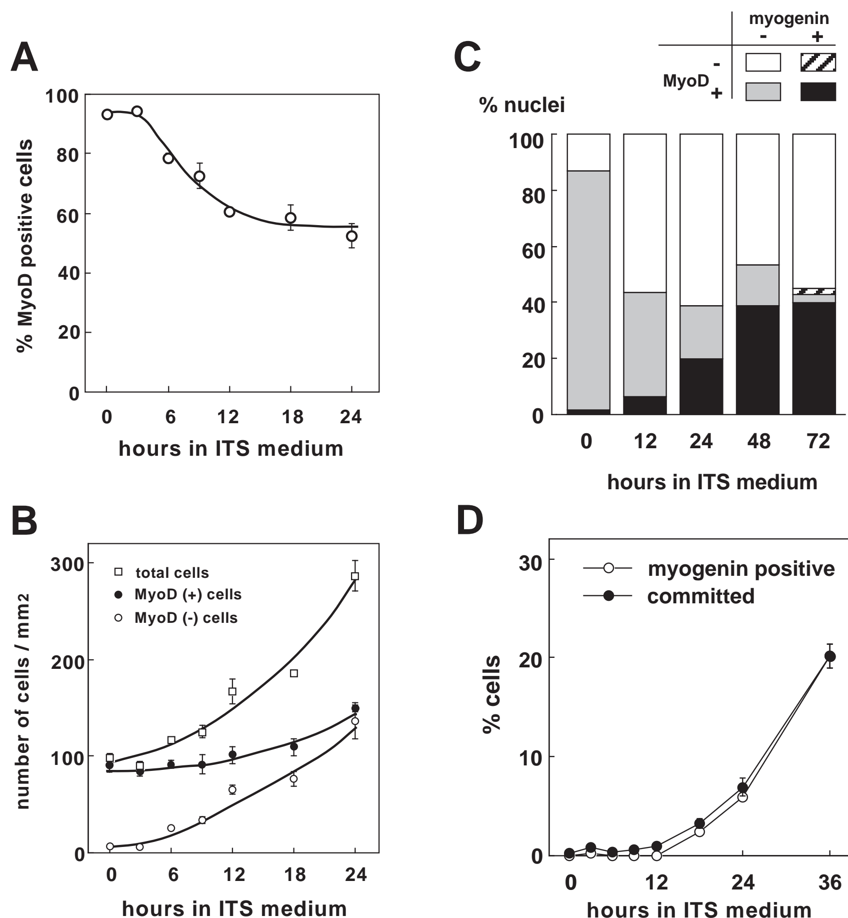

Fig. 7. (A,B) Detailed time course of MyoD expression in differentiating C2C12 culture. Cells were induced to differentiate in ITS medium, fixed after various incubation periods and stained for MyoD. Three randomly selected fields of 1 mm^2 each were counted for MyoD-positive and -negative nuclei. Percentage of the MyoD-positive cells (A), and the actual numbers of total cells, MyoD-positive or -negative cells (B) are shown. Bars indicate s.e.m. (C) Expression of MyoD and myogenin. Cells induced to differentiate in ITS medium were fixed at different time points, subjected to double immunofluorescence staining with anti-MyoD pAb and anti-myogenin mAb. Nuclei in three randomly selected fields were counted for their expression. Percentages of MyoD- and/or myogenin-positive nuclei were counted against the number of total nuclei at each time point. (D) Kinetics of myogenin expression and irreversible commitment to terminal differentiation. Duplicate C2C12 cultures were induced to differentiate in ITS. After various incubation times in ITS, one culture was fixed and tested for myogenin expression. The other was switched back to growth medium for a total of 36 hours after the change to ITS, and stained for myogenin to detect the irreversibly committed cells at that point. Both myogenin-positive and committed cells are presented as percentages in total cells ± s.e.m. (bars), from three random fields of 1 mm^2 each.

cells coincided with that of the myogenin-expressing cells, indicating that each cell goes beyond the point-of-no-return at the time when they first express myogenin. Myogenin mRNA, which might accumulate earlier than protein, showed indistinguishable kinetics from that for the protein within the limit of time resolution (data not shown).

These results together illustrate the chronologically ordered events generating both differentiated cells and reserve cells. Heterogenization of MyoD occurred first as a result of its down-regulation in one cohort of cells, which lead to reserve cells. After that, up-regulation of myogenin, which marks irreversible commitment, progressed in the other cohort of cells that continued to express MyoD and differentiate.

Early cell heterogenization is a reversible event

Because MyoD expression diverges before the appearance of irreversibly committed cells, we postulated that this early heterogenization may be a reversible event. To test this idea, C2C12 cells that had been cultured in ITS medium for 10 hours and then returned to the growth medium were tested for MyoD expression (Fig. 8). In the initial growth condition, nearly all the cells were positive for MyoD and after 10 hours of incubation in ITS medium approximately half of the cells lost MyoD staining. When these cells were returned to the growth medium, most of them regained the MyoD staining. Throughout this experiment, myogenin expression was restricted to only a small population of cells. Therefore, the heterogenization which occurs early in the differentiation and is characterized by MyoD expression is a reversible event.

Down-regulation of MyoD is a causal event of the generation of reserve cells

Does the MyoD down-regulation play a causal role in the generation of reserve cells? To answer this question, we tried to reverse the MyoD down-regulation by two sets of ectopic expression experiments. Nearly 100% of the cells transiently transfected with a MyoD expression vector showed the differentiated phenotype indicated by positive troponin T staining after incubation in ITS medium, while about a half of the mock-transfected cells remained undifferentiated, as observed in the original population (Fig. 9). Because transient transfection produces a much larger amount of MyoD protein than does the native state, we also examined a stable C2C12 transformant, C2*lac*MyoD, that conditionally expresses wild-type mouse MyoD at a comparable level with endogeneous MyoD only in the presence of IPTG (Fig. 10D). Parental C2C12 cells and a mock transformant showed essentially the same kinetics of MyoD and myogenin expression after differentiation-induction. Addition of IPTG did not affect these features (Fig. 10A,B). Without IPTG, C2*lac*MyoD cells showed the identical expression patterns of MyoD and myogenin. When IPTG was added, a significantly higher percentage of cells became positive for MyoD protein in their nuclei, as expected and, as a result, a significantly larger number of cells became myogenin positive (Fig. 10C).

These results imply that those cells which were originally assigned to be reserve cells altered their fate and became differentiated as a consequence of the forced expression of MyoD protein, indicating that the early down-regulation of

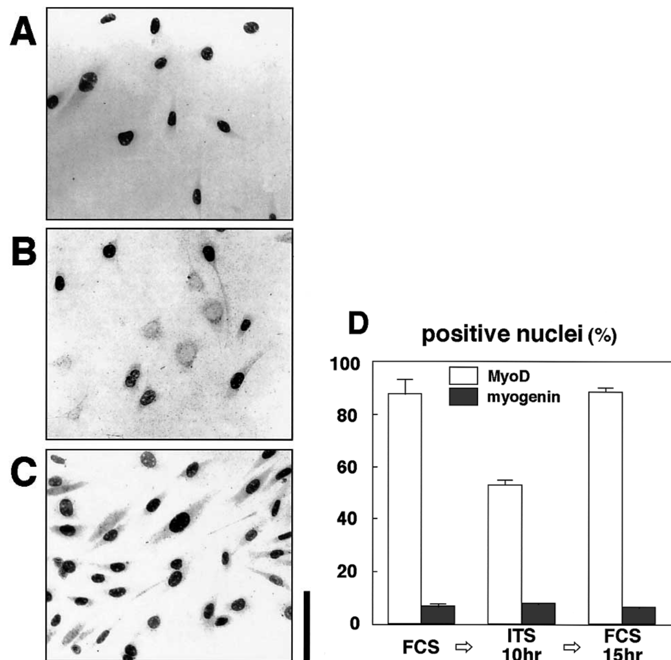

Fig. 8. The change in MyoD expression is reversible. C2C12 cells were induced to differentiation in ITS medium for 10 hours and then returned to the growth medium for another 15 hours. At each point, cells were fixed and stained for MyoD (mAb) by immunocytochemistry. MyoD stainings in growth medium (A), after 10 hours of culture in ITS medium (B), after another 15 hours in growth medium(C) are shown. Bar, 100 μm and representative fields are shown. (D) Quantification of the MyoD and myogenin-positive nuclei shown in A-C. Three randomly selected fields of 1 mm^2 each were counted to calculate the average percentage ± s.e.m.

MyoD protein is a causal event in the generation of reserve cells.

DISCUSSION

C2C12 myoblasts generate differentiated cells and reserve cells upon serum deprivation

As shown in this report, culture of C2C12 cells in differentiation-inducing conditions resulted in the formation of both differentiated cells and reserve cells, which were undifferentiated but retained their myogenic potential. Only the differentiated cells undertook a series of events associated with terminal differentiation, including myogenin expression, $p21^{WAF1}$ expression, cell cycle withdrawal, expression of contractile proteins and cell fusion (Miller, 1990; Halevy et al., 1995; Parker et al., 1995; Guo et al., 1995; Andres and Walsh, 1996). In contrast, the reserve cells did not initiate these events but remained undifferentiated and poorly proliferative. Interestingly, MyoD and Myf-5, two determination-class MRFs expressed in the proliferating myoblasts, were also down-regulated in these cells. Although observations that only a fraction of myoblast cells differentiates in differentiation-inducing conditions is reported in myoblast cell lines including C2C12 (Miller, 1990), detailed analyses of these undifferentiated populations with an emphasis on the expression of MRFs have not been done.

The cellular divergence in C2C12 myoblasts is not likely a consequence of the heterogeneity in the original cell population, because each one of the newly isolated subclones of the original cells resulted in a heterogeneous cell population when induced to differentiate (data not shown). Moreover, when a mixed population and a clonal growth of the isolated reserve cells were induced to differentiation again, both differentiating and non-differentiating cells appeared at similar ratios (Fig. 5). Therefore, the generation of two different subpopulations upon serum deprivation is caused by some intrinsic nature of the C2C12 cells.

That clonal, genetically homogeneous cells can produce two populations upon terminal differentiation is, we believe, an important feature. One population represents the differentiated phenotype irreversibly exited from the self-renewing cycles; the other is undifferentiated and retains the self-renewing and differentiation potentials that are characteristic of stem cells. It

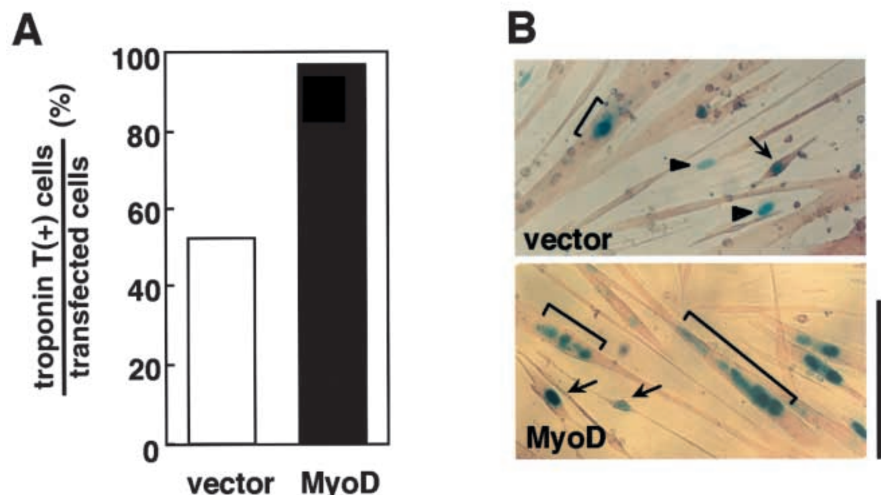

Fig. 9. Effect of transiently transfected MyoD on cell fate. Proliferating C2C12 cells were transfected with a MyoD-expression vector (pcDNA3/MMyoD) or the empty vector (pcDNA3) and incubated for 3 days in ITS medium. The transfected cells were stained for the β-galactosidase activity derived from the co-transfected plasmid (blue nuclear staining) and troponin T (brown cytoplasmic staining). (A) The number of troponin T-positive cells in randomly selected one hundred X-gal-positive (blue) cells were counted. In the case of syncytial myotubes, the number of transfected nuclei was counted assuming that a cluster of blue nuclei (brackets in B) contains only one transfected nucleus and β-galactosidase was transported into the neighboring untransfected nuclei. (B) Actual staining images. Bar, 100 μm. vector: pcDNA3-transfected cells; MyoD: pcDNA3/MMyoD-transfected cells.

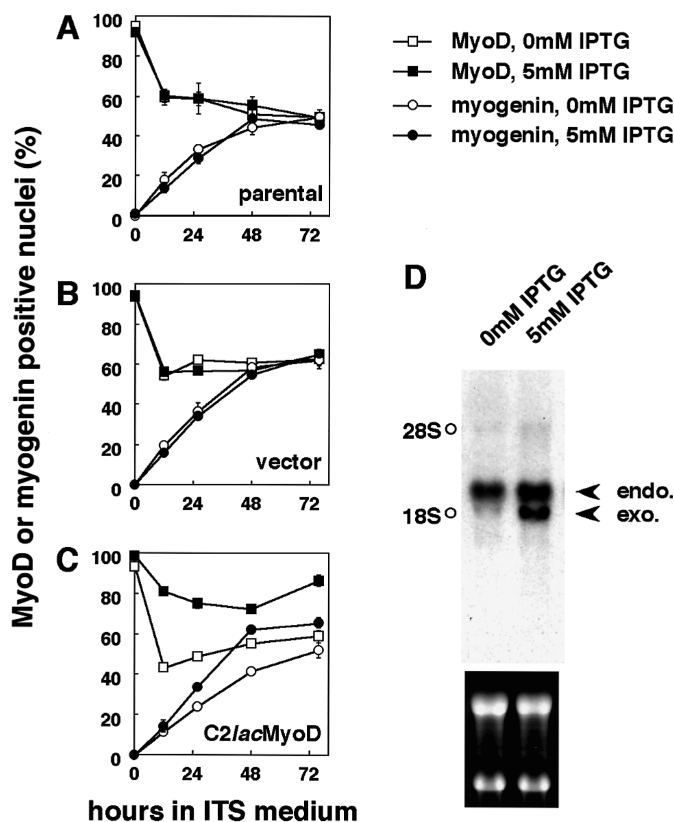

Fig. 10. Kinetics of MyoD and myogenin expression in C2C12 cells expressing ectopic MyoD. Parental C2C12 cells (A), a mock transformant with a vector (B), and C2lacMyoD (C) were seeded in growth medium as described and 5 mM of IPTG was added 6 hours later. After another 18 hours, cultures were changed to ITS medium containing IPTG. No IPTG was added to the control culture throughout the experiment. At different time points after switching to ITS, cells were examined for MyoD and myogenin expression. Three randomly selected fields of 1 mm^2 each were counted to calculate the percentage of MyoD or myogenin-positive nuclei. Bars indicate s.e.m. (D) Conditional expression of exogenous MyoD mRNA in C2lacMyoD cells. Total RNAs were isolated from C2lacMyoD cells cultured for 48 hours in ITS with or without IPTG and analyzed for MyoD mRNA by northern blot hybridization. endo., endogenous MyoD mRNA; exo., exogenous MyoD mRNA. Positions of 28S and 18S ribosomal RNAs are indicated. An ethidium bromide stained gel is shown in the bottom panel.

is still unknown whether all the cells each possess the identical features of a stem cell, or whether only a subset of them can act as stem cells and reconstitute the initial myoblast population.

Down-regulation of MyoD and Myf-5 specifies the cells to be reserve cells

Upon serum deprivation, C2C12 myoblasts, which all express MyoD, rapidly diverge into MyoD-positive and -negative populations. After that, the MyoD-positive cells become positive for myogenin and irreversibly committed to terminal differentiation while the other, MyoD-negative, cells remain undifferentiated resulting in the reserve cells. Adding to a chronological relationship, the difference of MyoD expression plays a causal role in specifying whether a cell is to differentiate or to be reserve; ectopic expression of MyoD in cells which should be negative for MyoD altered their fate to become differentiated cells.

Altogether, these findings reveal a unique aspect of terminal differentiation; when a myoblast culture is deprived of serum, down-regulation of MyoD occurred first, resulting in heterogeneity in their expression. The continuous MyoD (and possibly Myf-5) expression is a causal event for irreversible commitment and terminal differentiation. In contrast, down-regulation of MyoD is required for generation of the reserve cells. It is quite intriguing but remains to be revealed what mechanism is involved in this down-regulation of MyoD and Myf-5 upon deprivation of serum.

Similar events possibly govern the terminal differentiation of other cell types where proteins carrying the bHLH motif play important roles, such as in erythroid and neural differentiation. MEL cells and PC12 cells generate both differentiating and non-differentiating cells when induced to differentiate. In MEL cells, the non-differentiating cells were demonstrated to retain their differentiation potential as do the reserve cells in the myogenic system (Friend et al., 1971). SCL and MASH (mammalian achaete-scute homologue) are both transcription factors carrying bHLH motifs and are known to play a role in terminal differentiation of MEL and PC12 cells, respectively (Aplan et al., 1992; Johnson et al., 1990). Although their expression at the single cell level is not known, heterogeneities in the expression of these or other bHLH factors might cause the divergence of the cellular phenotypes in these cells upon terminal differentiation.

The reserve cells retain their myogenic potential in spite of their negative MyoD expression

MyoD and/or Myf-5 is implied to play a role in determination and/or maintenance of myogenic potential (Braun et al., 1992; Rudnicki et al., 1992, 1993; Weintraub, 1993). The C2C12 reserve cells maintain their myogenic potential, despite their undetectable amounts of MyoD protein and low or undetectable levels of Myf-5. Our experiments did not eliminate the possibility that a minute amount of MyoD and/or Myf-5 that could not be detected with the current experimental conditions might contribute to the maintenance of the myogenic potential. The other possibility is that reserve cells maintain their myogenic potentials independently of MyoD and Myf-5.

Similar situations have been reported. The expression of MyoD is extinguished in the BrdU-treated undifferentiated myoblasts (Tapscott et al., 1989) and none of the MyoD family members are detected in quiescent satellite cells (Smith et al., 1994; Yablonka-Reuveni and Rivera, 1994), both of which retain their myogenic potentials. In somite myogenesis, differentiated muscle cells that do not express detectable levels of MyoD family proteins have been reported to exist (Cusella-De Angelis et al., 1992; Smith et al., 1993). These observations and ours suggest the possibility that expression of the MyoD family might not always be required to maintain the myogenic potential.

Similarities between the reserve cells and the satellite cells

A number of properties of reserve cells described in this report are similar to those of the muscle stem cells, the satellite cells.

Satellite cells are predominantly dormant, mononucleated myogenic cells located between the extracellular matrix and the plasma membrane (Mauro, 1961). When activated, they proliferate and differentiate to participate in the myofibers of the growing neonatal/infantile muscle or regenerating muscle after injury (Moss and Leblond, 1971; Bischoff, 1975; Campion, 1984). Likewise, reserve cells are also mononucleated, predominantly quiescent, and can be stimulated by serum to proliferate while retaining a high differentiation potential.

In the quiescent state, satellite cells are negative not only for differentiation markers such as α-actin and myosin heavy chains, but also for all the MRFs (Smith et al., 1994; Yablonka-Reuveni and Rivera, 1994; Rantanen et al., 1995). In the activated state, they proliferate with high levels of MyoD protein in their nuclei (Fuchtbauer and Westphal, 1992; Koishi et al., 1995). In a later stage of regeneration, when satellite cells begin to form myotubes, myogenin staining appears (Smith et al., 1994; Yablonka-Reuveni and Rivera, 1994; Rantanen et al., 1995). This expression pattern of MRFs can be mimicked by reserve cells in vitro. MyoD-negative and poorly proliferative reserve cells became MyoD positive and myogenin negative when stimulated to proliferate in a high-serum condition. When these cells were deprived of serum, some of them began to express myogenin and differentiate (Fig. 5).

Since C2 cells are probably derived from satellite cells because they were originated from the cells in injured leg muscle of adult mice (Yaffe and Saxel, 1977), it is possible that C2C12 cells retain some of the characteristics of satellite cells. Recently, an in vitro experiment has shown that progenies of a single satellite cell generate both differentiated myotubes and quiescent cells that are undifferentiated but still myogenic, in differentiation-inducing conditions (Baroffio et al., 1996). Another in vivo study demonstrated that satellite cells contribute not only to the myotubes but also to the satellite cell pool (Schultz, 1996). These observations are similar to those for C2C12 cells shown in this paper. The expression of MRFs might change in a similar fashion in satellite cells to that observed in C2C12 cells, and might contribute to their diversification.

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