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Temporal differences in desmin expression between myoblasts from embryonic and adult chicken skeletal muscle

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Abstract

Desmin expression by myoblasts cultured from embryonic and adult chicken breast muscle was examined employing indirect immunofluorescence. The study was performed in conjunction with [³H]thymidine autoradiography and analysis of skeletal myosin expression in order to determine whether the desmin-expressing cells were terminally differentiated. Following 2 h of labeling with [³H]thymidine, 0.55%, 2.60%, and 15.10% of the cells in mass cultures from 10-day-old embryos, 18-day-old embryos and adults, respectively, incorporated [³H]thymidine and were desmin-positive but did not express skeletal-muscle-specific myosin. Using the same approach we determined that 0.07%, 1.25%, and 7.59% of the mononucleated cells in myogenic clones from 10-day-old embryos, 18-day-old embryos and adults, respectively, were desmin-positive, myosin-negative, [³H]thymidine-positive. We suggest that these desmin-positive, myosin-negative myoblasts are proliferating cells, and we conclude that the progeny of adult myoblasts exhibit more desmin-expressing cells of this type than embryonic myoblasts do.

Introduction

Desmin is the major subunit of muscle-type intermediate filaments and is expressed by skeletal, cardiac and most types of smooth muscle cells in both embryonic and adult tissues [2, 19, 21, 28]. In the skeletal-muscle lineage, desmin expression has been thought to be specific to postmitotic myoblasts and myotubes. This conclusion was based on studies of primary myogenic cultures employing immunofluorescence [2, 3, 16], protein analysis by gel electrophoresis [16], or mRNA analysis [5]. Other cell culture studies have demonstrated that during chicken myogenesis the expression of desmin precedes that of other muscle-specific proteins [11], and it has been suggested that replicating myoblasts can express desmin. A small number of cells that reacted with an antibody against desmin but not with antibodies against other skeletal-muscle-specific proteins was observed in myogenic cultures from embryonic chicken [11, 18]. At least some of these desmin-positive cells were replicating and not postmitotic [11]. We have demonstrated that desmin-positive, myosin-negative cells which also incorporate [³H]thymidine (³H-TdR; presumably cycling cells) can be detected not only in chick myogenic mass cultures but also in myogenic clones [35]. Although the frequency of such cells is low, clonal studies have indicated that these desmin-

positive cells are not merely nonmyogenic cells from the muscle cocultured with the myogenic cells but true descendants of myogenic precursor cells. More recent studies on rodent species have demonstrated that desmin expression precedes the expression of other muscle-specific proteins during myogenesis in vivo [15] and suggested that replicating myoblasts can express desmin [13, 20, 25].

The purpose of the present study was to measure the frequency of the desmin-positive, myosin-negative myoblasts in myogenic cultures from chicken skeletal muscle at different developmental stages and to establish whether these desmin-positive, myosin-negative myoblasts were replicating. Analyses were carried out on myoblasts from 10- and 18-day-old embryos and on myoblasts from adult skeletal muscle (satellite cells). We conclude that desmin-positive, myosin-negative myoblasts appear in cultures from all three developmental stages. However, the proportion of these cells and of desmin-positive, myosin-negative cells which also incorporate ^3H -TdR is much lower in embryonic muscle cultures and becomes more frequent in adult muscle cultures.

Methods

Source of cells

The breast muscles of White Leghorn chickens were used throughout the study. Embryos were either 10 or 18 days of age and adults were 10–12 weeks of age.

Cell isolation

Myogenic cultures from adult and 18-day-old embryonic chickens were established as previously described [35, 37]. Briefly, the tissue was subjected to a 45-min digestion with 0.2% collagenase (Sigma, St. Louis, Mo; type 1A, 482 unit/mg) followed by a 45-min digestion with 0.1% trypsin (Gibco, Grand Island, NY). Cultures from 10-day-old embryos were prepared using the same procedure but omitting the collagenase digestion [36, 38]. In some experiments the collagenase treatment was included for the 10-day-old embryos to verify that the results were independent of the use of collagenase. Following the enzymatic treatment, cell suspensions were subjected to Percoll density centrifugation [36, 37, 38]. This centrifugation step reduces the number of fibroblast-like cells and eliminates myofibril debris from older-stage preparations. Percoll was obtained from Pharmacia, Piscataway, NJ, USA.

Cell culture

Cells were plated into gelatin-coated, tissue culture dishes which were further preincubated for 2–3 h with 25% horse serum in Eagle's minimal medium (MEM) to promote cellular adherence [37]. The medium used consisted of 85 parts MEM, 10 parts horse serum, 5 parts chicken embryo extract, and antibiotics [37]. Cultures were maintained at 37.5° C in a water-saturated atmosphere containing 5% CO₂ in air. Mass cultures were plated at 2×10^5 cells per 35-mm dish. Clonal cultures were initiated with 100 cells per 60-mm dish [36, 37]. All cultures received fresh medium 20 h after initial plating and every other day thereafter.

Antibody staining

Cultured cells were fixed with 70% ethanol/formalin/acetic acid (20:2:1) and immunolabeled by indirect immunofluorescence as previously described [36]. Unless otherwise noted, the IgG fraction of a rabbit antiserum against chicken gizzard desmin and a guinea-pig antiserum against chicken muscle myosin heavy chain were used for immunohistochemical localization of desmin and myosin, respectively. The rabbit desmin antiserum [2, 12] was kindly provided by Dr. H. Holtzer (University of Pennsylvania, Philadelphia). The IgG fraction of this desmin antiserum was further purified in our laboratory using protein A-Sepharose and was shown to react only with the desmin band by polyacrylamide gel electrophoresis and immunoblot analysis [35]. This anti-desmin IgG fraction was used for immunofluorescence at 0.1 mg/ml as previously reported [35, 36]. The guinea-pig antiserum against chicken muscle myosin heavy chain was previously described [29,31] and was used at 1:60 dilution. Secondary antibodies were obtained from Cappel (Durham, NC) and were used at 1:60 dilution. Fluorescein-labeled goat anti-rabbit IgG was used to visualize the reaction of anti-desmin, and rhodamine-labeled goat anti-guinea pig IgG was used to visualize the reaction of anti-myosin. Cultures were exposed to a mixture of the anti-desmin and anti-myosin at room temperature for 1 h followed by a 1-h reaction with a mixture of the fluorescent secondary antibodies [31, 32, 35]. Negative control cultures were reacted with secondary antibodies only; with desmin antibody followed by anti-guinea-pig IgG; and myosin antibody followed by anti-rabbit IgG.

In some experiments, as indicated in results, we studied the expression of desmin and myosin using monoclonal antibodies. Monoclonal antibodies against chicken desmin were those originally prepared by Danto and Fischman (D3 and D76 [10]). Monoclonal antibody against all isoforms of chicken sarcomeric myosin heavy chain was that originally prepared by Bader et al. (MF20 [1, 40]). The hybridoma supernatants containing these three monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank which is maintained by a contract from NICHD (N01-HD-6-2915) and were used without any dilution (D3, D76) or at a 1:5 dilution (MF20). Fluorescein-labeled rabbit anti-mouse IgG (Cappel) was used at 1:50 dilution to visualize cells which positively reacted with the monoclonal antibodies. The time of appearance and the frequency of myosin-positive cells determined with the guinea-pig myosin antiserum were similar to those determined with the mouse monoclonal antibody MF20, indicating that all cells which expressed detectable amounts of myosin could be identified by the guinea-pig myosin antibody.

Autoradiography and photography

Cultures were exposed for 2 h to [³H]thymidine at a final concentration of 1 μ Ci/ml (³H-TdR; New England Nuclear, Boston, Ma; specific activity 6.7 Ci/mmol). The cells were fixed, reacted with the desired antibody, coated with NTB2 emulsion which was diluted 1:1 with water, stored in the dark for a week at 4° C, and developed with Microdol-X. Observations were made with a Zeiss microscope equipped for epifluorescence, and cultures were photographed with Kodak Ektachrome film (EL 135, 400 ASA, for slides). Both phase and fluorescence micrographs were taken with color film, which resulted in brown silver grains rather than black. For photography, the cells, rather than the grains, were in focus. This resulted in the final micrographs illustrated.

Results

Desmin expression in mass culture

The expression of desmin by single cells was studied in primary myogenic cultures from the breast muscle of 10- and 18-day embryos and from adult chickens (10–12 weeks old) using the rabbit desmin antibody. As shown in Table 1, desmin-positive cells could be detected in 42-h-old cultures from all developmental stages studied, but their frequency increased with the age of the animal. Two common markers of terminal differentiation of myoblasts are withdrawal from the cell cycle and the expression of sarcomeric myosin [26, 33]. In order to determine whether the desmin-positive cells were terminally differentiated by these criteria, 40-h-old cultures were pulse-labeled with ^3H -TdR for 2 h and immediately fixed and processed for immunofluorescent localization of both desmin (using the rabbit antibody) and skeletal myosin heavy chain (using the guinea-pig antiserum). Desmin-positive, myosin-negative cells (desmin⁺, myosin⁻) which also incorporated ^3H -TdR (^3H -TdR⁺) were detected at all developmental ages (Fig. 1, Table 1). The incorporation of ^3H -TdR and the absence of myosin expression indicate that the cells synthesized DNA but not myosin and therefore were not terminally differentiated. The proportion of total cells which were desmin⁺, myosin⁻, ^3H -TdR⁺ was 0.55% in cultures from 10-day-old embryos, 2.60% in cultures from 18-day-old embryos and 15.10% in cultures from adult chickens. This reflects an increase in the numbers of these desmin⁺ cells in cultures from older animals, since the proportion of *total* ^3H -TdR⁺ cells was similar for the cultures from animals of different ages (Table 1). In adult cultures desmin⁺, ^3H -TdR⁺ cells were present both singly and in small groups of closely associated cells (Fig. 1d, e). We previously reported the existence of these groups, and could distinguish terminally differentiated cells from dividing cells among such closely opposed cells only with the aid of histochemical markers [37]. These grouped cells could be differentiated if they accumulated some ^3H -TdR, withdrew from the cell cycle, and terminally differentiated during the 2-h pulse with ^3H -TdR. However, at the end of the 2-h period these cells did not express detectable amounts of skeletal myosin. Furthermore, reacting either the guinea-pig myosin antiserum or the mouse myosin monoclonal antibody (MF20) with adult cultures prepared as described under Methods, we have shown that the first myosin⁺ cells appear in such cultures only on the third day [17], whereas the desmin⁺, ^3H -TdR⁺, closely associated cells were observed in 42-h cultures. These data suggest that the closely associated, desmin⁺, myosin⁻, ^3H -TdR⁺ cells in satellite cell cultures were not terminally differentiated. The possibility that the closely associated cells were in the process of recognition or alignment, shown to precede fusion of myoblasts in embryonic myogenic-cultures [24], is also unlikely, because of the 30-h gap between the time these cells were first seen in adult cultures and the time that myosin⁺ cells or small myotubes were first observed. (Further discussion regarding the proliferative versus postmitotic nature of the cells is included under Discussion).

Desmin⁺, myosin⁺ cells which did not incorporate ^3H -TdR (presumably terminally differentiated myoblasts) were also detected in all cultures (Fig. 1a–c). In addition, desmin⁺, myosin⁻, ^3H -TdR⁻ cells were observed (Table 1). These cells were presumably cycling cells not in S phase when exposed to ^3H -TdR and/or cells which withdrew from the cell cycle prior to the addition of ^3H -TdR but were not yet expressing myosin. ^3H -TdR⁺ cells positive

for both desmin and myosin were very rare at all developmental stages; only five such cells were observed throughout this study.

Terminal myogenic differentiation, as determined by myosin expression, occurs earlier in cultures from 10-day embryos than in cultures from 18-day embryos or adults [17, 30]. These data suggest that the frequency of desmin⁺, ³H-TdR⁺ cells in cultures from 10-day-old embryos may have peaked before fixation at 42 h and not have been detected by our experimental design. Therefore, we measured the frequency of the desmin⁺, ³H-TdR⁺ cells in 20- and 30-h cultures from 10-day-old embryos. No higher frequency of desmin⁺, ³H-TdR⁺ cells was detected in such cultures (data not shown).

The rabbit desmin antibody reacted only with the desmin band on immunoblots [35]. Nevertheless, desmin expression was also determined using monoclonal antibodies in order to further verify that the increased frequency of desmin⁺ cells in cultured satellite cells was not a reflection of an additional antigen which is reactive with the anti-desmin rabbit IgG fraction. When 42-h cultures from adult skeletal muscle were stained with the desmin monoclonal antibody D3, the frequency of the desmin⁺ cells was similar to that observed with the rabbit desmin antibody. Desmin⁺ cells were also detected in 42-h adult cultures reacted with the desmin monoclonal antibody D76. However, the intensity of the fluorescence observed using this antibody was much lower than that obtained with D3 or the rabbit antibody. Parallel 42-h adult cultures reacted with MF20 did not demonstrate myosin⁺ cells (data not shown).

Expression of desmin in myogenic clones

The above experiments show that desmin⁺, ³H-TdR⁺ cells appear in myogenic cultures. Despite their bipolar appearance, some of the desmin⁺, ³H-TdR⁺ cells may not have been related to the skeletal myogenic lineage. To investigate the identity of the desmin⁺, ³H-TdR⁺ cells, primary clones were prepared from the breast muscle of 10- and 18-day-old embryos as well as from adults. After 7–9 days in culture the clones were pulsed for 2 h with ³H-TdR and immediately processed for antibody staining and autoradiography as described for mass cultures using the rabbit desmin antibody and the guinea-pig myosin antibody. Clones from all developmental stages were examined for the appearance of myotubes, expression of myosin and desmin, and incorporation of ³H-TdR (Table 2). A total of 70–90 clones in three independent experiments were examined from each developmental stage. The majority of the clones consisted of several hundred cells each, including cells fused into myotubes. Based on the appearance of myotubes and expression of skeletal muscle myosin we concluded that clonal cultures from 10-day-old embryos, 18-day-old embryos and adults consisted of 70%, 60%, and 99% myogenic clones, respectively. The myogenic clones contained many postmitotic, terminally differentiated cells which were desmin⁺, myosin⁺. The proportion of clones containing desmin⁺, myosin⁺, ³H-TdR⁺ cells varied between the different developmental stages and amounted to 5%, 50% and over 90% of clones from 10-day embryos, 18-day embryos and adults, respectively. The number of desmin⁺, myosin⁺, ³H-TdR⁺ cells in clones containing such cells was estimated as 1–2 cells/clone in clones from 10-day-old embryos, 1–4 cells/clone in clones from 18-day-old embryos, and 10–30 cells/clone in clones from the adult muscle. Representative areas from single

myogenic clones derived from 18-day embryos and adults are shown in Figs. 2 and 3, respectively.

Discussion

In this study, we detected cells which incorporated $^3\text{H-TdR}$ and expressed desmin, both in primary mass cultures and primary myogenic clones from embryonic and adult skeletal muscle. These $^3\text{H-TdR}^+$, desmin $^+$ cells did not express detectable amounts of skeletal-muscle-specific myosin heavy chain, a marker for terminally differentiated myoblasts. Only cells that were in S phase of the cell cycle during the 2-h exposure to $^3\text{H-TdR}^+$ contributed to the population of desmin $^+$, $^3\text{H-TdR}^+$ cells. Despite the use of Percoll density centrifugation for myoblast isolation, fibroblast-like cells were present, although in reduced numbers, in the cultures [36, 38]. These so-called fibroblasts were included in total cell counts, since no histochemical markers are currently available to distinguish these cells from myogenic cells. Hence, the data presented are minimal estimates of the desmin $^+$, $^3\text{H-TdR}^+$ cells at the different developmental stages. The frequency of desmin $^+$, $^3\text{H-TdR}^+$ cells was reduced in clones compared to mass cultures, but the appearance of such cells in the myogenic clones indicated that these cells were progeny of myogenic precursor cells. The frequency of desmin $^+$, $^3\text{H-TdR}^+$ cells increased with the age of the animal used for both mass cultures and clonal cultures. This result suggests that myoblasts from adult muscle and embryonic myoblasts are different at least with respect to the frequency of desmin $^+$, $^3\text{H-TdR}^+$ cells. Such cells could emerge during later stages of embryogenesis and cause the increase in desmin $^+$, $^3\text{H-TdR}^+$ cells in cultures from 18-day embryos. Previous studies by ourselves [17, 37, 39] and other investigators ([6, 8, 9], and reviewed in [7]) have characterized other differences between adult and embryonic myoblasts.

The desmin $^+$, $^3\text{H-TdR}^+$ cells are presumably proliferating cells, since they synthesized DNA during the 2-h pulse with $^3\text{H-TdR}$. Cell cycle studies on cultured chicken myoblasts from 10- to 12-day embryos indicated that the S, G2 and M phases of the cell cycle last for about 4–4.5 h, 2.5 h and 0.8 h, respectively [4, 22]. Withdrawal from the cell cycle, which precedes terminal differentiation of myoblasts, occurs during the G1 phase of the cell cycle [4]. If the above cell cycle parameters are valid for all developmental stages studied, then the desmin $^+$, $^3\text{H-TdR}^+$ cells could not withdraw from the cell cycle during the 2-h pulse period, and the cells had not yet differentiated. This would imply that proliferating myoblasts can express desmin and the frequency of these cells increases with the age of the animal. The findings that the number of desmin $^+$, cycling cells in myogenic cultures from embryonic chicken increases after addition of a tumor promoter [11], and that the substrate on which the cells are cultured can enhance the expression of desmin by proliferating cells in both rat and chick myogenic cultures [13, 23, 25], support the notion that the desmin $^+$, $^3\text{H-TdR}^+$ cells observed in the current study were indeed proliferating. Other studies on 40-h myogenic cultures from 11- to 12-day chicken embryos have, however, demonstrated, radiolabeled nuclei in myotubes following a 3-h pulse with $^3\text{H-TdR}$ [27]. Therefore, for all developmental stages studied, we cannot exclude the possibility that the desmin $^+$, myosin $^-$, $^3\text{H-TdR}^+$ cells are actually cells that synthesized DNA during the 2-h pulse and withdrew from the cell cycle during this period. This implies that the expression of desmin in chicken myoblasts occurs after withdrawal from the cell cycle but before the expression

of skeletal myosin. Furthermore, the higher frequency of $^3\text{H-TdR}^+$, desmin $^+$, myosin $^-$ cells in adult cultures could indicate that satellite cells initiate desmin synthesis earlier than embryonic myoblasts following cell cycle withdrawal. We still favor the notion that the desmin $^+$, $^3\text{H-TdR}^+$ cells were cycling rather than cells that had withdrawn from the cell cycle, because there was at least a 30-h lag between the time we detected the desmin $^+$, $^3\text{H-TdR}^+$ cells and the time that the first myosin $^+$ cells were detected in cultures from 18-day-old embryos and adults. Therefore, if the desmin $^+$, $^3\text{H-TdR}^+$ cells were cells which had withdrawn from the cell cycle, these cells terminally differentiated, according to anti-myosin staining, only 30 h later.

The frequency of desmin $^+$, $^3\text{H-TdR}^+$ cells was reduced for myogenic clones compared to mass cultures. This raises the possibility that some of the proliferating desmin $^+$ cells detected in mass cultures may not be related to the myogenic lineage but may be smooth muscle cells and therefore account for the higher proportion of desmin $^+$, $^3\text{H-TdR}^+$ cells in mass cultures compared to clones. Using an antibody against smooth muscle actin, we were able to detect actin $^+$ cells only at a very low frequency in the cultures, and none of the desmin $^+$ cells expressed smooth muscle actin [35, 36] indicating that the contribution of smooth muscle cells was minimal. Finally, capillary endothelial cells, which express desmin in the adult chicken [14], may account for a portion of the desmin $^+$ cells identified in mass cultures. However, no endothelial cells could be identified in the cultures using chicken endothelial cell markers [34].

After this work was completed, a study describing the identification of desmin $^+$, proliferating cells in primary *mass* cultures from mouse and embryonic chicken was published [20]. That study identified desmin $^+$, cycling cells at high frequency in mouse cultures, but at very low frequency in the embryonic chicken (0.1%–0.45% of total cells). The differences between the frequency of desmin $^+$, $^3\text{H-TdR}^+$ myoblasts observed in the present study (0.55% and 2.60% for 10-day and 18-day-old embryos, respectively) and the desmin $^+$, replicating myoblasts observed in the other study [20] might derive from differences in preparing and culturing the cells (we routinely enrich for myoblasts by density centrifugation) and in the method of detecting proliferating cells (using bromodeoxyuridine, Kaufman and Foster labeled replicating cells for 90 min and visualized the cells immunohistochemically with an antibody against the label).

In summary, this study confirms that chicken myoblasts are capable of expressing desmin prior to the expression of skeletal myosin and suggests that these desmin $^+$, myosin $^-$ cells are dividing myoblasts. The study further demonstrates that these desmin-expressing cells are rare in earlier embryos, but increase in frequency in more-advanced embryos and are more abundant among progeny of satellite cells compared to the embryonic stages examined.

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References

1. Bader DT, Masaki T, Fischman DA. Biochemical analysis of myosin heavy chain during avian myogenesis in vivo and in vitro. *J Cell Biol.* 1982; 95:763–770. [PubMed: 6185504]
2. Bennett GS, Fellini SA, Holtzer H. Immunofluorescent visualization of 100-Å, filaments in different cultured chick embryo cell types. *Differentiation.* 1978; 12:71–82. [PubMed: 86476]
3. Bennett GS, Fellini SA, Toyama Y, Holtzer H. Redistribution of intermediate subunits during skeletal myogenesis and maturation in vitro. *J Cell Biol.* 1979; 82:577–584. [PubMed: 383729]
4. Bischoff R, Holtzer H. Mitosis and the processes of differentiation of myogenic cells in vitro. *J Cell Biol.* 1969; 41:188–200. [PubMed: 5775784]
5. Capetanaki YG, Ngai J, Lazarides E. Characterization and regulation in the expression of a gene coding for the intermediate filament protein desmin. *Proc Natl Acad Sci USA.* 1984; 81:6909–6913. [PubMed: 6594672]
6. Chevallier A, Pautou MP, Harris AJ, Kieny M. On the non-equivalence of skeletal muscle satellite cells and embryonic myoblasts. *Arch Anat Microscop.* 1986; 75:161–166.
7. Cossu G, Molinaro M. Cell heterogeneity in the myogenic lineage. *Curr Top Dev Biol.* 1987; 23:185–208. [PubMed: 3330504]
8. Cossu G, Molinaro M, Pacifici M. Differential response of satellite cells and embryonic myoblasts to a tumor promoter. *Dev Biol.* 1983; 98:520–524. [PubMed: 6873465]
9. Cossu G, Eusebi F, Grassi F, Wanke E. Acetylcholine receptor channels are present in undifferentiated satellite cells but not in embryonic myoblasts in culture. *Dev Biol.* 1987; 123:43–50. [PubMed: 2442052]
10. Danto SI, Fischman DA. Immunohistochemical analysis of intermediate filaments in embryonic heart cells with monoclonal antibodies to desmin. *J Cell Biol.* 1984; 98:2179–2191. [PubMed: 6373792]
11. Dlugosz AA, Tapscott SJ, Holtzer H. Effects of phorbol 12-myristate 13-acetate on the differentiation program of embryonic chick skeletal myoblasts. *Cancer Res.* 1983; 43:2780–2789. [PubMed: 6342759]
12. Fellini SA, Bennett GS, Toyama Y, Holtzer H. Biochemical and immunological heterogeneity of 100 Å filament subunits from different chick cell types. *Differentiation.* 1978; 12:59–69. [PubMed: 86475]
13. Foster RF, Thompson JM, Kaufman SJ. A laminin substrate promotes myogenesis in rat skeletal muscle cultures: Analysis of replication and development using antidesmin and anti-BrdUrd monoclonal antibodies. *Dev Biol.* 1987; 122:11–20. [PubMed: 3297850]
14. Fujimoto T, Singer SJ. Immunocytochemical studies of endothelial cells in vivo. I. The presence of desmin only, or of desmin plus vimentin, or vimentin only, in the endothelial cells of different capillaries of the adult chicken. *J Cell Biol.* 1986; 103:2775–2786. [PubMed: 3539944]
15. Fürst DO, Osborn M, Weber K. Myogenesis in the mouse embryo: Differential onset of expression of myogenic proteins and the involvement of titin in myofibril assembly. *J Cell Biol.* 1989; 109:517–527. [PubMed: 2474551]
16. Gard DL, Lazarides E. The synthesis and distribution of desmin and vimentin during myogenesis in vitro. *Cell.* 1980; 19:263–275. [PubMed: 7188890]
17. Hartley RS, Yablonka-Reuveni Z. Temporal differences in myosin heavy chain expression between descendants of satellite cells and embryonic myoblasts in vitro. *J Cell Biol.* 1989; 109:261a. (Abstr).
18. Hill CS, Duran S, Lin Z, Weber K, Holtzer H. Titin and myosin, but not desmin, are linked during myofibrillogenesis in postmitotic mononucleated myoblasts. *J Cell Biol.* 1986; 103:2185–2196. [PubMed: 3536962]
19. Holtzer H, Bennett GS, Tapscott JM, Croop JM, Toyama Y. Intermediate-size filaments: Changes in synthesis and distribution in cells of the myogenic and neurogenic lineages. *Cold Spring Harbor Symp Quant Biol.* 1982; XLVI:317–329. [PubMed: 6955084]
20. Kaufman SJ, Foster RF. Replicating myoblasts express a muscle-specific phenotype. *Proc Natl Acad Sci USA.* 1988; 85:9606–9610. [PubMed: 3200845]

21. Lazarides E. Intermediate filaments: A chemically heterogeneous, developmentally regulated class of proteins. *Annu Rev Biochem.* 1982; 51:219–250. [PubMed: 6180679]
22. Lough J, Bischoff R. Differential sensitivity of 5-bromodeoxyuridine during the S phase of synchronized myogenic cells. *Dev Biol.* 1976; 50:457–475. [PubMed: 1278597]
23. Menko AS, Boettiger D. Occupation of the extracellular matrix receptor, integrin, is a control point for myogenic differentiation. *Cell.* 1987; 51:51–57. [PubMed: 3115595]
24. Nameroff M, Munar E. Inhibition of cellular differentiation by phospholipase C. II. Separation of fusion and recognition among myogenic cells. *Dev Biol.* 1976; 49:288–293. [PubMed: 815117]
25. Öcalan M, Goodman SL, Kühl U, Hauschka SD, von der Mark K. Laminin alters cell shape and stimulates motility and proliferation of murine skeletal myoblasts. *Dev Biol.* 1988; 125:158–167. [PubMed: 3334715]
26. Okazaki K, Holtzer H. An analysis of myogenesis in vitro using fluorescein-labeled antimyosin. *J Histochem Cytochem.* 1965; 13:726–739. [PubMed: 5325593]
27. O'Neill MC, Stockdale FE. A kinetic analysis of myogenesis in vitro. *J Cell Biol.* 1972; 52:52–65. [PubMed: 5006948]
28. Osborn M, Caselitz J, Weber K. Heterogeneity of intermediate filament expression in vascular smooth muscle: A gradient in desmin positive cells from the rat aortic arch to the level of the arteria iliaca communis. *Differentiation.* 1981; 20:196–202. [PubMed: 7040147]
29. Quinn LS, Nameroff M. Analysis of the myogenic lineage in chick embryos. III. Quantitative evidence for discrete compartments of precursor cells. *Differentiation.* 1983; 24:111–123. [PubMed: 6350088]
30. Quinn, LS.; Rhodes, LD.; Nameroff, M. Characterization of sequential myogenic cell lineage compartments. In: Stockdale, FE.; Kedes, LH., editors. *Cellular and Molecular Biology of Muscle Development.* Alan R Liss; New York: 1989. p. 37-46.
31. Robinson MM. Fixation and immunofluorescent analysis of creatine kinase isozymes in embryonic skeletal muscle. *J Histochem Cytochem.* 1987; 35:717–722. [PubMed: 3035007]
32. Robinson MM, Quinn LS, Nameroff M. BB creatine kinase and myogenic differentiation. Immunocytochemical identification of a distinct precursor compartment in the chicken skeletal myogenic lineage. *Differentiation.* 1984; 26:112–120. [PubMed: 6734985]
33. Stockdale FE, Holtzer H. DNA synthesis and myogenesis. *Exp Cell Res.* 1961; 24:508–520. [PubMed: 13917284]
34. Yablonka-Reuveni Z. The emergence of the endothelial cell lineage in the chick embryo can be detected by uptake of acetylated low density lipoprotein and the presence of a von Willebrand-like factor. *Dev Biol.* 1989; 132:230–240. [PubMed: 2783918]
35. Yablonka-Reuveni, Z.; Nameroff, M. Immunocytochemical studies on the expression of desmin by dividing cells from skeletal muscle. In: Emerson, C.; Fischman, D.; Nadal-Ginard, B.; Siddiqui, MAQ., editors. *Molecular Biology of Muscle Development.* Alan R Liss; New York: 1986. p. 47-60.
36. Yablonka-Reuveni Z, Nameroff M. Skeletal muscle cell populations: Separation and partial characterization of fibroblast-like cells from embryonic tissue using density centrifugation. *Histochemistry.* 1987; 87:27–38. [PubMed: 3038797]
37. Yablonka-Reuveni Z, Quinn LS, Nameroff M. Isolation and clonal analysis of satellite cells from chicken pectoralis muscle. *Dev Biol.* 1987; 119:252–259. [PubMed: 3025033]
38. Yablonka-Reuveni Z, Anderson SK, Bowen-Pope DF, Nameroff M. Biochemical and morphological differences between fibroblasts and myoblasts from embryonic chicken skeletal muscle. *Cell Tissue Res.* 1988; 252:339–348. [PubMed: 2838171]
39. Yablonka-Reuveni, Z.; Bowen-Pope, DF.; Hartley, RS. Proliferation and differentiation of myoblasts: The role of platelet-derived growth factor and the basement membrane. In: Pette, D., editor. *The Dynamic State of Muscle Fiber.* Walter de Gruyter; Berlin: 1990. p. 693-706.
40. Zadeh BJ, González-Sánchez A, Fischman DA, Bader D. Myosin heavy chain expression in embryonic cardiac cell cultures. *Dev Biol.* 1986; 115:204–214. [PubMed: 2422070]

Fig. 1.

a–f. Phase and fluorescence micrographs of 42-h primary myogenic cultures in which the incorporation of [^3H]-thymidine (^3H -TdR) was determined by autoradiography and the coexpression of desmin and myosin was examined immunohistochemically. **a–c** Cultures from 10-day-old embryos. **d–f** Cultures from adults. Cells were pulsed with ^3H -TdR and then double-labeled with rabbit antibody against desmin and guinea-pig antibody against myosin heavy chain. Anti-desmin was indirectly labeled with fluorescein-conjugated goat anti-rabbit IgG and anti-myosin was indirectly labeled with rhodamine-conjugated goat anti-guinea-pig IgG. **b, e** Cultures labeled with anti-desmin. **c, f** Cultures labeled with anti-myosin. Arrows indicate ^3H -TdR-labeled cells which express desmin. Arrowheads indicate ^3H -TdR-positive cells which are negative for desmin. Bar, 30 μm ; note that the appearance of the silver grains as white/gray bodies in these and all other figures is a result of using color slide film for original photography (see comment under Methods)

Fig. 2.

a–d. ^3H -TdR incorporation and desmin expression by cells in representative areas of a single myogenic clone isolated from 18-day-old embryos. **a, c** Phase micrographs. **b, d** Corresponding fluorescence micrographs of cells reacted with the desmin antibody. Cells were pulsed with ^3H -TdR and then double-labeled with anti-desmin and anti-myosin heavy chain as described in Fig. 1. The results with the myosin antibody are not shown but are discussed in the text. *Arrows* indicate ^3H -TdR-positive cells which express desmin but not myosin. *Arrowheads* indicate ^3H -TdR-positive cells which are negative for desmin; *bar*, 30 μm

Fig. 3.
a–f. ^3H -TdR incorporation and desmin expression by cells in representative areas of a single myogenic clone isolated from adult muscle. **a, c, e** Phase micrographs. **b, d, f** Corresponding fluorescence micrographs of cells reacted with the desmin antibody. Cells were pulsed with ^3H -TdR and then double-labeled with anti-desmin and anti-myosin heavy chain as described in Fig. 1. The results with the myosin antibody are not shown but discussed in the text. *Arrows* indicate ^3H -TdR-positive cells which express desmin but not myosin. *Arrowheads* indicate ^3H -TdR-positive cells which are negative for desmin; *bar*, 30 μm

Table 1
Frequency of desmin-positive, myosin-negative, [³H]thymidine (³H-TdR)-positive cells in myogenic mass cultures

Source of muscle ^a	cells assayed (n)	H ⁺ cells ^b (%)	D ⁺ cells (n) (%)	D ⁺ M ⁺ H ⁺ cells (n) (%)	D ⁺ M [±] H ⁻ cells ^c (n) (%)
E10	4200	55	118 (2.80)	23 (0.54)	95 (2.26)
E18	1662	52	155 (9.32)	43 (2.58)	112 (6.74)
Adult	1000	58	374 (37.40)	151 (15.10)	223 (22.30)

^a E10, 10-day embryos; E18, 18-day embryos; *adult*, chickens 10–12 weeks of age

^b H⁺, ³H-TdR⁺; D⁺, desmin-positive; M⁺, myosin-positive; M[±], myosin-positive or negative; H⁻, ³H-TdR-negative

^c This population consisted of cells positive for desmin and myosin which were terminally differentiated cells, and cells positive for desmin and negative for myosin which were not in S phase during the 2-h pulse with ³H-TdR

Table 2

Frequency of desmin-positive, myosin-negative, ³H-TdR-positive cells in myogenic clones

Source of muscle ^a	Myogenic clones (%)	Myogenic clones containing D ⁺ M ⁻ H ⁺ cells ^b (%)	No. of cells assayed ^c	No. of D ⁺ M ⁻ H ⁺ cells per clone	total No. of D ⁺ M ⁻ H ⁺ cells	D ⁺ M ⁻ H ⁺ cells ^d (%)
E10	70	5	10189	1-2	7	0.07
E18	90	50	11245	1-4	141	1.25
Adult	98	90	11081	10-30	842	7.59

^a E10, 10-day embryos; E18, 18-day embryos; adult, 10- to 12-week chickens

^b D⁺, desmin-positive; M⁻, myosin-negative; H⁺, ³H-TdR-positive

^c Total no. of cells reflects only mononucleated cells in clones. About 70-90 clones from three independent experiments were assayed for the different developmental stages. The clone size varied in the range of 50-400 cells per clone, and about 40%-60% of the cells were fused into myotubes, as was determined on a separate group of 12 clones from each developmental stage

^d Calculated as percentage of total mononucleated cells