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IMMUNOCYTOCHEMICAL STUDIES ON THE EXPRESSION OF DESMIN BY DIVIDING CELLS FROM SKELETAL MUSCLE[1]

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ABSTRACT Using a combination of [^{3}H]thymidine autoradiography and immunofluorescent staining with a desmin antibody, we examined mitotically active cells in primary cultures established from the breast muscles of 18-day-old chick embryos. In mass cultures about 5% of the [^{3}H]thymidine labeled cells were desmin positive. Skeletal myosin was not detected in the vast majority of the mitotically active, desmin positive cells observed in these cultures. In addition, some, but not all of the desmin positive, cycling cells expressed the BB isozyme of creatine kinase. In tissue sections from the breast muscles of 18-day-old embryos, the desmin antibody fluorescently stained muscle fibers and smooth muscle cells in blood vessel walls. To determine whether the [^{3}H]thymidine labeled, desmin positive cells were in the skeletal myogenic or another lineage, similar studies were carried out on myogenic clones established from the breast muscles. Again, desmin positive, cycling cells were observed in many of the clones which contained myotubes. These desmin positive cells may therefore reside in the myogenic lineage. Alternatively, these results could indicate that common stem cells may give rise to skeletal myogenic cells and to other cell types.

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INTRODUCTION

Desmin is the major subunit protein of muscle type intermediate filaments. The expression of this subunit by skeletal, cardiac and most types of smooth muscle is well documented in both embryonic and adult tissues (see Ref. 1 for review). In the skeletal muscle lineage, desmin expression has been thought to be specific to postmitotic myoblasts and myotubes. This conclusion has been drawn from studies on primary myogenic cultures using immunofluorescence techniques (2,3) and radiolabeling of proteins (3). The dividing cells that were examined by the fluorescence approach appeared to lack desmin (2). Similarily, using the biosynthetic approach, very low amounts of desmin were detected in extracts from myoblast cultures and were attributed to contaminating myotubes (3).

In contrast to the above-mentioned studies, we were able to detect dividing cells which expressed desmin using primary mass cultures from chicken embryonic breast muscles and employing a combination of [^{3}H]thymidine autoradiography and immunofluorescent staining with a desmin antibody. Although similar findings have been reported previously for primary myogenic cultures, the lineage identity of the cycling, desmin positive cells could not be determined (4). In the present study, we have detected the [^{3}H]thymidine labeled nuclei in desmin positive cells not only in mass cultures but also in individual myogenic clones. Some of our findings regarding these cells are described here. The fate of these desmin positive cells and their role, if any, in the myogenic lineage is still unknown.

METHODS

Myogenic cultures were established from the breast muscles of 18-day-old chick embryos as described previously (5) except that the tissue was exposed to 0.2% collagenase for 45 min prior to the trypsin digestion. To eliminate myofibril fragments which are present in muscle cell preparations from older embryos and adult chickens and prevent accurate cell counting, the muscle cell preparation was subjected to Percoll density centrifugation (6, and Yablonka-Reuveni, in preparation). Cells were grown in gelatin-coated, horse serum-treated tissue culture dishes (5). For mass cultures, cells were plated at

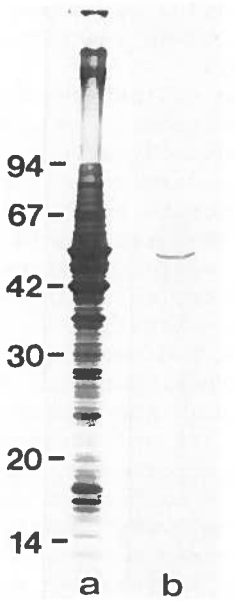

Figure 1. Immunoblot analysis of desmin antibody. a) Extract of a well fused muscle culture (4-day-old) was electrophoresced on a 10-20% SDS polyacrylamide gel and stained with Coomassie brilliant blue, b) Sample as in a) was further blotted onto nitrocellulose and the desmin band was visualized using the desmin antibody and the indirect immunoperoxidase technique.

vimentin, prepared from chicken erythrocyte ghosts according to published methods (14), and tested by the immunoblot technique (data not shown). As shown in Figure 2, when the anti-desmin was tested on a well fused myogenic culture (4-day-old), the myotubes reacted with the antibody whereas the majority of the mononucleated cells did not react. The desmin antibody was also tested on fixed sections from embryonic breast muscles as shown in Figure 3. Bundles of muscle fibers as well as smooth muscle cells in blood vessel walls were fluorescently stained. The connective tissue matrix in these muscle sections appeared to be lacking cells which were positive for desmin.

$2x10^5$ cells per 35 mm culture dish. For clonal cultures, cells were plated at 50-100 cells per 60 mm culture dish. Culture conditions and clone identification were as previously described (5,7).

A rabbit antiserum against chicken gizzard desmin was obtained from Dr. H. Holtzer. The preparation and characterization of this antibody have been reported (2,8). The IgG fraction of the desmin antiserum was obtained in our laboratory using protein A-sepharose (9). A guinea pig antiserum against skeletal muscle myosin heavy chain and a rabbit antiserum against BB-creatine kinase were prepared in our laboratory as described elsewhere (5,10). Single- and double-immunolabeling of cells in cultures were performed with the indirect immunofluorescence technique (10). In some experiments the desmin antibody was directly fluorescinated in the prescence of fluorescein isothiocyanate-celite (11) and direct:indirect immunostaining of cells was performed in sequence as previously described (3). Indirect immunofluorescent staining of tissue sections from the breast muscles of 18-day-old chick embryos was performed according to a recently described method (12, and Robinson et al., in preparation). Briefly, the tissue is fixed in a 4% solution of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (CDI), infiltrated with sucrose and sectioned with a cryostat. Indirect immunoperoxidase staining was used to visualize specific proteins separated by SDS polyacrylamide gel electrophoresis (10).

Cultures exposed to [^{3}H]thymidine were processed for autoradiography subsequent to antibody staining (10).

RESULTS

Characterization of the desmin antibody.

The desmin antibody which we used in this study is well characterized (2,8). However, due to the nature of our studies it was very important to reensure that the antibody reacts only with desmin. Analysis of the antibody by the immunoblot technique (13) is shown in Figure 1. The only protein that reacted with the antibody had a molecular weight of 51,000 which is in agreement with molecular weights reported for desmin (1,8). In addition, the antibody did not react with partially purified

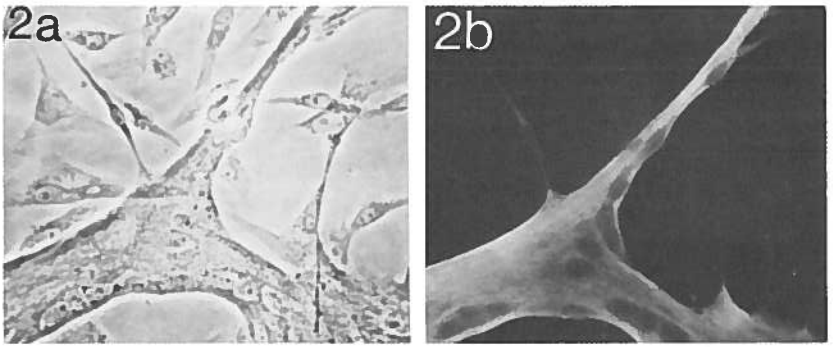

Figure 2. A phase and corresponding fluorescence micrographs of a 4-day primary muscle culture. a) Phase, b) Immunofluorescent staining with anti-desmin (0.1 mg/ml) indirectly labeled with fluorescein-conjugated goat anti-rabbit IgG (1:60). Micrographs were taken with a 40x objective and enlarged to an equivalent degree.

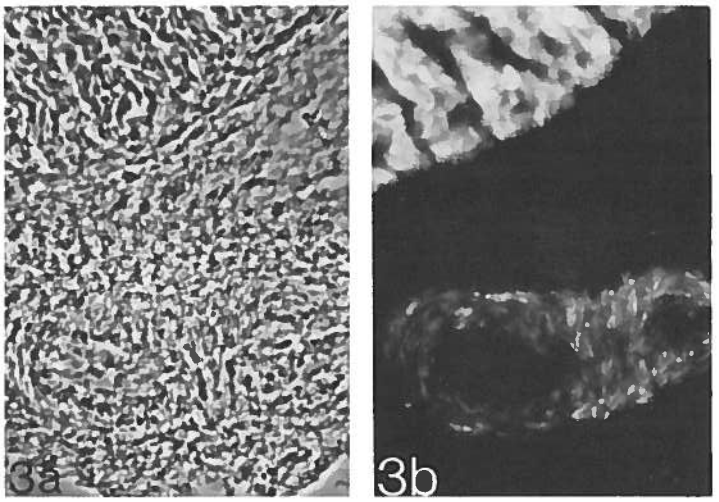

Figure 3. A phase and corresponding fluorescence micrographs of a tissue section from the breast muscles of an 18-day-old chicken embryo. a) Phase, b) Immunofluorescent staining with anti-desmin, details as in Figure 2.

Desmin expression in mass cultures.

To test the possible expression of desmin by mitotically active cells, primary myogenic cultures, 40 hr after plating, were pulsed for 2 hr with 1 μCi/ml [^{3}H]thymidine (specific activity 6.7 Ci/mmol) and immediately fixed and processed for immunofluorescent localization of desmin and skeletal myosin heavy chain, or desmin and BB-creatine kinase, and autoradiography. Selected microscopic fields are shown in Figure 4. The percentage of the total cells that incorporated the [^{3}H]thymidine was about 55%. Of these [^{3}H]thymidine labeled cells, about 5% were desmin positive. Desmin positive, postmitotic cells were also detected. Less than 0.1% of the [^{3}H]thymidine labeled cells were positive for both desmin and myosin (Figure 4, a-f). It has been suggested that the appearance of the BB-creatine kinase in cycling myogenic cells is a biochemical marker for the precursor compartment that gives rise to terminally differentiated myoblasts in the chicken skeletal myogenic lineage (10). Hence, the co-expression of desmin and BB-creatine kinase in dividing cells was investigated in order to determine whether the cycling, desmin positive cells are also in this lineage compartment (Figure 4, g-l). Desmin positive, cycling cells which are also BB-creatine kinase positive were detected. In addition, some of the desmin positive, mitotically active cells did

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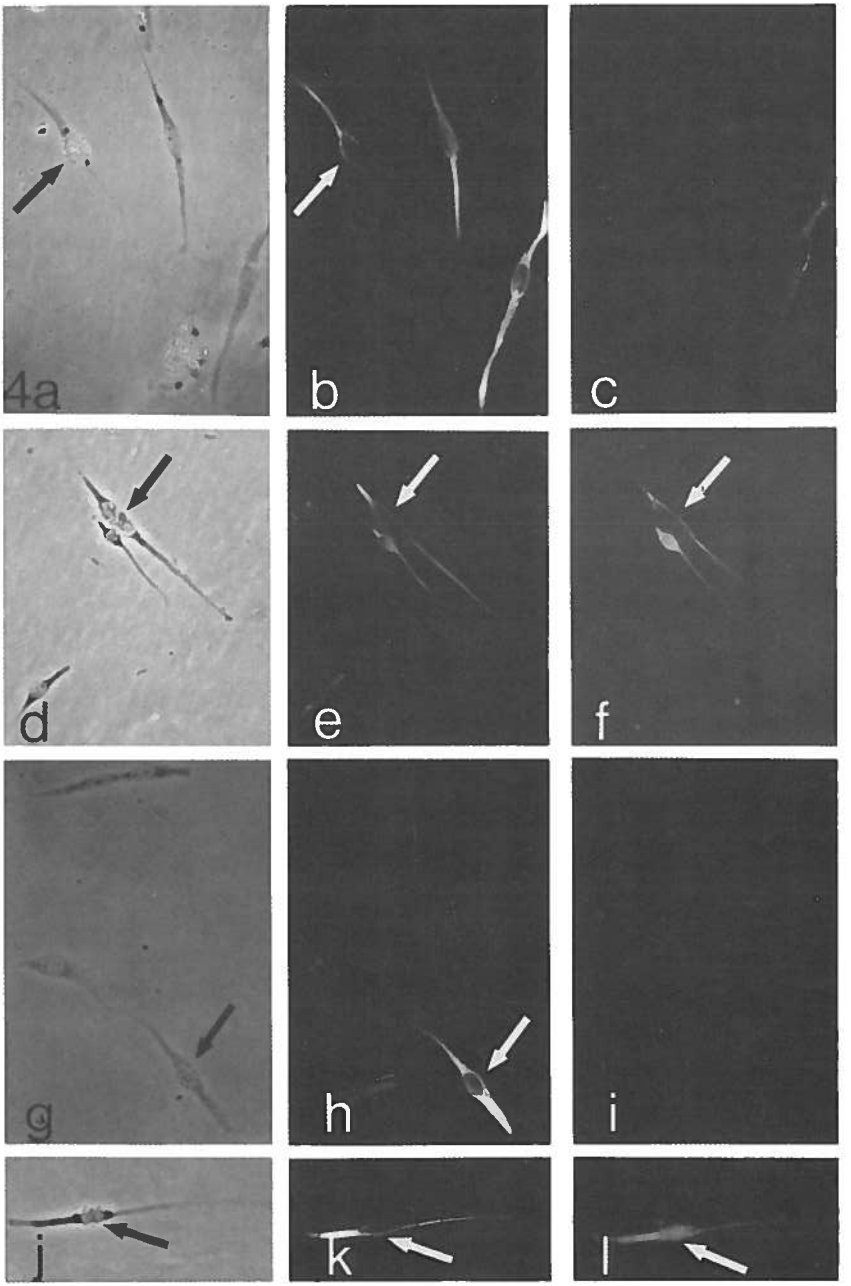
Figure 4. Micrographs of 2-day-old primary muscle cultures in which cycling cells were labeled with [^{3}H]thymidine. a) Phase, b,c) Double immunofluorescent staining of the same field with b) Anti-desmin (0.1 mg/ml) indirectly labeled with fluorescein-conjugated goat anti-rabbit (1:60), c) Anti-myosin (1:60) indirectly labeled with rhodamine-conjugated goat anti-guinea pig (1:60). d) Phase, e,f) The same field stained as described in b) and c), respectively. g) Phase, h,i) Double immunofluorescent staining of the same field with h) Anti-desmin (0.1 mg/ml) directly labeled with fluorescein, i) Anti-BB creatine kinase (1:50) indirectly labeled with rhodamine-conjugated goat anti-rabbit (1:60). j) Phase, k,l) The tively. All micrographs were taken with a 40x objective and enlarged to an equivalent degree. Arrows indicate [^{3}H]thymidine labeled nuclei.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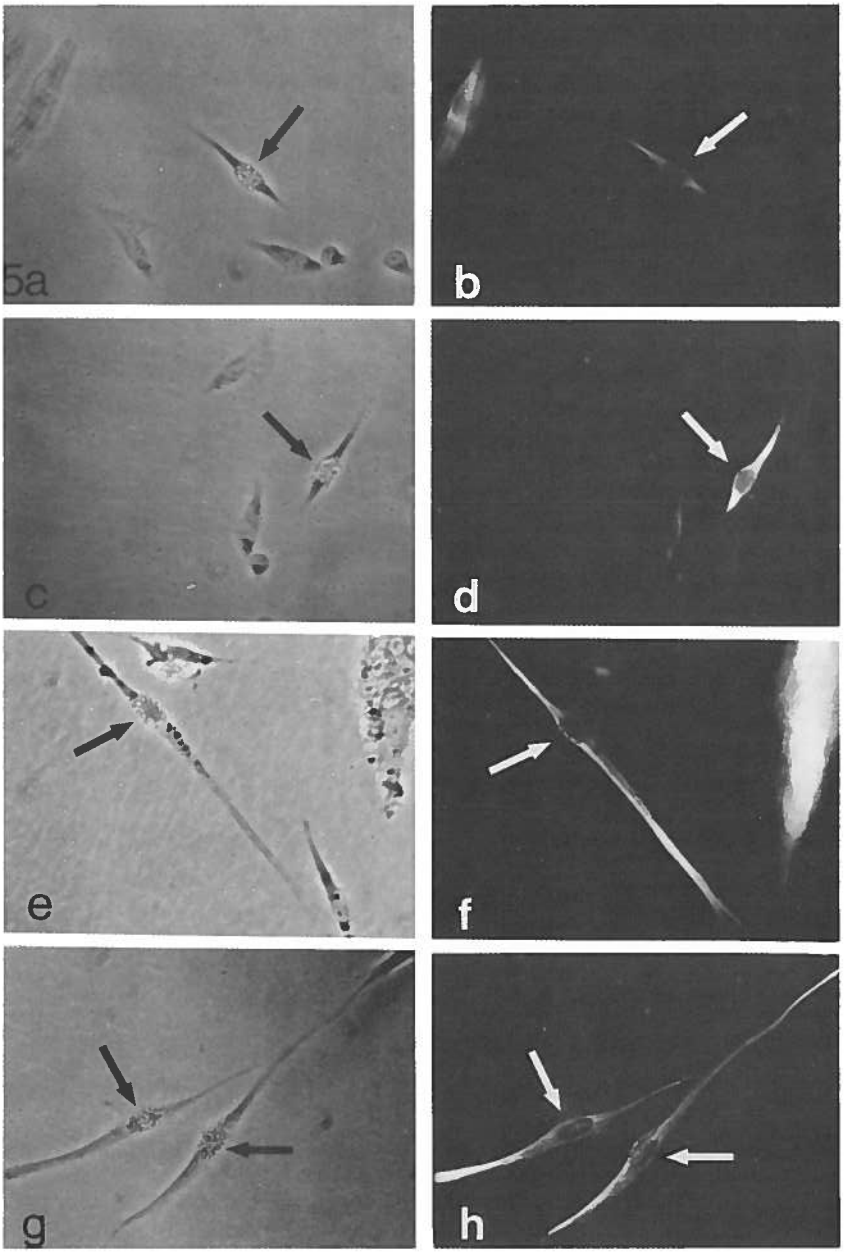
not express the BB-creatine kinase. Also, not all of the BB-creatine kinase positive, cycling cells expressed desmin. Of the postmitotic cells, the majority, but not all, of the "myoblast-looking", desmin positive cells were BB-creatine kinase positive.

Desmin expression in myogenic clones.

The above experiments showed that there was a biochemical heterogeneity among the desmin positive, cycling cells in respect to BB-creatine kinase expression. One possible interpretation of these results was that some of the desmin positive, cycling cells, despite their skeletal myoblast appearance, are not residing in the skeletal myogenic lineage. For example, smooth muscle cells which express desmin and are present in the muscle tissue at this embryonic age (Figure 3) could be isolated and cultured along with the skeletal myogenic cells. The same argument is true for the BB-creatine kinase positive, cycling cells, especially since the BB-creatine kinase isozyme has been, biochemically, detected in smooth muscle and other tissues (15). Immunofluorescent studies on BB-creatine kinase in smooth muscle cells are lacking. To determine whether the desmin positive, mitotically active cells were, at least in part, progeny of skeletal muscle precursor cells we grew primary clones from cells prepared from the breast muscles of 18-day-old chicken embryos. After 8 days in culture the clonal cultures were pulsed with [^{3}H]thymidine and immediately processed for antibody staining and autoradiography as described above for mass cultures. About 90% of the clones contained myotubes and mononucleated cells of which about 50% were still mitotically active. These clones are referred to as myogenic clones. The remaining 10% of the clones contained only mononucleated cells which did not react with antibodies against muscle specific proteins. The myogenic clones contained many postmitotic

Figure 5. Phase and corresponding fluorescence micrographas of selected microscopic fields of 8-day-old myogenic clones in which cycling cells were labeled with [^{3}H]thymidine. a,c,e,g) Phase, b,d,f,h) Immunofluorescent staining with anti-desmin, details as in Figure 2. Arrows indicate [^{3}H]thymidine labeled nuclei.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cells which were desmin positive and myosin positive. In about 50% of the myogenic clones we detected 1 to 4 [^{3}H]thymidine labeled cells which also expressed desmin. Less than 0.1% of these desmin positive, cycling cells were positive for skeletal myosin. Figure 5 demonstrates several desmin positive, myosin negative, cycling cells from different myogenic clones. Whereas most of these cells resemble myoblasts (Figure 5 a-d), some show a very peculiar appearance (Figure 5, e-h).

DISCUSSION

In the skeletal muscle lineage, desmin expression has been thought to be specific to postmitotic myoblasts and myotubes (1-3). More recently, the existence of small numbers of desmin positive, cycling cells in primary myogenic cultures was described (4). However, the lineage identity of these cells was not determined. The present study detected mitotically active cells which express desmin in both primary mass cultures and primary myogenic clones from embryonic skeletal muscle.

The presence of [^{3}]thymidine labeled, desmin positive cells in myogenic mass culture is not sufficient to suggest that these cells are precursors of skeletal muscle cells. These cells can represent smooth muscle cells which were isolated from the muscle tissue along with the skeletal myogenic cells. However, the heterogeneity among mitotically active cells in myogenic cultures with respect to the expression of desmin and BB-creatine kinase demonstrates that these cells do not constitute a homogeneous cell population. This finding suggests, as previously discussed (10), that caution should be used when biochemical and molecular data from extracts of myogenic cultures are being interpreted.

The finding of desmin positive, mitotically active cells in individual myogenic clones clearly demonstrated that at least some of the cycling cells which express desmin are descendants of cells that give rise to terminally differentiated skeletal muscle cells. Hence, these desmin positive cells may represent cells that reside in the skeletal myogenic lineage. If the latter is true, several explanations can be suggested for the appearance of these desmin positive, cycling cells. These include: a). The expression of desmin by cycling cells may reflect an event which normally occurs in chicken

skeletal muscle cells at a low rate where the synthesis of muscle specific proteins (e.g., desmin) and cell cycle withdrawal are separable. In fact, it has been shown that the addition of the tumor promotor PMA (phorbol 12-myristate 13-acetate) to muscle cultures causes an increase in the number of desmin positive, cycling cells. Hence, the frequency of this event can be manipulated. It has been suggested that the synthesis of muscle-specific proteins and cells cycle withdrawal are separable, non-interdependent events for quail myoblasts (16). Arguments against the separation of these two events as a regular pathway in chicken skeletal myogenesis were detailed in a recent publication from our laboratory (10). b) Desmin may be synthesized in all muscle precursor cells prior to the terminal M phase but accumulates to a degree that can be fluorescently detected only in some cells (i.e., cells that have prolonged G_2 phase, cells that can not enter or complete the final M phase). c) These cycling cells which express desmin are abnormal (i.e., dying cells) and might have initiated the differentiation program prior to its normal onset. Some of these suggestions have been also raised by others (4).

An alternative explanation to the appearance of cycling, desmin positive cells in skeletal myogenic clones is the existence of common stem cells which give rise to skeletal myogenic cells and to other cell types that express desmin, such as smooth muscle cells and cardiac cells. In a set of experiments, whose results are not shown here, we attempted to determine on primary myogenic mass cultures whether some of the desmin positive, cycling cells are actually smooth muscle cells. For this, we doubly labeled 2-day-old cultures with anti-desmin and a monoclonal antibody against smooth muscle-specific actin (actin was prepared from chicken gizzard, see Ref. 16 for details). None of the desmin positive, cycling cells reacted with the anti-smooth muscle-specific actin. It is noteworthy that some cells in the muscle culture (much less than 0.1% of the mononucleated cells) did react with the anti-smooth muscle-specific actin. These cells were spread out and almost unrecognizable when examined by phase microscopy, having the morphological appearance of smooth muscle cells in tissue culture. Fluorescence microscopy of these cells following staining with the anti-actin antibody, revealed a network of stress fibers. Surprisingly, these actin

positive cells did not express desmin as judged by double immunofluorescence. We also detected in mass cultures, at low frequency, cells that are very spread out and flat and which reacted with the anti-desmin but were negative for smooth muscle actin. In view of the above findings, the fact that the desmin positive, cycling cells did not react with the anti-smooth muscle actin is not sufficient to rule out the idea that these desmin positive, mitotically active cells may be smooth muscle cells. However, none of the desmin positive, cycling cells in the myogenic clones resembled the spread out presumed smooth muscle cells.

To further investigate the nature of the desmin positive, cycling cells, we are currently studying their appearance in skeletal muscle mass cultures and clones from different embryonic ages and from adult skeletal muscle, while also investigating the existence of these cells in skeletal muscle tissue in vivo.

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