In ovo exposure to monochromatic green light promotes skeletal muscle cell proliferation and affects myofiber growth in posthatch chicks

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Halevy, Orna, Yogev Piestun, Israel Rozenboim, and Zipora Yablonka-Reuveni. In ovo exposure to monochromatic green light promotes skeletal muscle cell proliferation and affects myofiber growth in posthatch chicks. Am J Physiol Regul Integr Comp Physiol 290: R1062–R1070, 2006. First published November 3, 2005; doi:10.1152/ajpregu.00378.2005.—Our previous studies demonstrated that illumination of chicken embryos with monochromatic green light results in enhanced body and muscle weight at later posthatch stages. In the present study, we investigated the cellular and molecular basis of this phenomenon. First, we showed that on day 6 posthatch, myofibers were more uniform in the in ovo illuminated group than in the control group incubated in the dark, with respect to the number of myofibers displaying diameter values within the range of the mean value. Second, we tested the hypothesis that in ovo illumination causes an increase in the number of myoblasts; this in turn can promote posthatch muscle growth. Indeed, a significant increase in the number of skeletal muscle cells isolated from pectoralis muscle was observed in the in ovo illuminated group on days 1 and 3 posthatch relative to the control group. This increased cell number was accompanied by higher expression levels of Pax7 and myogenin proteins on posthatch days 1 and 3, respectively. A parallel analysis of proliferating cells in the intact muscle further demonstrated a significant increase in the number of cells positive for proliferating cell nuclear antigen in muscle from the in ovo illuminated group. Third, we demonstrated that the transition from fetal- to adult-type myoblasts, normally occurring in late stages of chicken embryogenesis, is initiated earlier in embryos subjected to in ovo green-light illumination. We suggest that the stimulatory effect of in ovo illumination on posthatch muscle growth is the result of enhanced proliferation and differentiation of adult myoblasts and myofiber synchronization.

satellite cells; Pax7; myogenin; myoblasts; chicken

IN VERTEBRATES, HISTOGENESIS of skeletal muscle begins early in embryonic development and continues through adulthood. Early in development, muscle progenitors undergo myogenic determination, giving rise to myoblasts (reviewed in Refs. 5 and 27). These myoblasts first proliferate, then withdraw from the cell cycle, differentiate, and eventually fuse into multinucleate fibers. Under normal conditions, once nuclei are within a myofiber, they do not replicate DNA or divide (reviewed in Refs. 3 and 48). For most species, skeletal muscle fiber formation is essentially complete at birth or hatch, and the terminally differentiated myofibers are incapable of mitosis. The growth and repair of skeletal muscle in the postnatal stage are enabled primarily by mononucleated satellite cells, which are located beneath the basement membrane of muscle fibers (24). At birth or hatch, skeletal muscle consists of a high percentage of proliferating satellite cells; however, this number decreases rapidly toward the end of the growth period, reaching ~5% of the nuclei present within the muscle fibers in a quiescent state (19, 22).

Evidence from several laboratories studying avian, rodent, and human myoblasts has indicated that developing muscles consist of multiple myogenic populations (i.e., embryonic, fetal, and adult; see Refs. 9, 21, 41, and 48). In the chicken, based on characteristics of isolated muscle cells in culture, embryonic myoblasts are most abundant on embryonic day (E) 3, whereas fetal myoblasts are most abundant between E8 and E12 (40, 41). The onset of adult myoblasts correlates with the time that individual myofibers become encased by a basement membrane during late embryogenesis, a period at which the distinction of satellite cells by their morphology and location first becomes possible (48). Hence, adult myoblasts likely represent satellite cell progeny.

Myogenesis is tightly regulated by the MyoD family members (MyoD, Myf5, myogenin, and MRF4), all of which are basic helix-loop-helix transcription factors that act in concert with the myocyte enhancer factor-2 proteins (reviewed in Ref. 26). MyoD and myogenin are not expressed in quiescent satellite cells. However, upon activation of those cells (because of muscle injury or when the cells are cultured), MyoD expression is induced followed by the expression of myogenin (8, 47, 49).

The paired-box transcription factor Pax7 is selectively expressed in quiescent and proliferating satellite cells in rodents (1, 35, 37, 47, 48) and may be required for their self-renewal (28, 35, 51). Recently, we have shown that Pax7 is an early marker of myogenesis during posthatch muscle growth and that its expression is maintained by satellite cells in adult chicken muscle (19, 47). Our studies further proposed that reserve Pax7+/MyoD− cells can be renewed from proliferating Pax7+/ MyoD+ cells (19).

The light spectrum affects growth in birds; for example, chicks selected for growth that were reared under blue or green fluorescent lamps have significantly higher body weight than birds reared under red or white light (44). Similar results were obtained in a study on quails (29). Recently, we reported that illuminating chicks from day 1 posthatch with blue or green monochromatic lights increases their body and muscle weight compared with chicks reared under red or white light (30). These significant effects were observed as early as on day 3 posthatch and were highly correlated with enhanced satellite...
cell numbers in the chicks (13), suggesting that blue and in particular green light illumination enhances early posthatch muscle development.

In ovo illumination also affects embryonic development. Under regular conditions in hatcheries, eggs are incubated in darkness. Early studies indicated that white-light illumination accelerates embryonic development of several avian species (6, 7, 38, 46). Eggs incubated under white light hatched ~1 day earlier than those incubated in the dark (7, 38, 45). Continuous green-light illumination reduced embryo mortality in chicks (36). Whereas in the aforementioned studies heat effect could have been a contributory factor to the observed accelerated development, in our recent studies (31, 32) heat effect has been avoided by the use of intermittent lighting. We have demonstrated that intermittent lighting with monochromatic green-light during embryonic incubation increases posthatch body weight and absolute breast muscle weight in turkey pouls (31) and chickens (32).

In the present study, we investigated the cellular and molecular basis underling skeletal muscle development in embryos and posthatch chicks in response to in ovo green-light illumination. We report that green-light illumination during embryogenesis enhances proliferation and subsequent differentiation of adult myoblasts as reflected by alterations in the expression of Pax7 and myogenin and influences myofiber growth as evident by a higher number of uniform myofibers.

MATERIALS AND METHODS

All experimental procedures were approved by the Animal Welfare Committee of the Faculty of Agricultural Food and Environmental Quality Sciences of the Hebrew University of Jerusalem.

Birds

Embryos. Embryonated eggs (Cobb Strain) were preweighed and selected for an average weight of 68 g (range = 65–70 g). Eggs were divided randomly into two groups: the first (n = 250) received artificial light from E5 until hatch (termed Green group), and the second, control group (n = 250) was kept in the dark for the entire period (termed Dark group). Dark condition is used in commercial and experimental hatcheries. The monochromatic lighting system included light-emitting diode (LED) lamps that provided 560 nm light (half-band of 535–585 nm) at an intensity of 0.1 W/m² at eggshell level. To eliminate heat generation, lighting was intermittent (15 min light, 15 min dark) from E5 to E14; thereafter, lighting was continuous (32). On E10, all eggs were candled, and infertile eggs were removed. On E19, the eggs were transferred to hatching trays, the light-stimulated group to ones equipped with 560-nm LED lamps and the Dark group to ones kept in the dark. Incubation conditions were 56% humidity and 37°C.

Hatching and posthatch period. Eggs from different treatments were observed for hatching between E19 and E21 every 6 h. Chicks were considered hatched when they had completely emerged from the shell and pooled from those hatched during a narrow window of time of 6 h. Upon hatching, chicks were weighed and males were transferred to brooders with free access to commercial diet and water and were grown under normal light. At various days posthatch, pectoralis muscles were removed, cleaned from bones, and weighed. Each experiment was repeated four times.

Cell Cultures

 Cultures of muscle cells from posthatch chicks were prepared as described in Haley et al. (16). Muscle cell cultures from embryos were prepared according to a modified procedure of Hartley et al. (21) and Haley et al. (16). In brief, finely chopped pectoralis muscle tissue was enzymatically dispersed using 0.03% (wt/vol) pronase for 30 min, followed by 0.1% (wt/vol) trypsin for another 30 min at 37°C. DNase (0.006% wt/vol) was added for an additional 10 min. An enriched population of myogenic cells was recovered from the suspension by a low speed, and the pellet was resuspended in minimal essential medium (MEM) containing 1% (vol/vol) antibiotic-antimycotic solution and supplemented with 10% (vol/vol) horse serum (HS) and 3% (vol/vol) chicken embryo extract (CEE; GIBCO). Preplating procedure was as described (17). On all days, cell cultures were prepared from either 1.5 or 6 g muscle (for embryos and posthatch, respectively) sampled from a pool of chopped muscles from 10 embryos or chicks.

Cells were counted using a hemocytometer, and the number of cells per gram of muscle tissue, as well as the total number of cells, was determined for each group. Myoblasts isolated from fetal or posthatch muscle were collectively termed muscle cells. Cells were seeded on 10% HS-containing DMEM (for posthatch chicks). Cells were maintained at 37°C in a humidified atmosphere (95% air-5% CO2). An enriched population of myogenic cells was recovered with <5% of these cells being nonmyogenic. The coefficient of variation of cell preparations was ~5% (14). Each cell preparation was repeated in four independent experiments.

Muscle Sampling for Western Blotting and Histology

Muscle samples were excised from the superficial regions of the proximal one-half of the left pectoralis major of each chick. Each sample was ~0.5 × 0.5 × 1.2 cm in size. The long axis of each sample was parallel to the direction of the muscle fibers.

Western Blot Analysis

Western blot and densitometric analyses were performed as described in Haley et al. (16, 19). Equal amounts of protein of muscle extracts were separated by SDS-PAGE and transferred to nitrocellulose filters (Bio-Rad, Hercules, CA). Membranes were incubated overnight at 4°C with the appropriate antibodies and then washed and incubated for 1 h with horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgG (Zymed, San Francisco, CA). The primary antibodies for myogenin (rabbit polyclonal) and Pax7 (mouse polyclonal) were previously described (19). Band intensity in each lane was normalized to the levels of α-tubulin as an internal standard (19).

Myofiber Diameter Analysis

Myofiber diameter was determined by analyzing the lesser-myofiber diameter values, as we recently described (19). Muscle samples were fixed in 4% paraformaldehyde, embedded in paraffin, and deparaffinized as described previously (19). At least 10 arbitrary fields in two to three serial sections of each muscle sample were photographed using a ×40 objective. Myofiber diameter was then determined with Adobe Photoshop software. In each muscle sample, the lesser-fiber diameter was measured for individual myofibers, analyzing 500 fibers per sample and a total of 4,500 fibers (9 chicks) per treatment. A calibrated length (i.e., micrometer slide) was photographed and processed under the same conditions as the histological images, permitting the transformation of the digitized data from pixels to a standard length unit. Because there were no statistically significant differences among chicks, all data for the same treatment from individual experiments were pooled for further analysis.

Proliferating Cell Nuclear Antigen Immunostaining

Muscle sections were deparaffinized and immunostained with an antibody against proliferating cell nuclear antigen (PCNA, a marker
for dividing cells), using a commercial kit from Zymed followed by counterstaining with hematoxylin as previously described (14, 16). Control slides, where the primary antibody was omitted, were processed in parallel. Three chicks were analyzed per each group; five sections were studied per each chick, monitoring five random fields per each section. Analysis of positive cells was performed based on digitized images as previously described (16). Only nuclei within the myofiber perimeter were included in this analysis (using Abode Photoshop free hand tool); regions rich with connective tissue were not included.

**Double Immunolabling for Myosin Heavy Chain of Cell Cultures**

Embryonic and ventricular isoforms of sarcomeric myosin heavy chain (MHC) were detected by double-immunofluorescence labeling as described by Hartley et al. (20, 21) with modifications. Myoblasts were grown for 2 days, fixed in ice-cold AFA solution (70% ethanol-formaldehyde-acetic acid 20:2:1) for 1 min, and then incubated with a blocking solution [1% (vol/vol) goat serum in PBS] for 1 h. Cells were incubated with HV11 mouse monoclonal antibody against ventricular MHC (IgG1, ascites fluid; 1:1,000; a gift from E. Bandmann, University of California, Davis, CA) for 2 h, followed by incubation with a biotinylated anti-mouse IgG (Fab fragment, 1:250; Jackson, West Grove, PA) for 1 h and Texas red-Streptavidin (1:300; Jackson) for 30 min. EB165, a mouse monoclonal antibody against embryonic MHC, was added to the cells for 2 h (IgG1, ascites fluid, 1:1,000; kindly provided by E. Bandman), followed by incubation with an FITC-conjugated anti-mouse IgG antibody (1:250; Jackson) as the secondary antibody. Nuclei were detected by counterstaining with DAPI. The number of myosin-positive cells was determined by quantifying the nuclei within cells reacting with the myosin monoclonal antibodies. At least 600 nuclei in 15 or more fields were counted. Experiments were repeated two times.

**Statistical Analysis**

For representative experiments, data were analyzed by one-way ANOVA followed by t-test. For data based on four independent experiments, each experiment was considered a random block, and the variables were the light conditions. Data were analyzed by two-way ANOVA using a randomized block test. All data and statistical analyses were conducted using JMP (34). For myofiber analysis, data were evaluated using a t-test (assuming unequal variance; \( P < 0.05 \)). For comparisons of the distribution of data, chi square tests were performed; \( P \) values <0.05 were considered significant.

**RESULTS**

**Myofibers of In Ovo Illuminated Chicks Are More Uniform During Early Posthatch**

We have previously shown that green-light illumination of chick embryos has a significant enhancing effect on absolute muscle weight (i.e., %muscle of total body wt) from hatch until day 42 of age (32). Here, we further analyzed whether green-light illumination affects the myofibers themselves by investigating myofiber diameter in the pectoralis muscle. We chose to focus on muscle from 6-day-old chicks based on our earlier study in which we found a significant increase in myofiber diameter between day 3 and 6 of age (19). Figure 1A demonstrates that pectoralis muscle weight is significantly higher in the Green vs. Dark chicks on day 6 (2-way ANOVA using a randomized block test, \( P < 0.05 \)). Figure 1B depicts the results of the lesser-diameter analyses performed for parallel Dark and Green groups. The range of myofiber diameter values was similar for Dark and Green group myofibers (i.e., 0.5–16 \( \mu \)m). Mean myofiber diameter was 7.57 ± 2.20 \( \mu \)m for the Dark group muscle and 7.10 ± 1.89 for the Green group muscle (t-test, \( P < 0.0001 \)). An analysis of distribution of myofibers into quartiles (detailed in the legend to Fig. 1) further demonstrated a significant difference between the Green and Dark groups.

In both groups, myofibers showed a similar distribution in the lower-diameter bins, ranging between 0.5 and 5 \( \mu \)m (i.e., 19.55 and 19.48% of total myofibers for Green and Dark groups, respectively). However, when the ≥5.5-\( \mu \)m-diameter bins were compared, the distribution of the Green group myofibers showed a shift from the largest to the middle size bins (Fig. 1B), resulting in a more uniform distribution of myofibers in the Green group with respect to the number of myofibers displaying average or near-average diameter values (Chi square = 120.22; \( P < 0.001 \)). In the Green group, 51.11% of total myofibers fell within the diameter range of 5.5 to 7.5 \( \mu \)m, whereas in the Dark group, only 39.13% of total
fibers fell within this range. The Green group systematically displayed fewer myofibers in the higher-diameter bins, starting with a diameter of 8 μm through 16 μm (29.40% of the Green group fibers vs. 55.53% of the Dark group fibers).

In Ovo Green-Light Illumination Affects the Number of Muscle Cells in the Early Posthatch Period

The altered myofiber distribution seen in day 6 posthatch chicks after in ovo green light illumination could have resulted from changes in the myogenic cell pool. To further examine this possibility, we evaluated the number and proliferative state of cells within the pectoralis muscle of the two treatment groups.

Muscle cells were prepared from pectoralis muscle derived from chicks at various days posthatch and were counted immediately after isolation (Fig. 2). The number of cells per gram of muscle was higher in the Green group than in the Dark group on days 1 and 3 of age (P < 0.05). In both groups, the number of cells peaked on day 3 and then declined; however, the decline was slightly greater in the Green group. A stimulatory effect of in ovo green light illumination on muscle cell proliferation was further observed by immunohistochemical staining for PCNA in muscle sections (Fig. 3). The number of PCNA-expressing cells was significantly higher in muscle from the Green group than in the Dark one on day 3 and lower on day 6 (Fig. 3 and Table 1; P < 0.05).

Expression Levels of Pax7 and Myogenin at Early Posthatch Period Are Affected by In Ovo Green Lighting

To assess the effect of in ovo illumination on myogenic progression, we analyzed the expression of Pax7 and myogenin proteins as respective indicators of myoblasts at the predifferentiation and differentiation states during posthatch chicken muscle development (19, 47). Muscle samples were taken in parallel from four individual chicks per Green/Dark group at various days, and each individual sample was evaluated for protein expression levels by Western blot analysis followed by densitometry (Fig. 4A). The kinetics of Pax7 and myogenin protein expression was similar in muscles derived from both Dark and Green (Fig. 4A) groups; Pax7 levels were the highest on day 1, after which they declined. In both groups, myogenin levels peaked on day 3 and had declined by day 6.

In view of the large number of samples per each time point and the multiple in vivo repeats, samples harvested at the same time were analyzed in parallel on the same blot for each day. To normalize the results for all time points and experiments, protein expression levels in the Green and Dark groups are compared on each day and expressed as a percentage of the Dark group. When compared between groups, Pax7 levels were higher in the Green group than in the Dark group on all days examined (Fig. 4B; P < 0.05). The largest differences were observed on days 3 and 6 of age, reaching up to ninefold. Myogenin levels were significantly higher, up to fourfold, in the Green group than in the Dark one at hatch and on day 1 (Fig. 4C; P < 0.05). Myogenin levels did not differ between the two groups on day 3, but on day 6 posthatch, these levels were significantly lower in the Green vs. Dark groups (P < 0.05).

In Ovo Illumination and Myoblast Proliferation in the Embryo

The enhancing effect of in ovo illumination on muscle cell numbers in the posthatch period led to the possibility that this effect was manifested during embryogenesis. Moreover, we have reported that pectoralis muscle percentage of total body wt is significantly higher in the Green group embryos compared to the Dark group. To further support our observations, we decided to count the total number of muscle cells in pectoralis muscle cross sections prepared from Dark (a and c) and Green (b and d) groups at 3 and 6 days of age. Sections were immunostained for PCNA (dark brown nuclei) and counterstained with hematoxylin (blue nuclei).

Table 1. Effect of in ovo illumination on PCNA-expressing cells in chick breast muscle

<table>
<thead>
<tr>
<th>Age</th>
<th>Dark</th>
<th>Green</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 3</td>
<td>17.4±1.4</td>
<td>28.0±2.1*</td>
</tr>
<tr>
<td>Day 6</td>
<td>9.4±1.3</td>
<td>6.5±1.4*</td>
</tr>
</tbody>
</table>

Results are means ± SE; n = 3 chicks in each group. Five sections were studied/dechick, monitoring 5 random fields/each section. PCNA, proliferating cell nuclear antigen. Data were analyzed by two-way ANOVA using a randomized block test where each chick was considered a random block. Variables were the light conditions. *P < 0.05 vs. Dark group at the same age.

![Fig. 2. No. of muscle cells per gram of pectoralis muscle from Dark and Green group chicks on various days posthatch. Pectoralis muscles were removed from the experimental chicks, pooled within each group, and weighed. Myoblasts were isolated in parallel from each group and counted using a hemocytometer. Results are means ± SE (n = 5) and are representative of 4 independent experiments. *P < 0.05 vs. Dark group at the same age.](image)

![Fig. 3. Representative proliferating cell nuclear antigen (PCNA) staining of breast muscle cross sections prepared from Dark (a and c) and Green (b and d) groups at 3 and 6 days of age. Sections were immunostained for PCNA (dark brown nuclei) and counterstained with hematoxylin (blue nuclei).](image)
pared with the Dark group embryos on nearly all embryonic days between E11 and E21 (32). No major difference was observed in the total myoblast number per gram of muscle derived from the experimental embryos on E15 and E17 (Fig. 5). Although on E19 there were slightly more myoblasts per gram muscle in the Green group than in the Dark group, this difference was not statistically significant.

**DISCUSSION**

Green-light illumination of chicks, either during the incubation period (32) or immediately posthatch (30), results
in enhanced body and absolute muscle weight. The present study was aimed at defining the cellular and molecular events associated with the effect of in ovo green illumination on muscle growth. We focused on several characteristics of skeletal muscle development during embryonic and early posthatch stages in chicken. We conclude that in ovo illumination has a stimulatory effect on the proliferation and differentiation of satellite cells and a promoting effect on the uniformity of the muscle fibers in the early posthatch period.

In ovo green-light photostimulation enhanced the number of myoblasts per gram of muscle on early days posthatch. This effect was even more pronounced when the number of myoblasts was calculated per total pectoralis weight; the increase in cell number between days 1 and 3 in the Green group was more than twofold than that in the Dark group (data not shown).

Moreover, higher cell proliferative activity of muscle cells on day 3 in the Green vs. Dark group was evident in vivo, as indicated by the higher number of PCNA-positive cells within the perimeter of the myofibers. Indeed, the higher levels of Pax7 on day 3 and on day 6 (the day on which a decline in muscle cell number was observed in both groups) in the Green vs. Dark group (data not shown). Moreover, higher cell proliferative activity of muscle cells on day 3 in the Green vs. Dark group was evident in vivo, as indicated by the higher number of PCNA-positive cells within the perimeter of the myofibers. Indeed, the higher levels of Pax7 on day 3 and on day 6 (the day on which a decline in muscle cell number was observed in both groups) in the Green vs. Dark group (Fig. 4B) support the presence of higher reservoir of myogenic progeny resulting from in ovo green-light illumination. The importance of Pax7 as an indicator of the proliferation capacity of satellite cells has been recently shown in turkey poults that were held from food early posthatch; Pax7 expression was dramatically reduced in parallel to a decline in cell proliferation (18).

Our findings suggest that in ovo illumination does not affect the timing of the transition from proliferation to differentiation.

Table 2. Distribution of myogenic cells derived from E15 embryos, reacting with V⁺ and/or E⁺ MHC antibodies

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Treatment</th>
<th>V⁺E⁺ Cells</th>
<th>V⁺E⁻ Cells</th>
<th>Total Positive Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Dark</td>
<td>295</td>
<td>20</td>
<td>315</td>
</tr>
<tr>
<td></td>
<td>Green</td>
<td>224</td>
<td>48</td>
<td>272</td>
</tr>
<tr>
<td>II</td>
<td>Dark</td>
<td>485</td>
<td>2</td>
<td>487</td>
</tr>
<tr>
<td></td>
<td>Green</td>
<td>387</td>
<td>64</td>
<td>451</td>
</tr>
</tbody>
</table>

Cells were prepared from pectoralis muscle and cultured for 2 days. Cultures were fixed and double immunolabeled as described in MATERIALS AND METHODS. Nuclei were counterstained with DAPI, and the nuclei within all myosin-expressing cells were counted. E15, embryonic day 15; V⁺ E⁺, cells positive for both embryonic and ventricular myosin (corresponding to fetal myoblasts); V⁺E⁻, cells expressing only ventricular myosin (corresponding to adult myoblasts).
but enhances the number of cells available for proliferation and subsequently for differentiation. First, the kinetics of myogenin expression was similar in both nonstimulated and in ovo photostimulated chicks (Fig. 4A). Second, the relative levels of myogenin were higher in the Green group than in the Dark group at hatch and on day 1 (Fig. 4C). Third, the fact that in both groups myogenin levels peaked on day 3 whereas those of Pax7 were higher in the Green vs. the Dark group implies at least two different cell populations (1 that is still proliferating and 1 that is undergoing differentiation). Our earlier findings that the percentage of pectoralis muscle of total body weight in in ovo illuminated chicks is significantly higher than that in chicks incubated in the dark at all posthatch days analyzed (through day 42; 32), together with higher Pax7 levels (i.e., higher reservoir of quiescent/proliferative satellite cells) found in this study, imply an enhanced hypertrophy potential of muscle because of in ovo green-light illumination.

The immediate outcome of more differentiating muscle cells would be manifested in changes in posthatch myobiber development. We observed an overall wide range of myofiber diameters in both Green and Dark group muscle samples on day 6 posthatch. This wide range during the early postnatal growth phase could reflect the different “birth dates” of myofibers during embryogenesis because myofibers are formed during the embryonic phase in two main waves of primary and secondary myofibers that continue to mature postnatally (40). The finding that there are fewer myofibers at the trailing end of the larger-diameter fibers in the Green group compared with the Dark one may imply that in ovo green-light illumination slows the growth process for at least some of the myofibers. Nevertheless, in ovo green-light illumination enhances the uniformity of the myofiber; although the mean diameter value was lower for the Green group myofibers, those fibers were more uniform with respect to the number of myofibers displaying diameter values within the range of the mean value (Fig. 1B). Because the muscle weight is higher in the Green group while the mean myofiber diameter is smaller in samples from 6-day-posthatch chicks, we conjecture that there is an increase in the number of myofibers resulting from green-light illumination. We believe that these unified myofibers in the Green group are formed during the later stages of muscle development (possibly even during early posthatch days) and therefore have a prolonged developmental capacity still manifested in the posthatch phase. This is reflected by the increased number of myoblasts and the enhanced Pax7 and myogenin expression described in the present study. Moreover, our view that green light supports myobiber formation at the later stages of muscle development fits well with a study by Tidyman et al. (43) that focused on the temporal expression of sarcomeric myosin isoforms in the pectoralis muscle at various stages in pre- and posthatch chicken development. The transient reexpression shortly after hatch of the embryonic myosin isoform that is typically expressed by midembryogenesis suggested an additional round of new myofiber formation in late embryogenesis (43). In addition, previous studies have shown the appearance of myofibers exhibiting an embryonic phenotype in the anterior latissimus dorsi and in pectoralis muscles in growing birds, suggesting de novo myobiber formation during the early posthatch period (4, 25). Although a comparison of myofiber numbers within the pectoralis muscle of Green and Dark groups would provide the most direct demonstration that in ovo illumination leads to an increase in myofiber numbers, the fact that the pectoralis muscle is large, flat, and runs in multiple directions has presented a technical challenge for myofiber number analysis in connection with the current study.

Our findings regarding the stimulatory effect of in ovo illumination on satellite cell proliferation and differentiation during the chick’s early posthatch period led us to examine whether this treatment is already affecting satellite cells in the embryo. Indeed, higher numbers of adult-type myoblasts (presumably reflecting satellite cells; see Ref. 48) were detected in cultures derived from the Green group muscles than in those from the Dark group muscles on E15 (Table 1). Therefore, it is conceivable that in ovo green-light illumination increases the rate of adult myoblast proliferation in the embryo, or perhaps accelerates their appearance.

How do external signals such as in ovo lighting affect intracellular events underlying proliferation and differentiation of muscle cells throughout embryogenesis and posthatch muscle growth? It is possible that the monochromatic green light penetrates the eggshell and has a direct effect on muscle in the embryo. Although short wavelengths are more likely to be effective at the dermal level, blue light has been found to penetrate the abdominal wall of rats to a depth ≥2 mm (39). However, we were unable to detect any proliferative effect of monochromatic green light on cultured myoblasts derived from standard (nonilluminated) E17 embryos and 3-day-old chicks (Piestun Y., Rozenboim I., and Halevy O., unpublished data). A more likely explanation is that green light indirectly affects myoblast proliferation via the endocrine system; the latter receives photic cues from the retina or pineal photoreceptors. Indeed, we have shown a higher expression of growth hormone (GH) receptor mRNA in satellite cells derived from green light illuminated chicks (13). In addition, plasma GH levels, and IGF-I levels in muscle tissue, were higher in the Green group relative to the Dark one in early posthatch (Haleyev, O., Piestun Y., Hoizman, O., and Rozenboim, I., unpublished results). GH induces myogenic cell proliferation (15, 33), whereas IGF-I induces proliferation, differentiation, and fusion of muscle cells (11, 12). Another possible explanation for the in ovo photostimulation effect on adult myoblasts, at least while they populate the muscle during late embryogenesis, could be the secretion of extracellular signals by the surrounding tissues either because of a direct light effect or indirectly via the endocrine system. Previously, we reported that primary myogenic cells from E19 cultures bind much more platelet-derived growth factor (PDGF) than do those from E10 cultures, suggesting that PDGF may be a modulator for adult but not fetal myoblast myogenesis (50). Therefore, green light illumination may create a competent environment (e.g., extracellular signals) for the expansion of adult myoblasts, whereas under normal incubation conditions (dark), these myoblasts are shut off until later stages in development.

Both retinal and pineal cells are photosensitive in the embryo; however, this seems to be a rather late event in chick embryogenesis, since the long-wavelength cone opsins, red and green, are first detected in the retina on E14 (2), and pineal cells become sensitive to light from E16 onward (23). The late development of the retinal and pineal photoreceptors may explain the effect of green light specifically on satellite cells that become the major myoblast population during that period (48). Therefore, we have analyzed the number of myoblasts from
E15 onward, we cannot rule out the possibility that in ovo illumination has an earlier effect on fetal myoblast recruitment/proliferation. Indeed, nonretinal, nonpineal deep brain photoreceptors are expressed during the early stages of mammalian embryonic development (42). The question of whether these photoreceptors play a role in muscle development remains to be addressed.

In conclusion, the results shown in this study indicate a stimulatory effect of in ovo green-light illumination on skeletal muscle development in the chick during both late embryogenesis and the posthatch period. The stimulatory effect is specific to adult myoblasts, enhancing their proliferation and differentiation and consequently altering the pattern of myofiber formation.

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