Efficacy of myonuclear addition may explain differential myofiber growth among resistance-trained young and older men and women

John K. Petrella,1,2 Jeong-su Kim,1,2 James M. Cross,3 David J. Kosek,1,2 and Marcos M. Bamman1,2

1Department of Physiology and Biophysics, The University of Alabama at Birmingham; 2Geriatric Research, Education, and Clinical Center, Muscle Research Laboratory, Veterans Affairs Medical Center; and 3Department of Surgery, The University of Alabama at Birmingham, Birmingham, Alabama

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Petrella, John K., Jeong-su Kim, James M. Cross, David J. Kosek, and Marcos M. Bamman. Efficacy of myonuclear addition may explain differential myofiber growth among resistance-trained young and older men and women. Am J Physiol Endocrinol Metab 291: E937–E946, 2006. First published June 13, 2006; doi:10.1152/ajpendo.00190.2006.—Skeletal muscle stem (satellite) cells supporting growth/regeneration are thought to be activated and incorporated into growing myofibers by both endocrine and locally expressed autocrine/paracrine growth factors, the latter being load sensitive. We recently found that myofiber hypertrophy with resistance training is superior in young men (YM) vs. young women and older adults (Kosek DJ, Kim JS, Petrella JK, Cross JM, and Bamman MM. J Appl Physiol 101: 531–544, 2006). We hypothesized that the advanced myofiber hypertrophy in YM is facilitated by myonuclear addition in response to a milieu promoting stem cell activation. Twenty-six young (27.0 ± 1 yr, 50% women) and 26 older (63.7 ± 1 yr, 50% women) adults completed 16 wk of knee extensor resistance training. Vastus lateralis biopsies were obtained at baseline, 24 h after one bout, and after 16 wk. Muscle stem cells were identified immunohistochemically with anti-neural cell adhesion molecule (NCAM+)–. Muscle transcript levels of IGF-I and mechanogrowth factor (MGF) were determined by RT-PCR. Serum IGF-I, IGF-binding protein (IGFBP)-3, IGFBP-1, total and free testosterone, sex hormone-binding globulin (SHBG), and androstenedione were assessed by radioimmunoassay. Myofiber hypertrophy was twofold greater in YM vs. others, and only YM increased NCAM+ cells per 100 myofibers (49%) and myonuclei per fiber (19%) (P < 0.05). IGF-1Eα mRNA was higher in young and increased acutely (29%) with summation by 16 wk (96%) (P < 0.05). MGF mRNA increased only in young after one bout (81%) and by 16 wk (85%) (P < 0.001). Circulating IGF-I was twofold higher in young, whereas IGFBP-1 was lowest in YM (P < 0.05). Among men, free testosterone was 50% higher in YM (P < 0.01). Myonuclear addition was most effectively accomplished in YM, which likely drove the superior growth.

MECHANISMS OF REPAIR/REGENERATION in human skeletal muscle have been shown to be largely dependent on the activation of muscle stem cells, termed satellite cells (29, 44). Irradiation studies strongly suggest that satellite cell recruitment is not only important but perhaps requisite during load-mediated hypertrophy (1, 55), although nonmuscle stem cells may also be involved (4). The normally quiescent satellite cell can be induced to divide and contribute additional myonuclei as a result of muscle damage or increased activation (4). It is suggested that, within a myofiber, each myonucleus controls transcription and consequent protein synthesis over a finite volume of cytoplasm (16, 17), a concept known as the myonuclear domain (reviewed in Ref. 4). A present debate in muscle biology is whether the myonuclear domain is fixed during growth in adult myofibers, thus mandating nuclear addition during hypertrophy.

There are several indications (27, 31, 36, 37) that myofiber atrophy, including age-related atrophy (sarcopenia), exceeds the rate at which nuclei are shed, leading to contraction of the myonuclear domain. A growth stimulus applied to atrophied muscle would thus presumably lead to some growth prior to nuclear addition (i.e., protein accretion resulting in expansion of the myonuclear domain back to its original size) (4, 25). It is well known that load-mediated hypertrophy of adult myofibers results from an expanded fiber volume as protein content increases. In some instances, functional overload and resistance training have been shown to significantly increase the number of myonuclei concurrent with hypertrophy (3, 30), and thus there may be a limit on the amount of expansion a myonuclear domain can undergo during hypertrophy (i.e., a domain ceiling size) (37).

The mechanisms by which skeletal muscle stem cells are activated and incorporated into growing myofibers are modulated by both endocrine anabolic factors and locally expressed autocrine/paracrine growth factors, with the latter shown to be load sensitive (38, 39). The satellite cell pool appears to be maintained in humans into the seventh decade (30, 56) but declines thereafter (36, 53). The proliferative life span of these cells (to senescence) in humans decreases markedly across the first two decades of development but appears to be fairly stable throughout adulthood (22, 51). Importantly, satellite cells have shown blunted activation and proliferation responses to injury and to changes in activity with old age (18, 27). The blunted response of older muscle in vivo appears to be mediated at least in part by circulating endocrine anabolic factors (19) as well as the surrounding local milieu (12, 14). We have previously shown that a single bout of resistance loading upregulates the expression of factors thought to promote satellite cell activation to a greater degree in young vs. sarcopenic older adults [e.g., mechanogrowth factor (MGF), myogenin] (38, 39). Young men also realize a greater downregulation of myostatin mRNA expression than sarcopenic older women in response to acute resistance loading (38). Furthermore, we have recently found that the hypertrophic adaptation to long-term resistance...
training is superior in young men compared with young women and older adults (40).

The purpose of this study was to directly quantify the myonuclear domain (myofiber area per nucleus) and number of skeletal muscle stem cells in situ, along with a host of circulating and locally expressed growth/mitogenic factors, in young and older men and women undergoing resistance training to determine whether differences exist by age, sex, or training state (before and after 16 wk of training). We hypothesized that the advanced myofiber hypertrophy seen in young men is facilitated by the addition of new nuclei in response to a milieu promoting stem cell activation.

**METHODS**

**Subjects.** Fifty-two adults were recruited from the Birmingham, AL, metropolitan area into two age groups. Age ranges were 60–75 yr for the older group (13 men and 13 women) and 20–35 yr for the younger group (13 men and 13 women). All older subjects passed a physical exam and graded exercise stress test, whereas young participants were screened by health history questionnaire. Subjects were free of any musculoskeletal or other disorders that might have affected their ability to complete testing and/or resistance training. Subjects were not obese (BMI <30), nor did they have any leg resistance training experience within the past 5 yr. None of the subjects were being treated with exogenous testosterone or other pharmacological interventions known to influence muscle mass. The study was approved by the Institutional Review Boards of both the University of Alabama at Birmingham (UAB) and the Birmingham Veterans Affairs Medical Center. Written informed consent was obtained prior to participation.

**Resistance training.** Progressive resistance training of the knee extensors occurred 3 days/wk for 16 wk. Subjects warmed up on a cycle ergometer or treadmill for ~5 min or until warm (light sweat) prior to each training session. Training consisted of three exercises: knee extension, leg press, and squats. Each exercise was performed for three sets of 8–12 repetitions using resistance exercise stations or plate-loaded stations (barbell squats and linear 45° leg press). The amount of rest between sets was standardized at 90 s. Initially, training loads were based upon 80% of baseline one-repetition maximum (1-RM) strength and progressed to maintain 8- to 12-RM loads. We have previously shown little to no age difference in specific strength (1 RM/thigh muscle mass) in subjects from this population (48), indicating that training loads set at 80% of 1-RM established similar work intensities across the groups. Adherence to the 3 days/wk program averaged >90% and did not differ between groups.

**Muscle biopsy and tissue preparation procedures.** Percutaneous needle muscle biopsies were performed by a surgeon in the Pittman General Clinical Research Center at UAB. Muscle samples were collected from the m. vastus lateralis with established techniques using a 5 mm Bergstrom biopsy needle under suction (26). The baseline biopsy was taken from the left leg, the acute (24 h) postload biopsy was taken from the right leg after a rest period of 8 h, and the third biopsy 16 wk postresistance training was taken 24 h after the final training session. Immediately after the biopsy, visible connective and adipose tissues were removed with the aid of a dissecting microscope, and portions used for immunohistochemistry were mounted cross-sectionally on corks in optimal cutting temperature mounting medium mixed with tracaganth gum and frozen in liquid nitrogen-cooled isopentane. Portions of each sample (~35 mg) were used for RNA isolation were weighed and snap-frozen in liquid nitrogen.

**Myofiber size assessment.** We routinely assess myofiber type distribution (I, Ia, Ix) and type-specific myofiber size via myosin heavy-chain (MHC) isoform immunohistochemistry and have published these methods in detail elsewhere (39, 40). Mean fiber area (MFA) is computed as the average cross-sectional area (CSA) of the three fiber types weighted by the percentage of distribution of each type. For the 52 subjects in this study, myofiber type distribution was determined from 930 ± 33 myofibers in cross section at baseline and 818 ± 30 myofibers posttraining. MFA was used to assess mean fiber hypertrophy and the myonuclear domain.

**Quantification of muscle stem cells and myonuclei.** NCAM/CD56 is a cell surface protein expressed on quiescent, active, and proliferating satellite cells of human skeletal muscle (reviewed in Ref. 29) and has been used to successfully identify satellite cells in numerous studies of human skeletal muscle (15, 20, 24, 33–37, 42, 43, 52, 58, 59). NCAM/CD56 positive cells were revealed immunohistochemically on 6-μm sections in series with those used for myofiber typing/sizing. Sections were fixed for 45 min at room temperature in 3% neutral-buffered formalin and blocked with 5% goat serum in PBS for 30 min at room temperature. Sections were incubated for 1 h at 37°C with anti-NCAM/CD56 mouse mAb (1:200 in 1% goat serum; Becton-Dickinson Biosciences) followed by 30 min of incubation at room temperature in biotinylated goat anti-mouse secondary Ab (BA-9200, 1:200 in 1% goat serum; Vector Labs). After Vectastain ABC reagent was applied for 1 h at room temperature, NCAM+ cells were revealed by DAB substrate (ElitePk-6102; Vector Labs). Sections were rinsed briefly with PBS followed by 5 min in deionized distilled water (ddH2O). Nuclei were counterstained with Mayer’s hematoxylin for 5 min followed by 5-min rinses in warm, running tap water and ddH2O.

The protocol results in myonuclei stained blue and NCAM+ cells identified as any nuclei localized to the membrane of a myofiber and stained brown or with a brown rim, as shown in Fig. 1 (36, 37). Slides were mounted with Aqua-Mount (Lerner Labs) and stored at 4°C.

**High-resolution (48-bit TIFF) bright-field images were captured at ×20 magnification, and image analysis was performed using Image Pro Plus 5.0 software (Media Cybernetics).** Myonuclei, NCAM+ cells, and fibers were counted by the same analyst blinded to the age, sex, or time point of the sample. The total number of nuclei, NCAM+ cells, and myofibers were counted, and myonuclear per myofiber, myonuclear domain (fiber area per nucleus), NCAM+ cells per 100 fibers, and relative number of NCAM+ cells (no. of NCAM+ cells per total nuclei) were determined from 375 ± 6 cross-sectional myofibers before training and 362 ± 7 myofibers posttraining.

**Serum hormone concentrations.** Insulin-like growth factor I (IGF-I), IGF-binding protein (IGFBP)-3, IGFBP-1, testosterone (total and free), androstenedione, and sex hormone-binding globulin (SHBG) serum concentrations were determined in fasted morning serum samples withdrawn before and after 16 wk of resistance training (7). Ten milliliters of blood were withdrawn from an antecubital vein, and serum was aliquoted (500 μl) and frozen at ~80°C. Samples between subjects for a given hormone/binding protein were assayed in random order. All analyses were conducted by the UAB General Clinical Research Center Hormone/Substrate Core Laboratory. IGF-I, IGFBP-3, IGFBP-1, and SHBG were assessed by immunoradiometric assays (Diagnostic Systems Laboratories, Webster, TX). The interassay coefficient of variation (CV), average intra-assay CV, and assay sensitivity for each hormone/binding protein measured by immunoradiometric assay were (respectively) as follows: IGF-I (9.43%, 3.48%, and 4.89 ng/ml); IGFBP-3 (2.95%, 3.30%, and 200 ng/ml); IGFBP-1 (9.36%, 9.51%, and 0.42 ng/ml); and SHBG (8.21%, 7.69%, and 6.50 nM). Total testosterone and androstenedione were determined by solid-phase radioimmunoassay (Diagnostic Systems Laboratories, Webster, TX). For total testosterone, intraassay CV = 2.56%, average intra-assay CV = 4.92%, and the average sensitivity (90% bound) was 7.12 ng/dl. For androstenedione, intraassay CV = 16.87%, average intra-assay CV = 7.04%, and sensitivity was 0.12 ng/ml. Free testosterone levels were computed from total testosterone and SHBG using an established formula (60).

**RT-PCR.** The procedure of RNA isolation has been described in detail previously (38). Briefly, RNA was extracted by homogenizing muscle samples (~35 mg) in the TRI Reagent (Molecular Research

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of this primer-competimer mixture was optimized in preliminary experiments and ranged from 1:20 to 1:100 for IGF-IeA and MGF, respectively.

Based on the number of PCR reactions, a PCR premix was prepared as described previously (38). For each PCR, 1 μl of RT product (cDNA) was added into 24 μl of premix and topped with 50 μl of mineral oil (Sigma-Aldrich, St. Louis, MO). PCR was carried out in a DNA Engine (PTC-200) Peltier Thermal Cycler (MJ Research, Waltham, MA), with an initial denaturing step of 3 min at 96°C, followed by specific cycles (29 cycles for IGF-IeA and 35 cycles for MGF) of 1 min at 96°C, 45 s at specific annealing temperatures (58°C for both MGF and IGF-IeA primers), 45 s at 75°C, and a final step of 3 min at 72°C. Immediately following PCR, 25 μl of PCR product (22 μl of the reaction mixture diluted with 3 μl loading buffer) were separated by electrophoresis (100 V constant) in a 2% agarose gel for 1.5 h. Gels were run with molecular weight markers (100 bp Hyper Ladder IV; Genesee Scientific, San Diego, CA) to confirm the expected size of each mRNA. To eliminate age group or sex bias, each 20-well gel contained samples for subjects within each group (i.e., young men, young women, older men, older women), with the different subject groups loaded in random order on each gel. Ethidium bromide (0.1 μg/ml) was premixed in the 2% agarose gel, and images were captured under UV light in a Bio-Rad ChemiDoc imaging system (Hercules, CA). Band densitometry was performed using Bio-Rad Quantity One software. Parameters for image development were described in detail previously (38).

Statistical analysis. All statistical analyses were performed using Statistica 6.1 (StatSoft, Tulsa, OK). Data are reported as means ± SE. Between-group differences in baseline variables were tested using age × sex ANOVA. All variables measured before and after resistance training were analyzed using age × sex × training repeated-measures ANOVA. For each ANOVA model with a significant main effect or interaction, Tukey’s HSD tests were performed post hoc to localize the effect(s). Statistical significance was accepted at P < 0.05 for all tests.

RESULTS

Descriptive characteristics of study participants are presented in Table 1. Typical sex differences were noted for height and weight as males were taller and weighed more than females (P < 0.05). Within age groups, there was no difference in age between males and females. No age-sex group showed a significant change in weight after 16 wk. During resistance training, a minimum adherence rate of 83.3% (equivalent to attending 5 of 6 consecutive sessions) was required. Average adherence was 90% with no differences in adherence rates between age-sex groups (young women, 91%; young men, 90%; older women, 92%; older men, 89%).

Histological findings. Before training, 950 ± 33 myofibers per sample were distributed as 38 ± 2% type I, 48 ± 1% type IIa, and 14 ± 1% type IIx. As expected, the resistance training...
program resulted in the type IIx to IIa MHC shift typically found after exercise training such that after training, 818 ± 30 myofibers per sample were distributed as 37 ± 2% type I, 62 ± 2% type IIa, and 1 ± 0.3% type IIx. We determined MFA from CSA measurements of 147 ± 3 myofibers per sample, measuring CSAs of a minimum of 60 type I and 60 type IIa myofibers. CSA assessments of type IIx myofibers were obviously limited in most posttraining samples, but we accounted for this in the MFA computation since MFA was determined on the basis of the weighted distribution of types I, IIa, and IIx myofibers within each sample pre- and posttraining.

MFA. Results for MFA are shown for each age-sex group in Fig. 2. Before training, a main sex difference was noted in MFA, as MFA was 23% smaller in women vs. men. With resistance training, an age × training interaction (P < 0.05) revealed greater hypertrophy in young (1,161 ± 100 µm²; 27%) vs. older (706 ± 61 µm²; 17%) adults. MFA improved (P < 0.05) in all age-sex groups (young women, 835 ± 56 µm²; young men, 1,488 ± 55 µm²; older women, 794 ± 52 µm²; older men, 619 ± 50 µm²). The increase in MFA among older men was significant only by one-tailed testing.

Myonuclei per fiber. There was a significant effect of sex on the number of myonuclei per fiber at baseline, with women having fewer myonuclei per fiber than men (2.2 ± 0.1 vs. 2.6 ± 0.1, P = 0.007; Fig. 3). A main training effect was noted, as the number of myonuclei per fiber increased overall from 2.4 ± 0.06 to 2.6 ± 0.07 after 16 wk of resistance training. The effect was driven primarily by young men, as post hoc tests revealed that they were the only age-sex group to increase the number of myonuclei per fiber (19%, P = 0.02). The values were unchanged in the other three age-sex groups (P = 0.98–0.99).

Myonuclear domain. As shown in Fig. 4, sex had a significant effect (P < 0.05) on myonuclear domain at baseline, with women having a smaller area controlled by each nucleus compared with men (1,630 ± 57 µm² vs. 1,869 ± 74 µm²). This effect was primarily driven by older women, who had a significantly smaller myonuclear domain compared with both young and older men (older women, 1,486 ± 74 µm²; young men, 1,890 ± 83 µm²; older men, 1,848 ± 125 µm², P = 0.03). Training had a significant effect on myonuclear domain, which expanded overall (baseline, 1,750 ± 49 µm²; posttraining, 1,975 ± 45 µm², P < 0.001). Post hoc testing revealed that young women significantly increased myonuclear domain by 272 µm² (P = 0.04), with older women also exhibiting a strong trend for increasing myonuclear domain 262 µm² (P = 0.051). An overall expansion of 184 µm² was found among men across the two age groups (P < 0.05); however, the individual groups of young and older men did not show significant increases with resistance training (P = 0.22–0.53). A noteworthy finding was that all groups except older women reached a myonuclear domain of ~2,000 µm² (2,009–2,097 µm²) after resistance training. The myonuclear domain of older women remained significantly <2,000 µm² posttraining (1,748 µm², P = 0.001).

Population of NCAM + muscle stem cells. We found no differences by age or sex at baseline for the number of NCAM + cells per 100 fibers (Table 2). There was a significant main effect of training with an overall increase from 10.8 to 14.2 NCAM + cells per 100 fibers (P < 0.001). This effect was again driven primarily by young men, the only age-sex group with a training-induced increase in the number of NCAM + cells per 100 fibers (49%, P = 0.004). No positive trend was noted in any of the other groups (P = 0.40–0.57). We also
found no differences between groups at baseline for the number of NCAM+ cells relative to total nuclei (Table 2). There was a main training effect on the relative number of NCAM+ cells, as all subjects slightly increased the relative number of NCAM+ cells from 4.3% to 5.1% of total nuclei (P < 0.001). However, the relative number of NCAM+ cells did not significantly increase within any of the four age-sex groups (P = 0.44–0.77).

Serum hormone concentrations. There were main age and sex effects for circulating IGF-I because it was twofold higher in young subjects and 31% higher in men (Table 3). No differences were detected for its primary binding protein in circulation (IGFBP-3), whereas the inhibitory IGFBP-1 was lower in all young subjects and in men (P < 0.05). Typical sex differences in total and free testosterone and SHBG were noted between men and women (P < 0.001). However, no differences in total testosterone were detected between young and older men (P = 0.96). Although not significantly different, SHBG was 1.75-fold greater in older than in younger men; thus free testosterone was significantly higher in young men (59%, P < 0.001). There was a main effect of age on androstenedione because it was 42% higher in young subjects (P < 0.001). Resistance training did not alter circulating levels of any anabolic factors or their binding proteins.

Muscle IGF-IEa and MGF transcript levels. IGF-IEa and MGF mRNA results are presented in Fig. 5, A and B, respectively. There were main age, sex, and training effects for IGF-IEa, as expression was 22% higher in young subjects and in women. IGF-IEa expression increased overall after one bout of loading (29%, P < 0.05), and summation was noted by 16 wk of resistance training (85%, P < 0.001) with an additional 51% increase over the acute bout of loading (P < 0.001). As shown in Fig. 5A, the overall acute response was not significant when parsed into the two age groups; however, the overall summation responses by 16 wk remained significant within each age group. Figure 5B shows the same time course of baseline compared with acute and 16 wk responses for the expression of MGF mRNA. We detected an overall training increase as well as a main age × sex interaction (P < 0.05), as young men had greater levels than young women. When parsed by age, only young adults significantly increased MGF mRNA expression with one bout of loading (81%, P < 0.001). In contrast, the apparent 44% increase among older adults was not significant (P = 0.10). MGF mRNA levels 24 h postexercise after 16 wk of resistance training were not different from the acute response to loading (P = 0.999). The magnitude of change in MGF mRNA expression after 16 wk was twofold higher in young adults (85%) compared with older adults (40%). Within the young subjects, males had a 1.6-fold greater increase in MGF mRNA expression than females after both one bout of loading (young males, ↑ 107% vs. young females, ↑ 66%) and 16 wk of resistance training (young males, ↑ 112% vs. young females, ↑ 68%).

Table 2. NCAM+ muscle stem cells per 100 fibers before and after resistance training

<table>
<thead>
<tr>
<th></th>
<th>Women 20–35 yr</th>
<th>Men 20–35 yr</th>
<th>Women 60–75 yr</th>
<th>Men 60–75 yr</th>
</tr>
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<tbody>
<tr>
<td>NCAM+ cells/100 fibers†</td>
<td>9.1 ± 0.7</td>
<td>11.1 ± 1.3</td>
<td>10.8 ± 1.4</td>
<td>12.4 ± 1.5</td>
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<tr>
<td>Baseline</td>
<td>11.6 ± 1.3</td>
<td>16.5 ± 2.1</td>
<td>13.6 ± 2.3</td>
<td>14.9 ± 1.5</td>
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<tr>
<td>16 wk</td>
<td>4.0 ± 0.4</td>
<td>4.3 ± 0.5</td>
<td>4.6 ± 0.5</td>
<td>4.4 ± 0.4</td>
</tr>
<tr>
<td>Relative NCAM+ cells, %†</td>
<td>4.6 ± 0.5</td>
<td>5.2 ± 0.5</td>
<td>5.5 ± 0.8</td>
<td>5.2 ± 0.5</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 13 per group; NCAM+, anti-neural cell adhesion molecule. Relative NCAM+ cells = [no. of NCAM+ cells/NCAM+ cells + myonuclei]) × 100. *Significantly different from baseline within group, P < 0.05; †main training effect, P < 0.05.

Table 3. Profile of circulating anabolic hormones and their binding proteins before and after resistance training

<table>
<thead>
<tr>
<th></th>
<th>Women 20–35 yr</th>
<th>Men 20–35 yr</th>
<th>Women 60–75 yr</th>
<th>Men 60–75 yr</th>
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<tr>
<td>IGF-I, ng/ml*†</td>
<td>n = 9</td>
<td>n = 13</td>
<td>n = 13</td>
<td>n = 13</td>
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<tr>
<td>Baseline</td>
<td>320.3 ± 37</td>
<td>405.7 ± 36</td>
<td>167.4 ± 17</td>
<td>194.5 ± 20</td>
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<tr>
<td>16 wk</td>
<td>304.4 ± 40</td>
<td>380.9 ± 29</td>
<td>169.3 ± 18</td>
<td>205.0 ± 27</td>
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<td>IGFBP-3, ng/ml</td>
<td>n = 10</td>
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<tr>
<td>Baseline</td>
<td>4,135.4 ± 212</td>
<td>4,379.8 ± 212</td>
<td>3,782.2 ± 193</td>
<td>4,027.8 ± 112</td>
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<td>16 wk</td>
<td>3,701.9 ± 471</td>
<td>4,144.0 ± 195</td>
<td>3,960.8 ± 222</td>
<td>4,036.8 ± 86</td>
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<td>IGFBP-1, ng/ml†</td>
<td>n = 8</td>
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<td>Baseline</td>
<td>38.7 ± 5</td>
<td>19.2 ± 5</td>
<td>61.6 ± 12</td>
<td>34.3 ± 9</td>
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<tr>
<td>16 wk</td>
<td>34.5 ± 7</td>
<td>25.0 ± 4</td>
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<td>Total testosterone, ng/dl†</td>
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<tr>
<td>Baseline</td>
<td>21.0 ± 3</td>
<td>480.6 ± 50</td>
<td>22.1 ± 5</td>
<td>431.9 ± 28</td>
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<td>16 wk</td>
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<td>442.1 ± 46</td>
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<td>205.9 ± 68</td>
<td>67.8 ± 8</td>
<td>178.7 ± 31</td>
<td>108.0 ± 10</td>
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<tr>
<td>16 wk</td>
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<td>61.4 ± 6</td>
<td>142.5 ± 20</td>
<td>117.8 ± 15</td>
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<td>Free testosterone, pM†‡</td>
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<tr>
<td>Baseline</td>
<td>4.9 ± 1</td>
<td>23.1 ± 24</td>
<td>4.4 ± 2</td>
<td>138.1 ± 11</td>
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<td>16 wk</td>
<td>6.4 ± 2</td>
<td>23.7 ± 32</td>
<td>7.1 ± 2</td>
<td>156.5 ± 24</td>
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<td>Androstenedione, ng/ml*</td>
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<td>Baseline</td>
<td>1.9 ± 0.3</td>
<td>2.2 ± 0.2</td>
<td>1.4 ± 0.1</td>
<td>1.7 ± 0.1</td>
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<tr>
<td>16 wk</td>
<td>2.0 ± 0.4</td>
<td>2.0 ± 0.2</td>
<td>1.1 ± 0.1</td>
<td>1.6 ± 0.1</td>
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Values are means ± SE. *Main age effect; †main gender effect, ‡age × sex interaction, P < 0.05. IGFBP, IGF-binding protein; SHBG, sex hormone-binding globulin.
myofiber growth resulted from the successful incorporation of new myonuclei (found in young men only). Furthermore, only young men expanded the NCAM+ muscle stem cell pool (~50%) while realizing the most robust increase in muscle-specific MGF mRNA (112%) and possessing the most pro-growth circulating hormone profile (highest IGF-I and free testosterone, lowest IGFBP-1 and SHBG). We suggest that this suite of factors drove the robust hypertrophy adaptation in young men, and failure to expand the stem cell pool and incorporate new myonuclei hindered the rate of myofiber growth in the other age-sex groups.

**Baseline age comparisons.** The MFAs reported at baseline are similar to those reported elsewhere (15, 28, 30, 31, 35, 37). Although we did not find significant age-related differences in MFA, it is well established that age-related myofiber atrophy is localized to the type II fiber population in the early stages of decline. In our ongoing studies, we have documented type II myofiber atrophy among older adults, which is more marked in the older women (39, 40). In this sample, we also noted baseline age-related type II atrophy (~19%, P < 0.001) with no age difference in type I size, and type II myofibers were 27% smaller among older vs. young women compared with only 14% atrophy in older vs. young men (data not shown). The number of nuclei per myofiber did not decline with age. These findings concur with previous data in aging rodents (27) and humans (36, 56), suggesting that either the gradual decline in type II fiber size with aging is not accompanied by shedding of myonuclei or the rate of nuclear loss occurs more slowly than the rate of atrophy. We also noted a strong trend for the myonuclear domain to decrease with age (P = 0.08), further suggesting that nuclear loss occurs at a slower rate than atrophy.

A number of studies have associated age-related declines in circulating anabolic factors, including IGF-I and testosterone, with the muscle atrophy of aging (10, 32, 41), suggesting that these serum factors are important for the constitutive maintenance of muscle mass. Although we and others have shown that serum hormone levels do not change by any appreciable amount over long-term resistance training (6, 45, 50) and return from transient elevations shortly after acute resistance exercise (5, 46), the likelihood remains that basal circulating concentrations of one or more factors partially determine the growth/regenerative capacity of muscle. This was clearly demonstrated in recent seminal work by Conboy and colleagues. (18, 19). Using a parabiosis model, these researchers showed that the circulating milieu plays an important role in the regenerative capabilities of skeletal muscle, including satellite cell recruitment, as regenerative capacity was restored in aged mice provided a young circulation (18, 19). In this context, the higher basal endogenous concentrations of circulating anabolic factors found among young subjects in this report may have been important in the promotion of superior hypertrophy. Not surprisingly, there were no age differences at baseline in muscle-specific expression of IGF-I or MGF mRNAs; however, we suggest it is the load-mediated expression of these local factors, particularly MGF, that may in part differentiate the age groups’ hypertrophic potential.

**Effects of resistance training on muscle stem cell activation and hypertrophy.** After 16 wk of resistance training, young men exhibited the largest magnitude of myofiber hypertrophy on both an absolute (1,488 μm²) and relative (32%) scale and

![Graph](image-url)
were apparently the only age-sex group to successfully activate, proliferate, and differentiate skeletal muscle stem cells into myoblasts to serve as nuclear donors. In comparison, older men and both groups of women experienced modest myofiber hypertrophy concurrent with an expansion of the myonuclear domain but not a significant addition of myonuclei per fiber. The increase in myonuclei per fiber unique to young men occurred in conjunction with the most marked increase in muscle MGF mRNA expression.

The resident number of quiescent satellite cells in resting muscle may not be rate limiting for myofiber hypertrophy in humans, even in the elderly (54). The satellite cell pool appears to be maintained in humans into the seventh decade (30, 56) but declines thereafter (36, 53). For example, Roth et al. (56) found similar satellite cell populations in healthy adults ~25 and ~68 yr of age. We also report no age differences at baseline in the frequency of NCAM+ cells between adults ~27 and ~65 yr of age. In contrast, adults approximately one decade older show fewer relative NCAM+ cells compared with young (36, 53). As opposed to limited availability of precursor cells in the untrained muscle of the 64-yr-old adults studied herein, a more likely limiting factor is the capacity to activate these cells with a loading stimulus. Animal models have shown that satellite cell activation in response to various growth/regeneration cues (including mechanical load) is impaired in aged muscle, resulting in a slowed regenerative/recovery process (8, 18, 21, 27, 47).

The work of Conboy and colleagues (18, 19) clearly shows that satellite cells of aging muscle are inhibited by the surrounding milieu, as chronic exposure to serum from young animals restored the proliferative and regenerative capacities of satellite cells in old mice (19). Furthermore, exogenous IGF-I applied to atrophied muscles in rats has been shown (14) to significantly increase muscle mass and satellite cell proliferation. Studies of muscle transplantation in mice showed that old-to-young transplanted muscle recovered to levels equivalent to the contralateral young limb, whereas young to old transplants did not show significant improvements (12, 13). Circulating IGF-I has been implicated in the fusion of new myonuclei with myofibers (62). Although serum concentrations of IGF-I, free testosterone, and androstenedione in the present study did not differ pre- to posttraining as expected, it should be noted that this circulating milieu constantly bathing the muscle microenvironment was most favorable overall for muscle growth in young adults, particularly young men.

Although NCAM+ staining has been used numerous times to identify satellite cells in human skeletal muscle (15, 20, 24, 33–37, 42, 43, 52, 58), there is some debate as to whether this single marker captures all satellite cells (23) and in an exclusive manner. The possibility of not identifying a satellite cell by NCAM immunostaining seems minimal, on the basis of the existing literature, as these cells appear to be NCAM immunoreactive while quiescent, proliferating, and up to the point of terminal differentiation (29). Because there remains a chance of missing some satellite cells, however, the NCAM+ cell population quantified in this study might be viewed with caution as a distinct subpopulation. However, our staining and image analysis procedures still enabled us to reliably test for age, sex, and training effects within this subpopulation, as suggested by others (23). Previous work (36) used anti-laminin to identify satellite cells by architectural locus, yet satellite cell counts do not differ in the presence or absence of laminin staining. There has been an attempt to double-stain these cells with anti-NCAM and either anti-M-cadherin or anti-CD34, but no usable results have been attained (24). We have also examined the efficacy of anti-dystrophin immunostaining and found no difference in our NCAM+ cell count with or without dystrophin revealed (unpublished observations). Overall, using our immunostaining and blinded image analysis techniques, we maintain that the NCAM+ cells identified in this study by a combination of immunoreactivity and architectural locus represented the majority of the skeletal muscle satellite cell population.

Studies of muscle-derived stem cells in primary culture show that myogenic precursor cells (NCAM+), when given appropriate environmental cues, are capable of transdifferentiation into adipogenic and osteogenic cells (59). Additionally, satellite cells from older animals have a higher incidence of adipogenic differentiation (61) and, therefore, may be less likely to differentiate to myoblasts to serve as myonuclear donors and instead increase the number of adipocytes. Myogenic stem cells in adipogenic media do enter the adipocyte lineage; however, when subjected to stretch, these cells transdifferentiate back to the myogenic lineage, thus showing the ability of mechanical stress to favorably alter the appropriate cues (2). Resistance loading markedly alters the local expression of factors known to influence satellite cell activation, presumably (favorably) altering the milieu of the satellite cell microenvironment (5, 11, 38, 49). We (38, 39) have previously shown that acute mitogenic and myogenic responses to one bout of loading are generally most favorable in young men whereas the muscles of older sarcopenic women are less responsive.

Myofiber growth over the course of long-term resistance training is thought to result from repeated acute responses to each loading bout. In this sample, we assessed mRNA expression of IGF-IEa and MGF as surrogate markers of the satellite cell microenvironment. IGF-IEa mRNA showed a generalized increase among all subjects that was summative over the 16 wk of training, and although no baseline age difference was noted, a main age effect indicated that IGF-IEa mRNA expression across time was 22% higher in young vs. older subjects. MGF mRNA was preferentially upregulated in response to acute loading in young adults, particularly young men. Potentially, the transient MGF transcript response to each loading bout follows a shorter time course than IGF-IEa, returning to baseline between each bout and realizing a similar response to each successive bout. Of course we can only speculate, because a new baseline biopsy was not collected posttraining. Barton (9) recently indicated that the IGF-I isoform differences lie only in the E peptide extension (propeptide), resulting in the same active IGF-I peptide. Barton also found that muscle IGF-I concentration and associated intracellular signaling processes were enhanced more by viral expression of MGF transcript than IGF-IEa transcript; however, the hypertrophy adaptation in growing mice was similar. Thus, although MGF mRNA expression may be more closely and transiently regulated by mechanical load, increased expression of the active peptide from either transcript isoform appears sufficient to promote hypertrophy.

The older women in this study did show some hypertrophy with training (24%), which, on a relative basis, was similar to
young women (21%). Furthermore, it should not be overlooked that in 16 wk these older women achieved the pretraining mean fiber size of women ~35 yr younger, suggesting restoration of fiber size. The growth pattern among all women and older men in the study was apparently one of protein accretion with no additional nuclei. Muscle protein synthesis is enhanced after a single bout of resistance loading and remains elevated for 24–48 h (54). This increased synthesis indicates that existing myonuclei in fibers have the ability to respond quickly to resistance loading by enhancing translational capacity. Repeated bouts under long-term training would presumably put existing nuclei under greater strain as fiber volume expands. We and others (37) suspect that this drives the addition of new myonuclei to facilitate further hypertrophy, provided the mechanisms are in place to activate nuclear donors (satellite or other stem cells). Because the magnitude of hypertrophy in the present sample was greatest in young men and these men experienced the largest overall increases in both mitogenic gene expression and myonuclear addition, we suggest the microenvironment plays a key role in the local activation of precursor cells. This process is likely required to facilitate a growth response beyond protein accretion within existing myonuclear domains.

Despite some evidence for satellite activation (57), most training studies in older adults have not shown a change in myonuclei per fiber but, rather, an expansion of the myonuclear domain during hypertrophy. Cycling has been shown to slightly increase MFA in older men with no change in number of myonuclei (i.e., expansion of the myonuclear domain) (15). Resistance training among older men has also led to significant myofiber hypertrophy with no increase in myonuclear number (30, 31), suggesting incorporation of nuclei lags behind the increase in fiber size, resulting in expansion of the myonuclear domain (31). Young muscles may be more adept at incorporating new nuclei than older muscles. Resistance training studies in young adults (30, 34) have shown hypertrophy with an increasing number of myonuclei to maintain myonuclear domain; however, others (37) show increased satellite cell content with no increase in myonuclear number. This discrepancy in the responses of young adults may be due to a ceiling on the myonuclear domain that is approached before the myofiber demands incorporation of new nuclei for continued hypertrophy.

Theory of myonuclear domain ceiling driving the addition of nuclei. Significant increases in the number of myonuclei per fiber have been reported (37) when fiber size increases >26% but not when hypertrophy is ≤15%. Thus some initial myofiber hypertrophy can expand the myonuclear domain as existing myonuclei increase their local protein synthesis to support moderate enhancements of cytoplasmic volume (37). However, once a certain limit in the myonuclear domain is reached, further myofiber hypertrophy might only be possible by the addition of new myonuclei. As a follow-up to this “ceiling theory” (37), we used cluster analysis on the basis of the percentage of mean fiber hypertrophy within each subject and detected two distinct clusters with a theoretical cut point of 27% on the basis of the lowest magnitude of hypertrophy among the “responders” (40.0 ± 2.3% hypertrophy) contrasted with “nonresponders” (9.7 ± 2.4% hypertrophy) (Fig. 6A). Our data agree with the hypertrophy threshold (26–27%) recently introduced into the literature (37), because this was the minimum magnitude of myofiber hypertrophy associated with an increase in myonuclei (responders). Using relative rather than absolute hypertrophy, the numbers of responders (n = 25) and nonresponders (n = 27) were not influenced by age among women (responders: 7 young, 6 older; nonresponders: 6 young, 7 older) but differed markedly among men (responders: 9 young, 3 older; nonresponders: 4 young, 10 older).

As shown in Fig. 6A, both responders and nonresponders increased the muscle stem cell pool; however, only the responders significantly increased the number of myonuclei per fiber. These data suggest that 1) muscle stem cell proliferation is a generalized response to loading in many individuals irrespective of growth rate, 2) incorporation of new myonuclei may be the rate-limiting step toward maximizing one’s hypertrophic potential, and 3) this myonuclear addition may not be necessary until the myonuclear domain approaches a ceiling. We also saw striking differences in mitogenic gene expression between responders and nonresponders (Fig. 6B). For both IGF-I and MGF transcripts, acute upregulation was noted only...
among responders, and transcript levels increased more in responders than in nonresponders by 16 wk.

Based on these data in 52 subjects, we propose a theoretical myonuclear domain ceiling of ~2,000 μm² that can be attained with protein accretion prior to requisite nuclear addition for further hypertrophy. Domain size expanded significantly to reach this ceiling (on average) in both responders (2,016 μm², P < 0.001) and nonresponders (1,937 μm², P < 0.05); however, this appeared to be a limiting constraint on the modest hypertrophy experienced by nonresponders, because no nuclei were incorporated to progress the hypertrophy process further. Within each age-sex group, only older women did not attain an average posttraining myonuclear domain of ~2,000 μm². It is possible that the modest hypertrophy in older women was limited more by protein accretion than nuclear incorporation. Whether nuclear incorporation would have eventually become a limiting factor among older women upon approaching the domain ceiling cannot be determined from our data.

In summary, progressive hypertrophy of human skeletal myofibers beyond the modest growth achieved by initial protein accretion appears to depend largely on muscle stem cell activation and the ability to incorporate new myonuclei, making the muscle stem cell pool and its microenvironment primary targets in combating atrophy due to aging or disuse. In this study, the ability to incorporate new nuclei (likely derived from satellite cells) was most effectively accomplished in young men. We suggest that there may be a ceiling size on the myonuclear domain of ~2,000 μm² that is approached before the addition of new nuclei is required to further progress myofiber hypertrophy. For older women with a markedly contracted domain size, this would allow a fair amount of growth (or “regrowth”) on the basis of protein accretion alone given sufficient training duration and/or optimal work-recovery intervals. Men with larger myofibers appear to operate closer to this ceiling in the untrained state than women, perhaps making nuclear addition a more important early hypertrophy adaptation in men than in women. The marked age differences among men in the ability to activate muscle stem cells and successfully achieve nuclear addition likely explain the substantially greater relative myofiber hypertrophy in young (32%) vs. older (13%) men. Future studies should examine the effects of longer-term training (beyond 16 wk) to determine whether nuclear addition would eventually occur in older women after protein accretion controlled by existing myonuclei nears a maximum.

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