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Sex, Fiber-Type, and Age Dependent In Vitro Proliferation Of Mouse Muscle Satellite Cells

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ABSTRACT

During postnatal growth and after muscle injury, satellite cells proliferate and differentiate into myotubes to form and repair musculature. Comparison of studies on satellite cell proliferation and differentiation characteristics is confounded by the heterogeneity of the experimental conditions used. To examine the influence of sex, age, and fiber-type origin on in vitro properties of satellite cells derived from postnatal muscles, fast extensor digitorum longus (EDL) and slow soleus (SOL) muscles were extracted from male and female mice of 1 week to 3 months of age. Myoblast proliferation and myogenic regulatory factor (MRF) expression was measured from cultures of freshly isolated satellite cells. Higher proliferation rate and elevated *Myod1* expression was found in male EDL and SOL derived cells compared with females at age of 40, 60, and 120 days, whereas inverse tendency for cell proliferation was apparent in EDL of juvenile (7-day-old) pups. *Myogenin* and *Mrf4* transcripts were generally elevated in males of 40 and 60 days of age and in female EDL of juveniles. However, these differentiation markers did not significantly correlate with proliferation rate at all ages. *Pax7*, whose overexpression can block myogenesis, was up-regulated especially in 40-day-old females where MRF expression was low. These results indicate that gender, postnatal age, and muscle fiber origin affect proliferation and muscle transcription factor expression in vitro. The results also support the view that satellite cells originating from slow and fast muscles are intrinsically different and warrant further studies on the effect of cell origin for therapeutic approaches. J. Cell. Biochem. 112: 2825–2836, 2011.

KEY WORDS: MUSCLE; SATELLITE CELL; PROLIFERATION; AGE; SEX; MUSCLE FIBER; CELL CULTURE

Sexual dimorphism affects various aspects of the human and animal life. Body size, life expectancy, and susceptibility to diseases differ in males and females [Deasy et al., 2008]. Skeletal muscle among other tissues presents unequal characteristics in the two sexes. Muscle fiber diameter and, therefore, muscle mass is generally higher in males, although no differences are found in terms of maximum force generated when normalized by muscle mass [Miller et al., 1993; Kanehisa et al., 1996]. On the other hand, female muscles posses consistently greater fatigue resistance than male muscles under submaximal contractions [Hicks et al., 2001]. Purely structural, mechanistic, or metabolic differences in the musculature do not sufficiently explain the underlying mechanisms for muscle mass differences in different sexes which, partly because of lack of animal model studies, still remain unclear.

Muscle mass is related to the fiber cross-sectional area, which is dependent on muscle stem cell activity as these cells are responsible for postnatal muscle growth and repair [Deasy et al., 2008]. The best documented muscle stem cells are satellite cells, committed mononuclear progenitors derived from the embryonic dermomyotome that reside between the basal lamina and the sarcolemma of the mature muscle fiber. Several molecular markers have been used to identify satellite cell population, paired-box transcription factor *Pax7* being the most widely accepted [Seale et al., 2000; Zammit et al., 2004; Shefer et al., 2006; Buckingham, 2007]. In adults, satellite cells are quiescent but become activated upon stimuli such as acute muscle injury, exercise, or denervation [Seale and Rudnicki, 2000]. The activation is associated with coordinated expression of myogenic regulatory factors (MRFs) [Smith et al., 1994; Cornelison

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and Wold, 1997; Cooper et al., 1999], muscle-specific basic helix-loop-helix (bHLH) transcription factors that heterodimerize with ubiquitously expressed bHLH transcription factors named E proteins to drive the expression of muscle specific genes. Upon activation satellite cells re-enter the cell cycle, downregulate *Pax7* [Olguin and Olwin, 2004; Zammit et al., 2004], upregulate the primary MRF *Myod1*, and perform several rounds of proliferation before upregulating a secondary MRF *myogenin* [Smith et al., 1994; Yablonka-Reuveni and Rivera, 1994], and subsequently *Mrf4* [Seale and Rudnicki, 2000]. In the end of this coordinated process, the proliferating satellite cells (also called myoblasts) begin to fuse with each other and differentiate into myotubes or, alternatively, fuse with existing muscle fibers to repair damage or to increase muscle mass [Hawke and Garry, 2001; Charge and Rudnicki, 2004].

Because of their central role in regulation of muscle-specific genes, it is possible that *Myod1* and *myogenin* expression may have direct effects on muscle bulk. In this scenario, increased muscle mass in males would be expected to be associated with higher expression of the MRF genes compared with females. However, comparison of MRF expression in rat anterior tibial muscle revealed no gender differences *Myod1* gene expression and even higher *myogenin* mRNA levels in females than in males [te Pas et al., 1999], suggesting that gender-related differences in the intact muscle cannot be primarily explained by MRF expression levels. Muscle cell lines derived from turkeys selected for increased muscle mass and from related but randomly bred control animals revealed higher in vitro proliferation and differentiation rates in the selection line, whereas there were no sex-dependent differences within the selected or control groups [Velleman et al., 2000]. Consistently, subsequent in vitro MRF expression analysis revealed no drastic sex-dependent differences in *Myod1* or *myogenin* expression, although there was an increment in the *Myod1* expression in highly proliferative (selected) cell line compared with the slower proliferating control cell line [Liu et al., 2005]. The authors suggest that this in vitro result may indicate prolonged proliferation of the satellite cells also in vivo which would, in turn, promote the formation of large myotubes with higher number of nuclei and ultimately result in increased muscle mass.

With respect to satellite cell content ex vivo, no sex differences have been reported in mouse gastrocnemius [Salimena et al., 2004] or in human vastus lateralis [Roth et al., 2000] and tibialis anterior muscle [Kadi et al., 2004], although age-dependent reduction has been reported in one study [Kadi et al., 2004]. Discrepancies also exist in reported differentiation capacity of satellite cells in cell culture. Higher differentiation rate of male myoblasts with respect to females was reported in turkey pectoralis major-derived cells, although the proliferation rate was similar in both sexes [Velleman et al., 2000]. In another study using the same muscle and age range [Doumit et al., 1990], no differences in proliferation or differentiation was found. Gender-dependent heterogeneity may also affect the regenerative efficiency of satellite cells when transplanted in muscle-compromized hosts. Although muscle derived stem cells from both genders have been used to characterize their in vivo regenerative capacity [Lee et al., 2000] direct comparisons of male and female derived stem cells have not been carried out.

Besides the sex of the donor, muscle fiber type has been reported to influence satellite cell properties [Rosenblatt et al., 1996; Huang et al., 2006; Rossi et al., 2010]. Two main types of muscle fibers exist based on their expression of myosin heavy chain isozymes. Type I fibers (slow-twitch fibers) exhibit slow myosin ATPase activity and oxidative metabolism, present high fatigue resistance and are implicated in postural maintenance and endurance. By contrast, type II fibers (fast-twitch fibers) express fast myosin ATPase activity and anaerobic (glycolytic) metabolism, present low fatigue resistance, and participate in fast corporal movements. Although most muscles are composed of a mixture of both fast and slow fiber types, the percentage is highly variable among the different muscles of the body. Fibers in slow-twitch muscle contain more satellite cells than those in fast-twitch muscles, as they are the first and most frequently recruited during muscle activity [Hawke and Garry, 2001; Collins et al., 2005]. However, the satellite cell content in the muscles composed of a mixture of slow and fast-twitch fibers show no fiber type-specific differences in humans [Kadi et al., 2006]. Throughout life, the composition of myofibers is not stable but may experience dynamic changes, for example, in response to exercise, injury, aging, or disease [Canepari et al., 2010].

The two most commonly used ''prototypic'' fast and slow muscles are extensor digitorum longus (EDL) and soleus (SOL), respectively. Comparison of rat EDL and SOL muscles at age of 1 month, 1 year, and two years revealed a continuous decrease in satellite cell number with age in EDL but an increase up till 1 year of age in SOL [Gibson and Schultz, 1983]. Not only the number of satellite cells is different in fast and slow-twitch fibers, but also their potential for proliferation and differentiation in vitro may differ. Studies of male rats 2 months of age indicated that SOL-derived satellite cells displayed a higher proliferative potential but lower differentiation rate compared to those derived from EDL [Lagord et al., 1998]. Moreover, the capacity of EDL and SOL satellite cells to regenerate fast (type II) and slow (type I) muscle, respectively, is not reversed by electrical stimulation resembling firing patterns of the opposite muscle type [Kalhovde et al., 2005]. This suggests that fast and slow skeletal muscle fiber associated satellite cells are intrinsically different. Differences in MRF expression in fast- and slow-twitch muscle fibers have been also described. *Myod1* is preferentially expressed in the fast EDL whereas *myogenin* is mainly distributed in the slow soleus muscle [Voytik et al., 1993]. However, the biological significance of this finding remains obscure.

The analyses of satellite cell numbers in young and aged humans [Renault et al., 2002], pigs [Campion et al., 1981], and rodents [Gibson and Schultz, 1983; Shefer et al., 2006] indicated a decline in satellite cell number with age, although in some cases muscle type-dependent increases in early life were found. I young mice, decrease in the number of EDL-associated satellite cells starts earlier that in SOL [Gibson and Schultz, 1983]. Comparison of EDL and SOL muscles from young and aging mice revealed that, in EDL, a major decline in satellite cell number occurs before 1 year of age, whereas in SOL this only occurs by the age of 2.5 years [Shefer et al., 2006], consistently with accelerated aging-related atrophy of fast muscles. The proliferation and differentiation rate of satellite cells in vitro has been also shown to decrease as function of age in pigs [Mesires and Doumit, 2002]. In turkeys, however, no age-associated differences in cell proliferation were found [Doumit et al., 1990]. In male mice, initial proliferative phase in vitro is retarded in satellite cells derived

from senile animals (29–33 months old) compared with young males (3–6 months), although differentiation capacity is not affected [Shefer et al., 2006].

Newborn or very young animals are commonly used as a source of muscle stem cells because they are present in higher numbers compared with adult muscle [Hawke and Garry, 2001]. However, little is known about potential differences in proliferation dynamics in newborn, pre-puberal, puberal, and young adult individuals. Notably, studies that investigate these parameters in parallel using both sexes and different muscle types do not exist. This bias may derive from the fact that at this stage, the musculature is in the growing phase and show altering satellite cell dynamics in vivo [White et al., 2010]. Additionally, changes in male and female sex hormones in the young animals may influence satellite cell function [Chen et al., 2005; Enns and Tiidus, 2008]. All evidence presented imply that the age and sex of the individual, as well as muscle fiber-type can strongly influence satellite cell physiology and may provide an explanation for some of the contrasting data obtained from seemingly similar experiments. Importantly, biased use of only one sex (normally males) in the studies of the muscle stem cell function or their therapeutic use should be discouraged [Check Hayden, 2010]. Hence, further research is necessary to clarify the number, proliferation, differentiation, and MRF expression of satellite cells as these conclusions may condition research and therapeutic approaches. The aim of this study was to investigate the in vitro proliferation rate and MRF expression of the mouse satellite cells during rapid postnatal growth and muscle maturation. Satellite cells extracted from fast and slow-twitch skeletal muscles of male and female mice were studied in parallel.

MATERIALS AND METHODS

All experimental procedures were approved by the Ethics Committee of Universidad de Zaragoza and followed the international and the institutional guidelines for the use of laboratory animals. Mice were housed under a 12 h light: 12 h dark cycle in 21–23°C with relative humidity of 55%. Food and water were available ad libitum. Animals were sacrificed by cervical dislocation.

SATELLITE CELL EXTRACTION AND CULTURE

Three male and female B6SJL mice per age group were sacrificed at 7 (neonatal), 40 (early-young), 60 (young adults), and 120 days of age (adults). The EDL and the SOL muscles from both sexes were collected and processed in parallel. Satellite cell extraction and culture was carried out as described [Montarras et al., 2005]. Briefly, pools of six muscles per age, sex, and muscle type were minced to a slurry with sterile dissection scissors and digested in F-12 + GlutaMAX nutrient mixture (Gibco) containing 0.1% trypsin and 0.1% collagenase (w/v) (Sigma–Aldrich) at 37°C for 30 min. The supernatant was collected and further enzymatic digestion was inhibited by addition of fetal bovine serum (Gibco). Three to four rounds of digestion were performed until the muscle bulk was digested completely. Pooled cell suspension from each group was filtered through a 70 μm diameter sterile strainer and centrifuged 1,800 rpm for 15 min at 4°C. Cell pellet was resuspended to DMEM + GlutaMAX (Gibco) and stained with 0.4% trypan blue (Sigma–Aldrich) for viable cell counting. For each well used for proliferation and gene expression analysis (see below), total 1,000 cells were seeded in 96-well plates covered with 0.1 mg/ml of Matrigel basement membrane matrix (Becton Dickinson SA). The culture medium consisted of 39% F-12 + GlutaMAX (Gibco), 39% DMEM + GlutaMAX (Gibco), 10% fetal calf serum (Gibco) and 2% Ultroser G (Pall-Biosepra). This medium was used as it supports both proliferation and differentiation of satellite cells without requirement for switch to differentiation medium. Cells were left to adhere and start proliferating for 3.5 days at 37°C and 5% CO_2. At least four replicate wells for proliferation assay and for PCR analysis were plated for each group of sex, muscle type, and age. The experiments were performed in parallel with the cell proliferation and the gene expression analysis.

CELL PROLIFERATION ASSAY

Starting 3.5 days after the plating, and repeatedly every 24 h till 7.5 days, cell proliferation plate was fixed with 10% neutral buffered formalin solution (Sigma–Aldrich) for 15 min and nuclei were stained with Hoechst 33342 (Sigma–Aldrich) for 5 min. After washing with 1× PBS, five fields per well were photographed at 20× magnification under an epi-fluorescence microscope (Nikon TE2000-E) at 325 nm. For each time point, the total number of cells from five random fields per replicate (total 20 fields) was counted from each sex, muscle type, and age. At each day of the culture, the data were compared with the corresponding sex and age group of interest (see below for the statistics). These data were also normalized so that the maximum value from each comparison is set to 100. The error bars represent the standard error as percentage of the maximum value of the comparison that has been set to 100. The *P* values for each data point are shown in Supplementary online materials (Table 1).

QUANTIFICATION OF MRF EXPRESSION

In parallel with cell proliferation plates, plates for RNA extraction were washed with cold 1× PBS and transferred directly on 96-well plates to −80°C to wait extraction. RNA was extracted and the cDNA was synthesized using the Cells-to-cDNA kit (Ambion) according to manufacturer's instructions. Plates were placed on ice and cells were lysed in 100 μl of ice cold cell lysis solution, followed by heat treatment at 75°C for 15 min to rupture cells and to eliminate endogenous RNase activity. Traces of genomic DNA were eliminated with Dnase treatment at 37°C for 15 min, followed by 5 min of heat inactivation at 75°C. cDNA synthesis from each RNA sample was performed in duplicates. Briefly, 2 μl of dNTPs and 1 μl of random hexamers were mixed with 5 μl RNA followed by incubation at 75°C for 3 min. Two microliters of master mix composed of 1 μl 10× reverse transcription buffer, 0.5 μl M-MLV retrotranscriptase, and 0.5 μl RNase inhibitor were added and the reaction was incubated at 42°C for 60 min followed by 95°C for 10 min. For QPCR, cDNA was diluted 1:10 in dH_2O and 2 μl were used as a template for each reaction (three replicates per cDNA sample) containing 2.5 μl Fast 2X TaqMan master mix (No AmpErase UNG) and 0.5 μl gene-specific TaqMan assays (Applied Biosystems) for *Pax7* (Mm00834079_m1), *Myod1* (mM00440387_m1), *myogenin*

(Mm00446194_m1), or *Mrf4* (*Myf6*, Mm00435126_m1) in a final volume of 5 μl. Reactions were run using StepOne Plus Real-Time PCR System (Applied Biosystems) using following cycle: 95°C for 10 min, followed by 47 cycles of 95°C for 15 s and 60°C for 30 s. Reaction efficiencies of the primer/probe sets were inside 100 ± 10% over 4-log dilution range of template RNA prepared in the above mentioned method. In the same conditions, reference gene amplification was performed using TaqMan assays for three reference genes (Applied Biosystems): 18S ribosomal RNA (4352930E), Gapdh (4352932E), and β-actin (4352933E). Geometric mean of these housekeeping genes was used for normalization and relative gene expression was determined using the $2^{-\Delta\Delta CT}$ method [Manzano et al., 2011 and references therein]. Ct values for the housekeeping genes and the calculated geometric mean are shown in Supplementary online material (Fig. 1). Because Ct value is exponentially related to copy number, the upper and lower error values for relative expression were estimated from mean change in Ct (ΔΔCt) plus standard deviation (ΔΔCt + SD) amd ΔΔCt minus standard deviation (ΔΔCt − SD) for each group (Figs. 6 and 7, Supplementary online material Table 2).

STATISTICAL ANALYSIS

Total cells from five photographic fields of a single well were counted and mean and standard error of mean (SEM) for each biological sample was calculated from the value of four replicate wells. Different groups were compared using Student's *t*-test (Statistic 5.0). Statistical differences were considered significant at $P < 0.05$.

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In order to ensure the myogenic origin of the studied cells, one well per group was fixed at day 4.5 of cell culture and immunostained for MYOD1, a marker for activated (proliferating) myoblasts. Cells were rinsed with PBS and fixed in 4% neutral buffered formalin solution (Sigma–Aldrich). Fixed cells were permeabilized with 0.2% (v/v) Triton X-100 (Sigma–Aldrich) in PBS and blocked using 0.2% (w/v) gelatine in PBS. Monoclonal antibody for MYOD1 (sc-304, Santa Cruz Biotechnology) was diluted 1:100 in *PBS* and the fixed/permeabilized cells were incubated with the primary antibody for 2 h at room temperature. The primary antibody was visualized with Alexa 546-conjugated anti-rabbit secondary antibody (Invitrogen) before mounting in DakoCytomation Faramount fluorescent mounting medium containing 1,000 ng/ml Hoechst 33342 (Sigma–Aldrich). All cultures used for the study showed myogenic (MYOD1 positive) cell content 70–80%.

RESULTS

To investigate potential sex- and muscle type-dependent variation of in vitro proliferation rate of satellite cells from young and mature wild-type mice, satellite cells were extracted from typical fast-twitch EDL and slow-twitch SOL muscles from both sexes at age of 7, 40, 60, and 120 days (see Materials and Methods Section for details). From all muscle, gender, and age groups, the cells were seeded at standard density immediately after the extraction to avoid potential effects from ex vivo amplification and cryopreservation. General proliferation rate was measured from the total number of Hoechst-stained nuclei per well every 24 h between 3.5 days (no differentiation evident) and 7.5 days (differentiation to myotubes evident) of culture (Fig. 1A). Immunostaining of fixed cells at day 4.5 revealed that 70–80% of the cells were expressing MYOD, a marker for activated myoblasts (Fig. 1B). In conditions used for these studies, differentiation into myotubes was observed in both sexes and all ages and muscle types.

In satellite cells (hereafter called myoblasts) derived from the muscles of 7-day-old mice, significantly faster proliferation was observed EDL-derived myoblast of females (Fig. 2A), whereas in SOL there were no consistent differences between sexes at any time point (Fig. 2B) (see Supplementary Table 1 for *P* values for each data point). However, fast-twitch EDL-derived cells proliferated consistently faster than slow-twitch SOL-derived cells, independently of gender (Fig. 2C and D), although in males the substantial sample variation precluded significance in last time points. In contrast, in myoblasts from 40-day-old animals a tendency for higher proliferation rate was observed in males compared to females and in both muscles under study (Fig. 3A and B). However, this difference was not significant at every day of culture due to sample variation between the four replicates. As in earlier age, EDL-derived cells tended to proliferate faster than those from the SOL, although this was only significant in females (Fig. 3C and D). At 60 days of age, myoblast proliferation was again higher in males compared with females in both muscles (Fig. 4A and B). Proliferation rate of EDL-derived myoblasts continued to be higher compared with that of SOL-derived cells in both sexes (Fig. 4C and D). Finally, like at 60 days of age, myoblasts from animals of 120 days of age proliferated faster in males than in females in both in EDL and SOL-derived cultures (Fig. 5A and B). However, unlike in younger animals, muscle type comparison from same gender revealed that EDL and SOL-derived myoblasts proliferate with similar rate and suggested possibly even higher proliferation rate in SOL-derived cells in females (Fig. 5C and D). Collective data from these primary myoblast proliferation experiments indicate that in early juveniles (7 days), male satellite cell derived myoblasts show equal or lower proliferation capacity compared with females. Later, however, their proliferation rate exceeds that of female-derived cultures. Cells from fast EDL muscle, on the other hand, are generally more proliferative that those of slow SOL muscle in vitro when derived from young animals up to at least 2 months of age. However, this difference is abolished (males) or possibly reversed (females) in more mature mice of 120 days.

Because our findings with respect to proliferation rate contradicts those previously reported in 60-day-old rats where SOL was found to proliferate faster than EDL [Lagord et al., 1998], we looked for further evidence for our findings on molecular level. Satellite cell proliferation that precedes the differentiation into myotubes in vitro and in vivo is characterized by up-regulation of *Myod1* mRNA [Megeney et al., 1996; Zammit et al., 2004]. Therefore, higher *Myod1* mRNA levels would be expected in myoblasts that proliferate faster. To investigate if the observed gender-specific modulation of myoblast proliferation may be reflected in the level of MRFs we analyzed *Myod1* expression at two time points of cell culture: At day

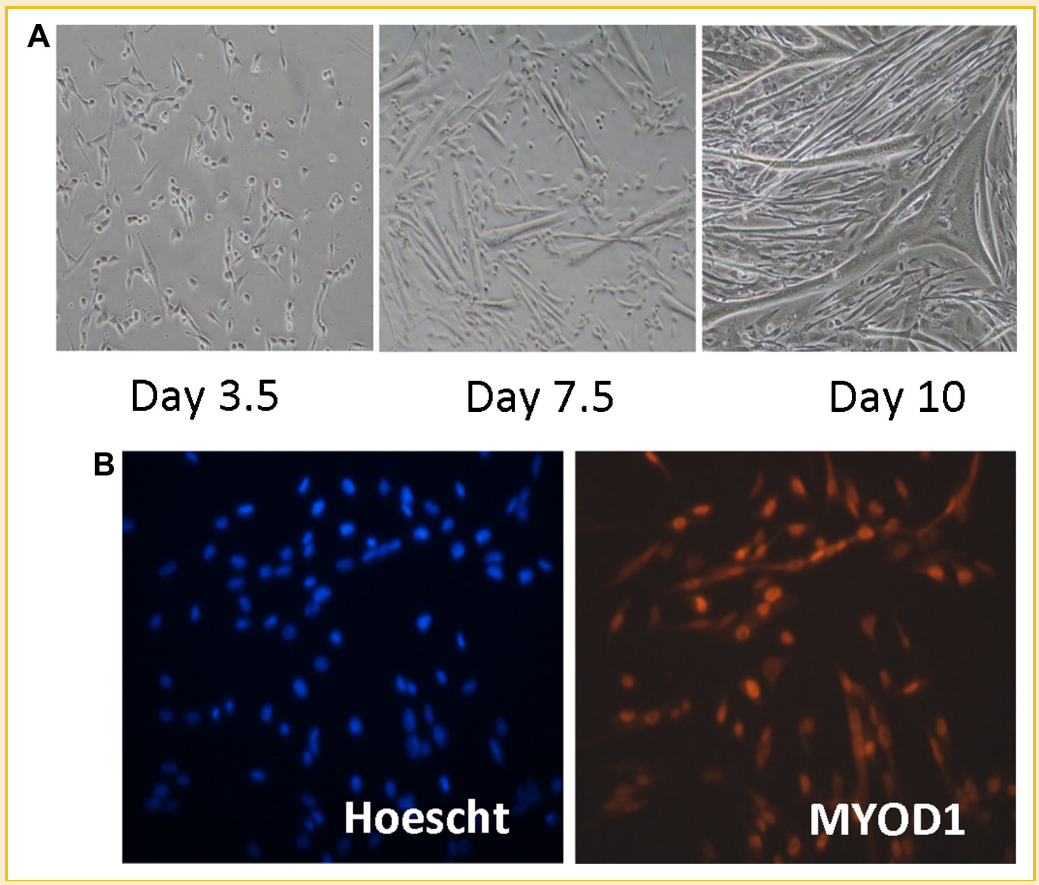

Fig. 1. Proliferation and differentiation process of mouse myogenic cell cultures. A: Appearance of cultures 3.5, 7.5, and 10 days postplating. B: Myogenic origin of the cells was assessed 4.5 days postplating by co-staining for Hoechst and immunocytochemistry for MYOD1. The percentage of cells that expressed MYOD1 at this stage was 70–80%. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

5.5 where proliferation was prominent without significant differentiation process and at day 7.5 where clear differentiation into myotubes was observed. In myoblasts from 7-day-old mice, no significant differences between sexes were found in *Myod1* expression at day 5.5 of culture in EDL (Fig. 6A) or SOL (Fig. 6B), and the same was true for day 7.5 of culture (Fig. 6C and D, respectively). However, starting from age of 40 days and up till 120 days, more actively proliferating male myoblasts expressed generally higher levels of *Myod1* than females in both EDL and SOL muscles (Fig. 6A–D, see Supplementary Table 2 for statistics). The same was observed consistently in days 5.5 and 7.5 of cell culture, despite the fact that in one point this did not reach significance (Fig. 6C, 60 days of age, $P = 0.094$), and in 120-day-old *EDL* females actually expressed more *Myod1* (Fig. 6C, $P = 0.002$). Despite this odd data point, these results are generally consistent with proliferation data that suggests increased activation and proliferative potential of male satellite cells commencing around 40 days of age. Therefore, contrasting results obtained from rats may represent true differences between these two rodents or, alternatively, derive from the use of extracellular matrix components (Matrigel) in our study compared with gelatin by Lagord et al. [1998].

Differentiation of myoblasts in vitro is regulated by many factors, including cell proliferation, migration to establish cell–cell contacts and cell fusion [Seale and Rudnicki, 2000]. Proliferation rate in restricted space, such as cell culture well, is inherently related to the cell density because faster proliferating cells will reach confluence sooner. Myoblasts are known to migrate towards developing myotubes which enhances the fusion process and, therefore, facilitates differentiation. Assuming no differences in differentiation potential per se (e.g., by aberrant cell cycle regulation) one would expect higher proliferation rate to lead to increased differentiation as the cells reach critical density faster. In our conditions, differentiation process was considered prominent at day 7.5 of culture as evidenced by frequent myotube formation was (Fig. 1A). At this timepoint, *myogenin and Mrf4*, markers of differentiation process, were determined. In myoblast cultures from mice of 7 days of age, *myogenin* expression was only significantly ($P = 0.039$) higher in EDL in females compared to males (Fig. 7A) consistent with a increased proliferation in females at this age (Fig. 2A). Expression of *myogenin* in SOL-derived cells, as the proliferation rate, was not affected at this age (Fig. 7B, $P = 0.493$). In contrast, *Mrf4* was not affected in EDL (Fig. 7C, $P = 0.429$) and was downregulated in SOL (Fig. 7D, $P = 0.014$). In 40 days of age, where *Myod1* was upregulated in both muscles of males, we found consistent increase in *myogenin* expression in male EDL ($P < 0.001$) and SOL ($P = 0.038$) (Fig. 7A–D, respectively), *Mrf4* was also

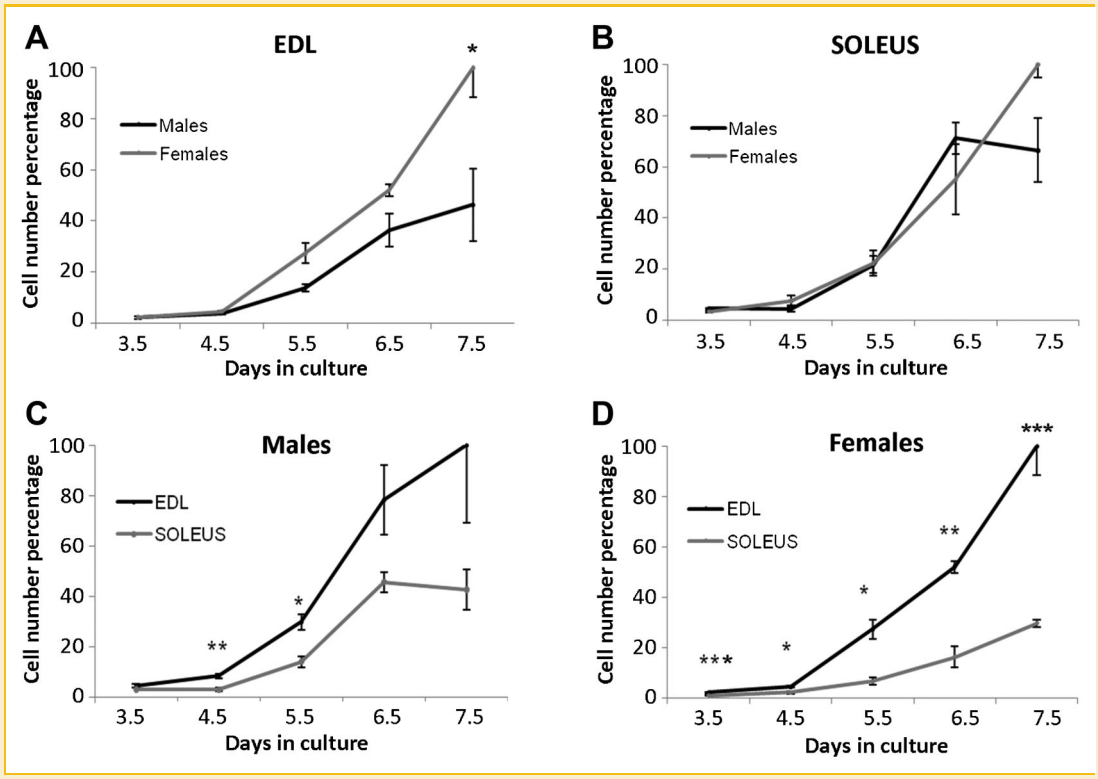

Fig. 2. Myoblast proliferation curves at 7 days of age. Pairwise comparison of male and female myoblasts from (A) EDL and (B) SOL muscles, and between EDL and SOL muscles in (C) males and (D) females. Asterisks denote a student *t*-test *P*-value <0.05 (*), <0.01 (**), or <0.001 (***).

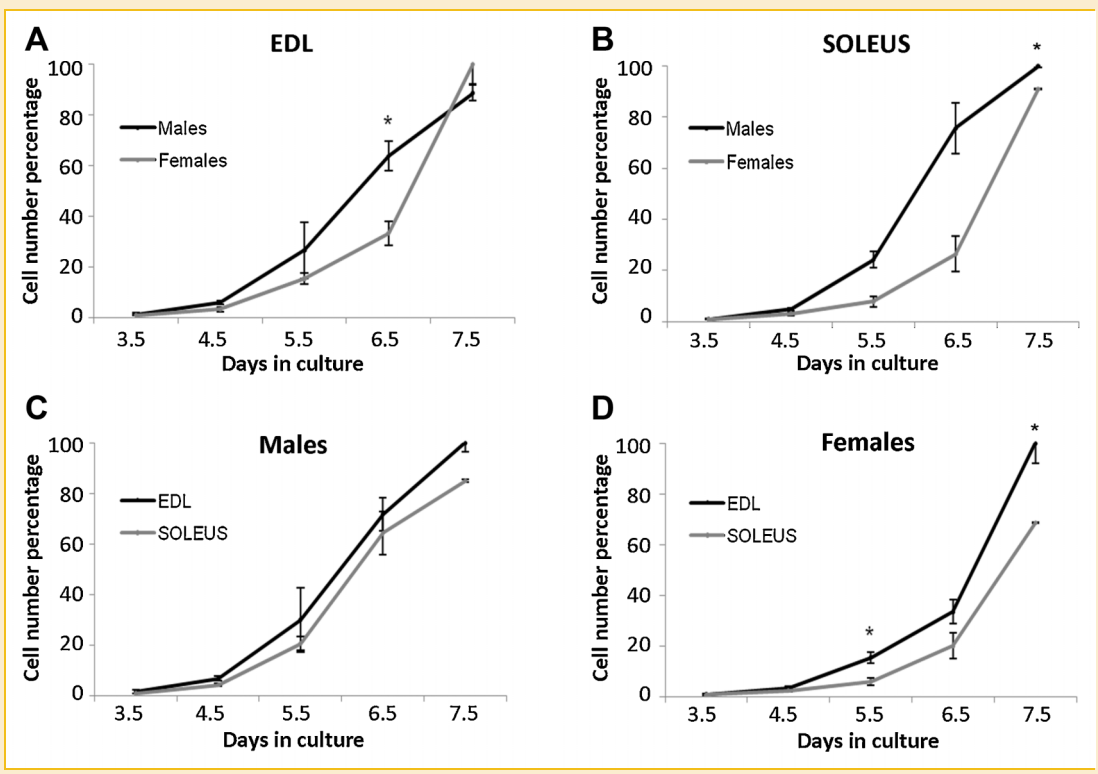

Fig. 3. Myoblast proliferation curves at 40 days of age. Pairwise comparison of male and female myoblasts from (A) EDL and (B) SOL muscles, and between EDL and SOL muscles in (C) males and (D) females. Asterisks denote a student *t*-test *P*-value <0.05 (*).

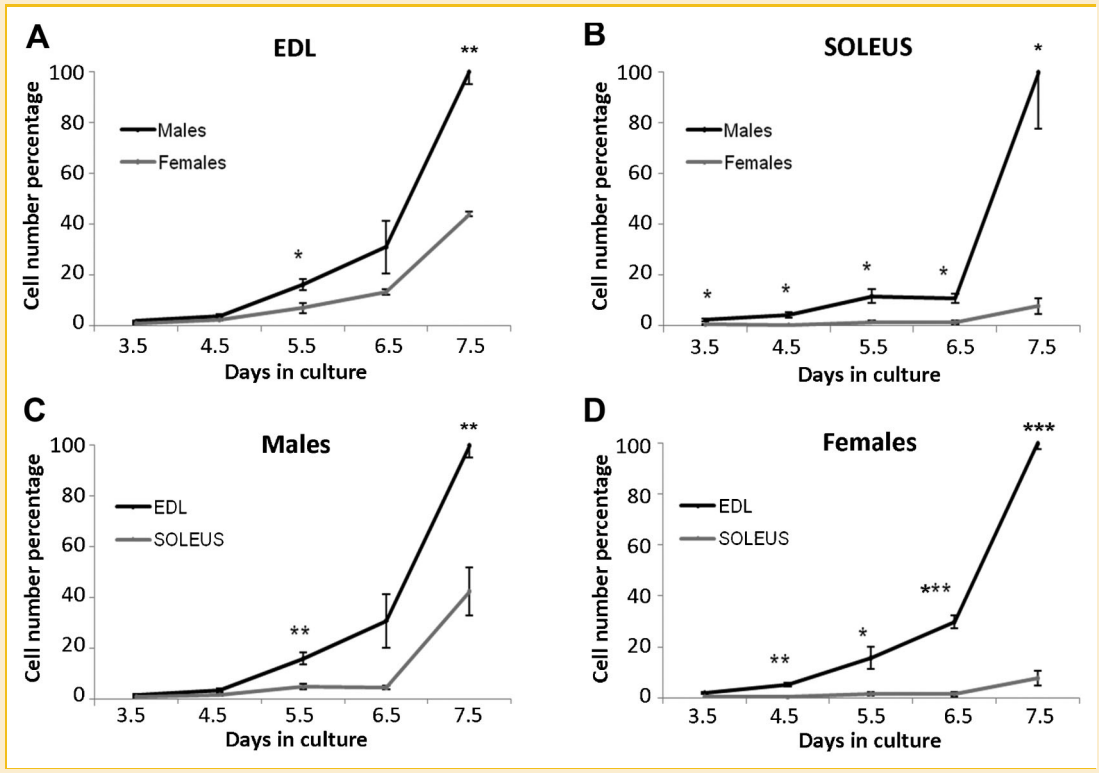

Fig. 4. Myoblast proliferation curves at 60 days of age. Pairwise comparison of male and female myoblasts from (A) EDL and (B) SOL muscles, and between EDL and SOL muscles in (C) males and (D) females. Asterisks denote a student *t*-test *P*-value <0.05 (*), <0.01 (**), or <0.001 (***).

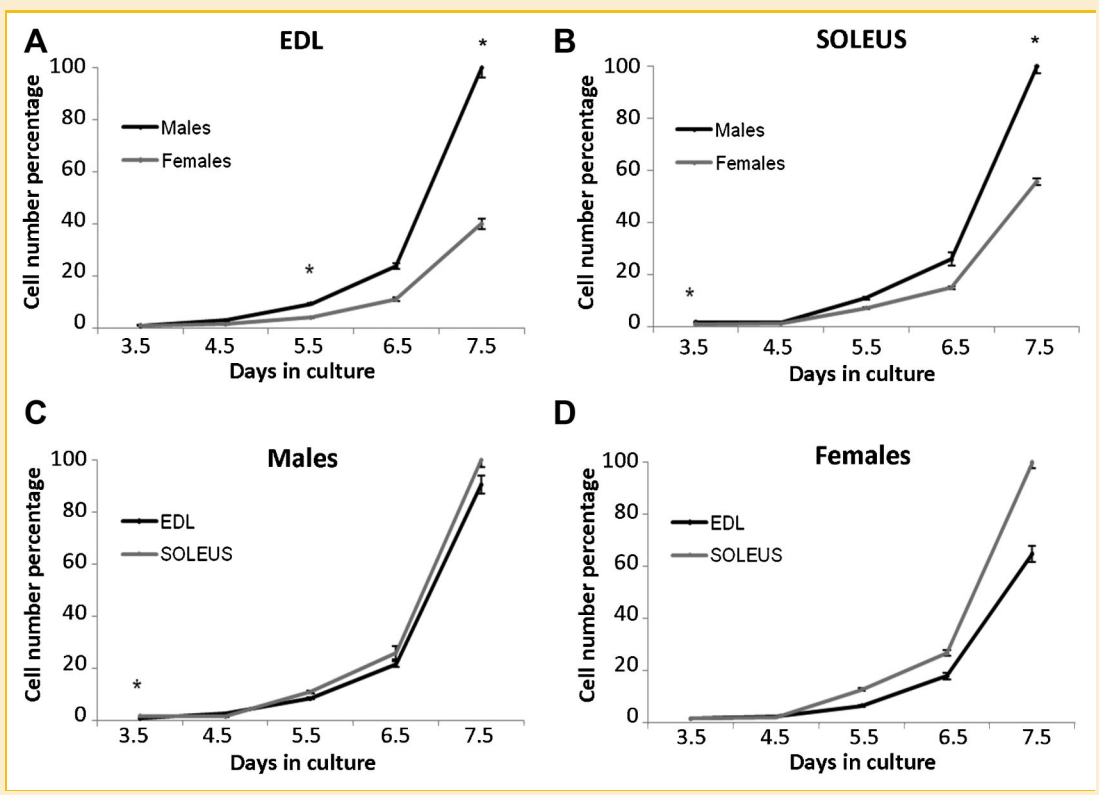

Fig. 5. Myoblast proliferation curves at 120 days of age. Pairwise comparison of male and female myoblasts from (A) EDL and (B) SOL muscles, and between EDL and SOL muscles in (C) males and (D) females. Asterisks denote a student *t*-test *P*-value <0.05 (*).

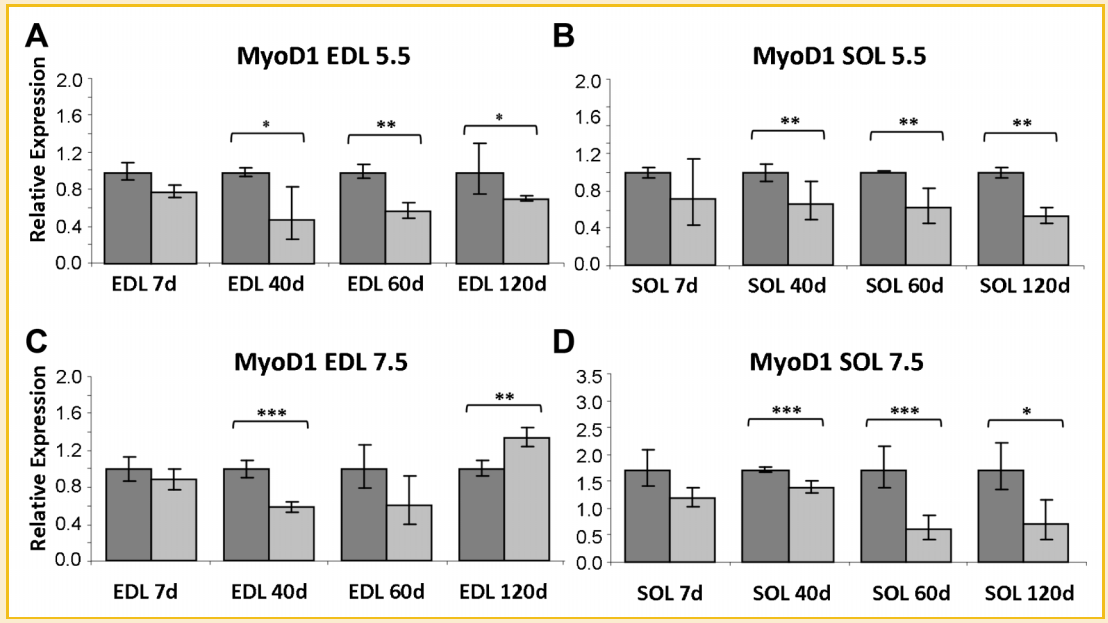

Fig. 6. *Myod1* expression in proliferating and differentiating myoblasts. *Myod1* transcript levels in male (dark gray bars) and female (light gray bars) myoblasts from mice of age 7, 40, 60, and 120 days, shown as relative expression compared with males at each age. (A) EDL and (B) SOL myoblasts at 5.5 days of culture. (C) EDL and (D) SOL myoblasts at 7.5 days of culture. Asterisks denote a student *t*-test *P*-value <0.05 (*), <0.01 (**), or <0.001 (***).

upregulated at this timepoint in EDL (Fig. 7C, $P=0.033$) although in SOL this did not reach significance (Fig. 7D, $P=0.328$). Although both cell proliferation data and increased *Myod1* were consistent with enhanced proliferation rate in males of 60 and 120 days of age, this was not consistently reflected in the level of *myogenin* (Fig. 7A and B) of *Mrf4* (Fig. 7C and D). These observations suggest that females older than 60 days of age, differentiation process may be *altered* in both EDL and SOL compared with males.

Pax7 is marker of quiescent satellite cells, its expression being gradually down-regulated in activated myoblasts as the differentiation process starts [Zammit et al., 2004]. *Pax7* overexpression blocks myogenesis by a mechanism involving regulation of MyoD protein stability [Olguin et al., 2007] and prevents *myogenin* induction promoting cell cycle exit [Olguin and Olwin, 2004]. Here, the analysis of *Pax7* expression in satellite cell/myoblasts of male and females EDL and SOL muscles at 5.5 days in culture revealed a tendency for an upregulation of *Pax7* transcripts in females compared to males. The most prominent and significant differences were found in cultures of 40 days old mice ($P<0.001$ and $P=0.004$ for EDL and SOL, respectively), the tendency was small but consistent in most ages under study and in both EDL and SOL muscles (Fig. 7E and F). The drastically decreased *Pax7* expression in males relative to females at 40 days of age is consistent with enhanced activation of male satellite at this age as measured by increased expression of *Myod1* (Fig. 6), *myogenin* and *Mrf4* (Fig. 7).

DISCUSSION

Distinct characteristics that depend on sex, muscle type, and age are frequently reported in skeletal muscle or muscle derived stem cells, which highlights their importance as a possible source of biological variation [Lagord et al., 1998; Velleman et al., 2000; Mesires and Doumit, 2002; Huang et al., 2006; Deasy et al., 2007]. Especially gender-dependent variation deserves consideration when new innovative approaches for experimental or therapeutic protocols are established since, even if unintentional, sex-biased basic research may lead to qualitatively different and possibly risky interpretations [Check Hayden, 2010]. Whereas cross comparison of studies using different species may already be difficult, additional level of complexity derives from various isolation methods and cell culture techniques carried out in different laboratories. While species-, gender-, or muscle-specific information from different laboratories can actually provide complementary or contrasting information that drives research forward, simultaneus studies of satellite cell proliferation comparing above mentioned parameters are rarely reported. Additionally, studies describing the age-dependent differences in muscle function are largely comparisons of young, old, and senile animals whereas less is known about modulation of muscle stem cell properties during early postnatal development, although two recent studies have elegantly addressed this issue lately ex vivo in mouse EDL [White et al., 2010] and in vitro in pooled hind-limb muscles of male rats [Suzuki et al., 2010]. This is important because most research carried out on muscle stem cells utilize material from relatively young animals, where possible complications with respect to postnatal muscle growth, hormonal status are largely undefined.

By providing myonuclei to postnatally growing muscle fibers satellite cells are essential building blocks for construction of adult musculature. In mice the number of myonuclei reach their adult levels by 3 weeks of postnatal life after which the muscle growth reflects solely increased cytoplasmic volume without alteration in

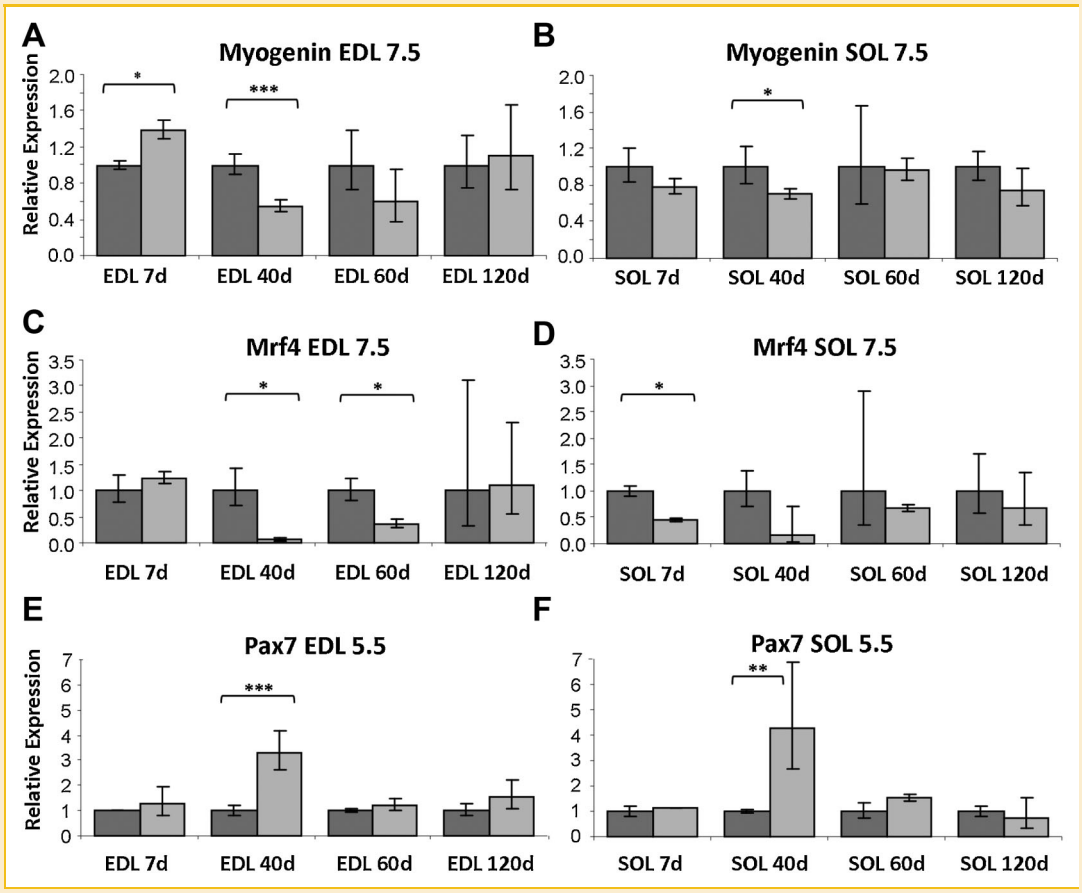

Fig. 7. *Myogenin*, *Mrf4*, and *Pax7* expression in myoblast cultures. Transcript levels in male (dark gray bars) and female (light gray bars) myoblasts from mice of age 7, 40, 60, and 120 days, shown as relative expression compared with males at each age. *Myogenin* expression in differentiating (A) EDL and (B) SOL myoblasts at 7.5 days of culture. *Mrf4* expression in differentiating (C) EDL and (D) SOL myoblasts at 7.5 days of culture. *Pax7* expression in proliferating (E) EDL and (F) SOL myoblasts at 5.5 days of culture. Error bars represent mean ± standard deviation converted to linear scale (Materials and Methods Section). Asterisks denote a student *t*-test *P*-value <0.05 (*), <0.01 (**), or <0.001 (***).

the number of myonuclei [White et al., 2010]. Consistently, satellite cell number in EDL reaches its adult level by age of 3 weeks [White et al., 2010] at which point their metabolic activity declines and they exit from the cell cycle. Although these results clearly imply that determinants of adult muscle in mice are defined in early postnatal life, similar studies have not been carried out yet in slow-twitch muscle, and the sex-dependency, if any, has not been adressed.

In the present work we have described in vitro proliferation potential and MRF transcript levels in mouse satellite cell-derived cultures of the two genders, in EDL and SOL muscles from individuals at 7, 40, 60, and 120 days of age. We found that myoblast proliferation rate of juvenile (7-day-old) mice is higher in fast-twitch EDL of females, whereas that of SOL-derived myoblasts is not affected by sex. However, male myoblasts show increased proliferation rate starting from 40–60 days of age and continuing at least till 4 months of age. In contrast to what has been found in male rats [Lagord et al., 1998], EDL-derived satellite cells from male and female mice generally proliferate faster in vitro that those from SOL muscle, although this difference is possibly abolished in more advanced age. The sex-specific proliferation data is supported by general increase in *Myod1* expression in actively proliferating male-derived cells. However, the expression of *myogenin* and *Mrf4* is not consistently higher in males, but is only significantly up in EDL and SOL of 40-day-old males. At this point, female cells also show greatly increase *Pax7* expression compared with males, which is consistent with the described role of *Pax7* to inhibit myogenesis and cell cycle progression [Olguin and Olwin, 2004] and suggests enhanced capacity to activate quiescent cells in male-derived cells.

These results are partially consistent with the fact that sex hormones may influence satellite cell proliferation and MRF expression. Androgen receptors [Sinha-Hikim et al., 2004] and estrogen receptors [Guo et al., 2010] are expressed in satellite cells. Testicular steroidogenesis in male mice increases dramatically between 30 and 60 days in mice and could possibly explain the lower proliferation rate in 7-day-old males, when testosterone levels are still low. Testosterone stimulation can induce *Myod1* expression in cultured multipotent mesenchymal cells [Singh et al., 2003] whereas *removal* of estrogen by ovariectomy causes similar increase in muscles of female mice [Rogers et al., 2010]. Although these studies may suggest that male and female sex hormones have

opposite effects on MRF expression, effects of testosterone on in vitro myoblast proliferation are contrasting [Chen et al., 2005] and also positive effect of estrogen on satellite cell activation has been described [Enns and Tiidus, 2008]. In any case, in conditions used in our study it seems unlikely that the potential hormonal effects would be persistent enough to carry out their influence through several days of in vitro culture.

Increasing evidence suggests that gender differences in satellite cell function may not be hormonal but innate. In an extensive study carried out in vitro and in vivo using mouse model of Duchennés muscular dystrophy (mdx), muscle-derived stem cells from females exhibited higher regenerative potential compared to males [Deasy et al., 2007]. Based on enhanced tendency of male derived cells to differentiate under oxidative stress in vitro, it was proposed that male and female cells may also exhibit distinct response to oxidative stress induced by muscle injury in mdx mice. In this model, male cells would rapidly activate, proliferate, and differentiate into myotubes whereas female cells would be less proliferative, being able to perform more rounds of proliferation and ultimately differentiate, fuse, and regenerate muscle effectively when acute inflammation is over [Deasy et al., 2007]. Alternatively, female satellite cells may have better survival when transplanted if their expression of *Myod1* is lower than that of males, as satellite cells lacking *Myod1* show increased survival when transplanted to regenerating muscle [Asakura et al., 2007]. Although these studies are consistent with our findings with respect to enhanced proliferation rate and *Myod1* expression of male cells in 40–120 days of age, as well as with increased *Pax7* expresion in 40-day-old females, Deasy et al. [2007] reported no gender differences for in vitro proliferation in the absence of oxidative stress. Several possible reasons could explain the differences, such as different muscle used, different method used to extract muscle derived stem cells, and different age of animals (21 days of age in Deasy et al., 2007]. Although we did not study mice at age of 20 days, it is worth repeating that no consistent sex-dependent effects on MRF expression was observed in juvenile mice (7 days of age), and the effect on proliferation was muscle-dependent.

Clonal analysis carried out recently [Rossi et al., 2010] indicates that satellite cells, even those derived from the same muscle fiber, may exhibit intrinsic differences in their ability to proliferate and differentiate. It was found that cells proliferating at low rate (LPC) spontaneously generate myotubes, whereas highly proliferative cells (HPC) may either differentiate into adipocytes or, when co-cultured with LPCs, eventually form myotubes. In this study, we cannot exclude that satellite cells from females and males may consist of uneven proportions of LPCs and HPCs. Higher proportion of HPCs in males could potentially explain their rapid rate of in vitro expansion. If male cells indeed have higher proportion of HPCs, which are likely to be more sensitive to oxidative stress [Rossi et al., 2010], this would possibly explain why oxidative stress in vitro would favor differentiation of male cultures [Deasy et al., 2007]. When transplanted, HPCs also show lower regenerative potential in vivo [Rossi et al., 2010], consistent with lower regeneration potential of muscle-derived stem cells from males [Deasy et al., 2007]. Although we cannot prove causality in our studies, these observations may suggest higher proportion of HPCs in male muscles, which is warrant further studies. If this is the case, however, differentiation of these cells is not negatively affected in our conditions as no gross defect in *myogenin* and *Mrf4* induction is observed. The possible sex differencas in LPC/HPC content warrant further studies in the future.

The comparison of the myoblast proliferation rate between the two muscle types indicated that cells from EDL, formed mainly of fast-twitch muscle fibers, proliferate faster than cells from SOL, consisting of slow-twitch fibers. This result was true from juvenile (7-day-old) to young, sexually mature mice (60-day-old) in both sexes. However, the situation was reversed in older mice, especially in females where SOL proliferated faster than EDL at age of 120 days. Fast and slow muscle-derived satellite cells have been shown to differ in physiology and, in vitro, are imprinted to produce muscle fibers with distinctive characteristics of their fibers of origin [Huang et al., 2006], that is, muscle fibers engineered in vitro from slow muscle satellite cells contract and relax slower than similar tissues engineered from the fast muscle. This demonstrates that satellite cells from slow and fast-twitch muscles are intrinsically different. Hence, it is not unreasonable that their ability to proliferate and differentiate in vitro is different. Fast-twitch muscle fibers are more abundant than slow-twitch in adult skeletal muscle. It could be hypothesized that during postnatal muscle growth when fast-twitch fiber supply is needed more to increase muscle bulk, cells derived from fast muscle would be capable of proliferating at a higher rate to build up muscular tissue. By contrast, adult slow-twitch fibers posses higher number of satellite cells than fast-twitch fibers [Hawke and Garry, 2001], probably because the first and most frequently recruited muscles during every day activity are slow-twitch type [Hawke and Garry, 2001; Kadi et al., 2006]. These data correlate to our finding that 120 days old SOL satellite cell cultures proliferate at similar (males) or higher rate (females) compared to EDL cultures, thus being capable of responding to typical requirements of adult muscle activity. It is clear that in vitro studies may not perfectly reflect satellite cell dynamics in situ, where their function is likely to be modified by complex interactions in their myofibrillar niche. However, positive switch in relative proliferation capacity of SOL-derived cells in more advanced ages is consistent with relatively higher amount of satellite cells in slow compared with fast muscle in adults.

Gender and muscle type-dependent variation in proliferation and MRF expression were modulated by postnatal age of the animal, perhaps suggesting that satellite cell properties, or relative abundance of high and low proliferating cells, may experience dynamic changes in growing postnatal muscle. In very young mice, no gender differences were found in proliferation and differentiation process in cells from slow-twitch SOL muscle. However, increased cell proliferation and *myogenin* (but not *Myod1*) expression was found in EDL-derived cultures from juvenile females. It is unknown why *Myod1* was not upregulated in these faster proliferating female cells. One potential explanation is that, as discussed above, these results may reflect higher proportion of spontaneously differentiating cells [Rossi et al., 2010] in juvenile females. In satellite cell cultures from 40-day-old mice, higher levels of *all* MRFs were found in males than in females, in both EDL and SOL muscles (although Mrf4 in SOL was only suggestive). Indeed, in

this postnatal age the sex-dependent effect on MRF expression were most consistently male biased, and increased *Myod1* and *myogenin* expression was correlated with relatively decreased *Pax7* expression. Collectively, this suggests depletion of quiescent satellite cell pool in fastly proliferating and differentiating male cells. This result is in concordance with higher in vitro myoblast fusion index in males compared to females in 7-week-old turkeys [Velleman et al., 2000]. Male cells continued to generally express higher levels of *Myod1* in myoblasts derived from 60 to 120-day-old muscles (except in EDL 7.5-day culture). However, *myogenin* and *Mrf4* were not consistently affected in EDL or SOL, although relatively high variation in the replicate cell cultured prevented significance in some cases. Increasing the number of replicates was not feasible because of high number of variables investigated (sex, age, and muscle type), and to avoid variation in the cell quality the priority was set to extract and process both muscles and both genders at the same time. Nevertheless, realtively lower *Myod1* in females coupled with similar levels of *myogenin* as in males may suggest that female cells may be prone to differentiate earlier in vitro. This early differentiation is not likely to depend on cell density as females at this stage proliferated much slower than male cells. These results encourage further research on potential differences on cell cycle regulators, as well as detailed characterization of differentiation capacity in different sexes and muscle types.

To our knowledge, this is the first study investigating the specific influence of muscle fiber-type muscle, sex, and postnatal age in the same experimental and cell culture conditions. We have concluded that male satellite cells exhibit higher proliferation rate compared to females in EDL and SOL muscles from young to adult mice, and in EDL muscle compared to SOL in both sexes from juvenile to young mice. The proliferation rates are generally reflected in the expression of MRF genes, although these changes are modulated by postnatal age of the animals. The results presented strongly support the notion that the satellite cell heterogeneity is not only externally induced but also an intrinsic character of these cells. Therefore, our study remarks the importance of gender, muscle type, and age as important factors to understand postnatal muscle growth and regeneration. These results may have special importance for research on regenerative medicine of muscle or neuromuscular disorders, where the source of the regenerative cells has to be appropriate to ensure the efficacy of the satellite cell-mediated therapy. Recently, effort has been taken to transplant intact or genetically modified satellite cells to alleviate neuromuscular disease in animal models [Lee et al., 2000; Montarras et al., 2005; Deasy et al., 2007]. Therefore, our results indicate that, to ensure unbiased interpretation before clinical application, further work should be carried out preferably in the two sexes and, if possible, using muscle types closely resembling those of the target tissue.

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