Journal of
Cellular
Physiology

# Exercise Running and Tetracycline as Means to Enhance Skeletal Muscle Stem Cell Performance After External Fixation

G. SHEFER,[1] E. CARMELI,[2] G. RAUNER,[1] Z. YABLONKA-REUVENI,[3] AND D. BENAYAHU[1]*

[1]*Department of Cell and Developmental Biology, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel*

[2]*Department of Physical Therapy, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel*

[3]*Department of Biological Structure, School of Medicine, University of Washington, Seattle, Washington*

Prolonged limb immobilization, which is often the outcome of injury and illness, results in the atrophy of skeletal muscles. The basis of muscle atrophy needs to be better understood in order to allow development of effective countermeasures. The present study focused on determining whether skeletal muscle stem cells, satellite cells, are directly affected by long-term immobilization as well as on investigating the potential of pharmacological and physiological avenues to counterbalance atrophy-induced muscle deterioration. We used external fixation (EF), as a clinically relevant model, to gain insights into the relationships between muscle degenerative and regenerative conditions to the myogenic properties and abundance of bona fide satellite cells. Rats were treated with tetracycline (Tet) through the EF period, or exercise trained on a treadmill for 2 weeks after the cessation of the atrophic stimulus. EF induced muscle mass loss; declined expression of the muscle specific regulatory factors (MRFs) Myf5, MyoD, myogenin, and also of satellite cell numbers and myogenic differentiation aptitude. Tet enhanced the expression of MRFs, but did not prevent the decline of the satellite cell pool. After exercise running, however, muscle mass, satellite cell numbers (enumerated through the entire length of myofibers), and myogenic differentiation aptitude (determined by the lineal identity of clonal cultures of satellite cells) were re-gained to levels prior to EF. Together, our results point to Tet and exercise running as promising and relevant approaches for enhancing muscle recovery after atrophy.

J. Cell. Physiol. 215: 265–275, 2008. © 2007 Wiley-Liss, Inc.

Skeletal muscle is a dynamic tissue that, according to the load of use or disuse, adapts by cellular and metabolic changes muscle mass and strength (Booth and Gollnick, 1983). For example, muscle disuse due to limb immobilization results in a progressive decrease in muscle mass, a process termed atrophy. Skeletal muscle loss occurs under additional various circumstances, including space flight, chronic health disorders, and aging. Loss of muscle mass and strength is generally ascribed to poor prognosis in disease (Jespersen et al., 2006) and to reduced functioning that may persist for months after immobilization (Hortobagyi et al., 2000). Age-associated muscle atrophy is termed 'sarcopenia', and often leads to disability and mortality in the elderly (Roubenoff, 2004; Karakelides and Sreekumaran Nair, 2005). Currently, there are no effective pharmacological agents that attenuate muscle atrophy or enhance recovery from it.

The deleterious effects of muscle disuse were mainly studied in hindlimb suspension models with most attention paid to signaling pathways that are involved in protein metabolism (Appell, 1990; Glass, 2003). Less is known about the involvement of skeletal muscle stem cells, satellite cells, in the etiology of muscle-disuse atrophy or in recovering from it, albeit the known association with the loss of myofiber nuclei, and reduced number of muscle precursor cells (Mitchell and Pavlath, 2001; Jejurikar and Kuzon, 2003).

The possible involvement of satellite cell function in atrophy was suggested in studies on limb immobilization or aging. Specifically, depletion of proliferating cells (presumably muscle precursor cells) by $\gamma$-irradiation of an immobilized muscle prevented the recovery from atrophy (Mitchell and Pavlath, 2001). It has been suggested that the regulation of satellite cell performance and pool size may be compromised in sarcopenia (Chakravarthy et al., 2000; Conboy et al., 2003; Shefer et al., 2006). Accordingly, we opted to elucidate the relationships between limb immobilization and satellite cells with the view that this will provide better understanding not only of the functional changes induced by lack of activity but also of muscle deterioration in general, regardless of etiology.

The essence of satellite cells for myofiber maintenance was demonstrated with Pax7 null mice where satellite cells were no longer present after early postnatal growth. Pax7 is expressed by satellite cells and their proliferating progeny (Seale et al., 2000; Collins et al., 2005; Shefer et al., 2006). In Pax7 null mice, all satellite cells die during postnatal growth and muscles show severe atrophy and extreme regeneration deficit after injury (Kuang et al., 2006; Relaix et al., 2006). The molecular control of satellite cell proliferation and differentiation during muscle regeneration is a tightly controlled process where the muscle regulatory transcription factors (MRFs) Myf5, MyoD, myogenin and MRF4 are key players (Perry and Rudnick, 2000). MRFs are upregulated in a chronological manner in satellite cells and their progeny, marking distinct phases of myogenic differentiation (Zammit et al., 2006). The proliferative phase of myogenesis is characterized by co-expression of Pax7 and MyoD. Induction of myogenin along with a decline in Pax7 mark the differentiation

*Correspondence to: Dafna Benayahu, Department of Cell and Developmental Biology, Sackler School of Medicine, Tel-Aviv University, Tel-Aviv 66978, Israel. E-mail: dafnab@post.tau.ac.il

Received 19 June 2007; Accepted 30 August 2007

DOI: 10.1002/jcp.21306

© 2007 WILEY-LISS, INC.

of satellite cell progeny (myoblasts), a phase followed by myoblast fusion into myotubes (Yablonka-Reuveni and Rivera, 1994; Seale et al., 2000; Zammit et al., 2006). Myf5 is expressed in satellite cells and in myoblasts, and is downregulated after the formation of myotubes (Beauchamp et al., 2000; Zammit et al., 2006; Day et al., 2007). In light of the crucial role of satellite cells in maintaining muscle mass and integrity, the purpose of this study was to examine how bona fide satellite cells are affected by (i) atrophic conditions induced by limb immobilization and (ii) pharmacological and physiological countermeasures.

To achieve relevance to clinical and physiological situations we used external fixation (EF) as a model of limb immobilization. EF is a procedure used to treat unstable bone fractures, ligament ruptures, and degenerative diseases of joints (LaBianco et al., 1996; Pacheco and Saleh, 2004; Zhang, 2004; Ferreira et al., 2006). EF is known to inflict sustained inflammation and atrophy that are accompanied with elimination of nuclei from myofibers (Vescovo et al., 2000; Liu et al., 2005; Dupont-Versteegden, 2006; Dupont-Versteegden et al., 2006). We therefore investigated the potential of pharmacological (tetracycline) and physiological (exercise running) measures to mitigate muscle loss and/or to stimulate re-growth after EF. The effect of an anti-inflammatory drug, tetracycline (Tet), was investigated based on previous studies showing that inflammatory processes associated with disuse atrophy, including EF, abrogated muscle regeneration by interfering or inhibiting muscle differentiation (Guttridge et al., 2000; Langen et al., 2004; Bar-Shai et al., 2005; Mourkioti and Rosenthal, 2005; Degens and Alway, 2006). For exercise running, we assumed that it will increase the number of satellite cells since it was proposed that exercise induces skeletal muscle hypertrophy by affecting satellite cells (Kadi et al., 2005). Altogether, the purpose of this study was to shed light on the involvement of satellite cells in processes that occur during muscle disuse and on the extent to which these cells are affected by pharmacological or physiological measures.

## Materials and Methods

### Animals

Male Long-evans rats (n = 60, 6 months old, 450–500 gr) were used for the experimental procedures conducted according to the Institutional Animal Care and Use Committee of the Tel-Aviv University (number M05-116). Rats were randomly assigned to either one of the following two experiments:

## Experimental Design

### Experiment 1: External fixation (EF) and tetracycline (Tet) treatment

This experiment comprised six groups (n = 5/group) in which the right hindlimb was externally fixated and rats treated or untreated with Tet. Groups that were externally fixated for periods of 1,3, and 4 weeks (EF-Tet groups). Three additional groups (n = 5/group) were treated with saline for the respective periods of EF (EF-Sal group). Tet 1 ml/1 kg (Engemycine 10%, Abic veterinary, Beit Shemesh products LTD, Israel) was injected into the non-immobilized hamstring muscle of the externally fixated limb 2 days after fixation procedure, and every other day thereafter. Matching volumes of saline solution were injected to the hamstring muscle of the contralateral (left) non-fixated hindlimbs at the same time schedule as Tet.

### Experiment 2: External fixation (EF) and mild daily exercise running

This experiment comprised 4 groups (n = 6/group) in which the right hindlimb was or was not externally fixated for 3 weeks. Three days after the removal of EF one externally fixated and one non-externally fixated group exercise ran on a treadmill (EF-Run, No EF-Run groups). Two additional groups remained in their home cages for the respective period of running (EF-No Run, No-EF-No Run groups). After 3 weeks of EF rats were anesthetized and the EF was removed as described below. Generally, the knee joint area of EF rats appeared healthy. Rats were allowed to recuperate for 2 days prior to the beginning of exercise running sessions.

### External fixation of the knee

To assess the effect of immobilization on skeletal muscles we used a model of unilateral (EF). Rats were anesthetized with isofluorane (Rhodic) using an anesthetic machine (Ohmeda) with medical-grade oxygen (2–3% vol), hindlimbs were shaved, disinfected with iodine, and the right knee of rats in the EF-groups was fixated in a 45° flexion position. To form a rigid frame, a Kirschner wire (0.8 mm diameter and 38 mm in length) was inserted to the lateral plane of the femur about 1.2 cm above the knee joint (femur wire); a second Kirschner wire was inserted about 1.5 cm below the knee joint, at the lateral plane of the tibia bone (tibia wire). Femur and tibia wires perturbed to the left and right sides of the knee joint (approximately 1.5 cm proximal and distal to the knee joint's transverse plane). Wire ends were then inserted into upper and lower longitudinal slots (13 mm) of threaded brass rods (4.8 mm in diameter and 33 mm in length). Wires were secured to brass rods by connector screws (Fig. 1). EF device weighed about 12 gr. The quadriceps and gastrocnemius muscles were immobilized, but there was weight bearing on the fixated limb. The gastrocnemius muscle was chosen to be further analyzed for two main reasons: (i) it affects both the knee and ankle joints (while the hamstring muscle affects only the knee joint) and (ii) it is relatively a homogeneous muscle in terms of fiber type, mainly composed of fast twitch fibers (compared to the mixed composition of fast- and slow-twitch fibers of the quadriceps muscle).

### External fixation removal

Before removal of the EF device, rats were anesthetized as described above, the screws securing the femur and tibia wires to the brass rods were unscrewed, and wires were pulled out of the knee joint. Rats were weighed; the gastrocnemius muscle was excised, cleaned from surrounding fat and connective tissues, and weighed.

### Treadmill, acclimation, and running protocols

A motorized low-noise treadmill (running area = 41 × 114 cm, Horizon ID 100) was adjusted to slow-down to a speed range of 0–2 km/h (at 0.1 km/h increment steps), a range that meets the physiological running capacity of rats. A custom-designed Plexiglas mount (treadmill enclosure) was placed 1 cm above the belt, dividing the belt surface into six compartments (12 × 45 × 20 cm each), allowing six rats to run simultaneously at the same belt speed (Fig. 1B). All the compartments were covered with a wire mesh, and a tray was placed at the rear side of the treadmill to collect the feces. The treadmill, with a horizontal belt, was placed in a quiet, temperature-controlled room.

To reduce stress that could be elicited by the experimenter or treadmill apparatus, a month prior to the beginning of the experiment each rat was daily held and pet for several minutes (handling). A week prior to the beginning of experiment rats that were assigned to the exercise running group were introduced daily to the treadmill compartments without operating the belt, allowing the rats to explore and habituate to the apparatus for 10 min. Then, treadmill was activated at a low speed (0.3 km/h, 0% grade) for 5 min. After

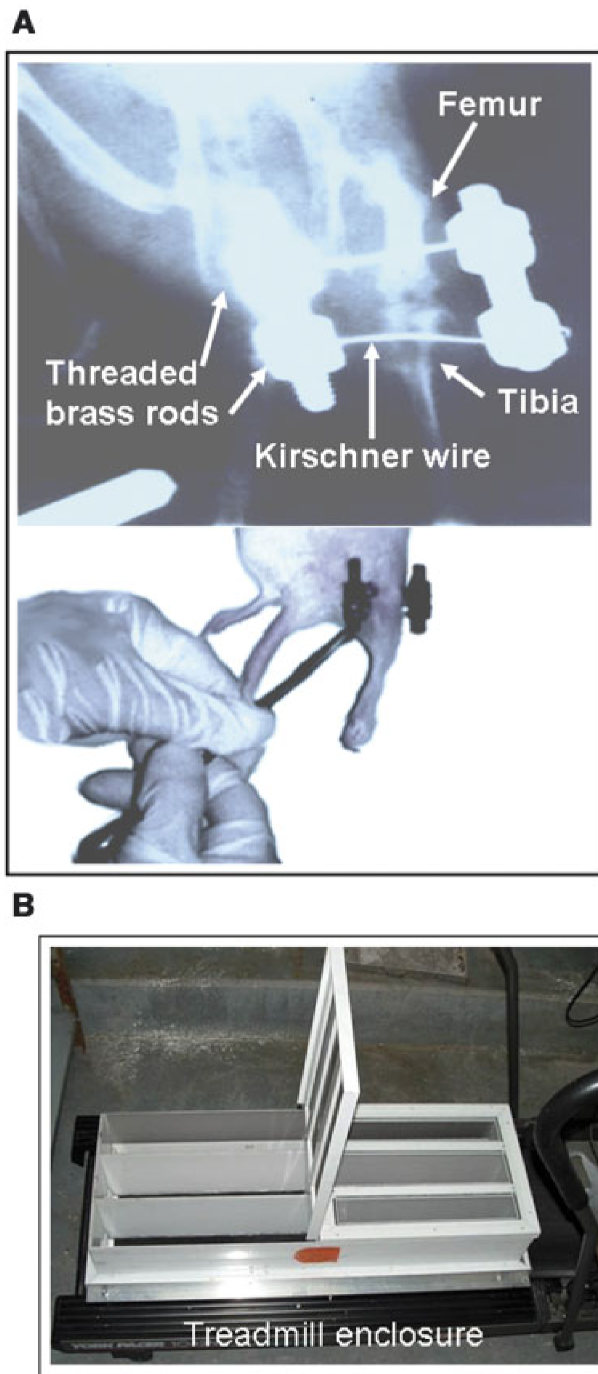


**Fig. 1. An X-ray image of the EF device attached to the right hindlimb of a rat (A). A six compartment custom-designed Plexiglass mount, treadmill enclosure, was placed 1 cm above the treadmill belt and allowed six rats to run simultaneously at the same belt speed. A wire mesh covers the compartments (B). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]**

acclimation period, running sessions began and rats were tested for 20 min at a speed of 15 m/min (0% grade). We chose a protocol of daily moderate running, since it inflicts myogenesis of satellite cells (Jejurikar and Kuzon, 2003) in a mechanism that resembled muscle re-growth after atrophy. Thus this type of running was assumed to accelerate natural processes of muscle recovery, although it is not suggested here that hypertrophy is simply the converse of atrophy. Running sessions were held at the same time of day, during the active phase of rat activity (dark), for 2 weeks (6 consecutive days/week). This running intensity was designed to correspond the 75% of VO2max that was specified in (Lawler et al., 1993) as high for senile animals, and as moderate for young-adult animals (Thomas et al., 2000). Non-running rats were exposed to the same environmental conditions (such as treadmill motor noise and vibration) during time other animals performed their daily exercise session.

### Muscle composition measurements

Rats were scanned in a dual energy X-ray absorptiometry (DEXA-Lunar PIXImus, Lunar Corp., Madison, WI) before and after EF and after exercise running. During DEXA scanning, animals were anesthetized with isofluorane. Total gastrocnemius mass was determined from the peripheral dual-energy X-ray absorptiometry images.

### Gastrocnemius muscle excision

After 2 weeks of exercise running rats were weighed, right and left gastrocnemius muscles were excised, weighed, and placed in enzyme solution (0.2% collagenase in DMEM). Enzyme solution containing the muscle was labeled, incubated and single fiber isolation proceeded as described below. Muscles from fixated and non-fixated contralateral limbs were analyzed separately. Single myofibers were isolated from the hindlimb gastrocnemius muscles to assess the abundance and differentiation potential of satellite cells. The isolated muscles were digested in enzyme solution for 90 min. Myofibers were either cultured individually in Matrigel-coated wells and fixed after initial adherence for 3 h as described in (Shefer and Yablonka-Reuveni, 2005; Shefer et al., 2006) or served for cloning resident satellite cells (Shefer et al., 2004, 2006).

### Gene expression analysis by PCR

After the EF removal, muscles were cleaned, weighted, and immediately frozen in liquid nitrogen for further molecular analyses. Total RNA was extracted from 30 mg gastrocnemius muscle tissue using SV total RNA isolation kit (Promega Z3100, Madison, WI). The quantity of RNA was determined using NanoDrop spectrophotometry (NanoDrop Technologies, Wilmington, Delaware). From each sample, 100 ng of total RNA was reverse transcribed to cDNA using avian myeloblastosis virus reverse transcriptase (AMV-RT) and oligo-dT (Takara Shuzo Co. Ltd., Japan) according to manufacturer's protocol.

*Semi-quantitative PCR* analyses were performed according to (Shur et al., 2001). In brief, we used PCR mix (Sigma-Aldrich, Rehovot, Israel) in a 10 μL total volume using 1 μL of cDNA and 10 pmoles of forward and reverse primers per reaction (Table 1). Cycling parameters were 95°C for 30 sec, 58°C for 30 sec, 72° for 1 min, with a final extension step of 72°C for 10 min. The number of amplification cycles was 24 for G3PDH and 30–33 for all other genes. PCR products were loaded on 1% agarose gels (SeaKem GTG, FMC, Rockland) in Tris Borate EDTA (TBE) buffer; gels were soaked in a 0.5 μg/ml ethidium bromide solution for 15 min to allow the visualization of DNA fragments on a UV transilluminator. The optical density of bands was measured by Bio-Imaging System, BIS 202D and analyzed using "TINA" software. PCR amplification with genes of interest and G3PDH was performed at least twice.

*Quantitative PCR* was performed as previously described (Shur et al., 2007) with a Stratagene MX 3000P$^{TM}$ real-time PCR system (Stratagene, La Jolla, CA). In Brief, to monitor target gene amplification we used Brilliant SYBR Green QPCR Master Mix kit (Stratagene). The thermal cycling conditions comprised an initial Taq heat-activation step at 95°C for 10 min and

TABLE 1. Primers used for PCR assays

| Gene | Primers | Product size (bp) | References |
|---|---|---|---|
| Pax7 | GAA AGC CAA ACA CAG CAT CGA<br>ACC CTG ATG CAT GGT TGA TGG | 466 | Day et al. (2007) |
| Myf5 | CAG CCA AGA GTA GCA GCC TTC G<br>GTT CTT TCG GGA CCA GAC AGG G | 440 | Kastner et al. (2000) |
| MyoD | CAC ACT TCC CCA CTA CGG TGC<br>CAC TGT AGT AGG CGG CGT CGT AG | 506 | Kastner et al. (2000) |
| Myogenin | AGT GCC ATC CAG TAC ATT GAG CG<br>GGG TGG AAT TAG AGG CGC ATT A | 631 | Kastner et al. (2000) |
| G3PDH | ACCACAGTCCATGCCATCAC<br>TCCACCACCCTGTTGCTGTA | 450 | Shur et al. (2004) |

45 cycles of 95°C for 30 s, 58°C for 1 min, and 72°C for 1 min. To differentiate between true (above background) amplification of a gene of interest from enhanced SYBR Green I fluorescence due to primer–dimers or non-specific product formation, a dissociation curve was generated. Dissociation curve comprised fluorescence measurements of PCR products that are subjected to a stepwise increase in temperature from 55°C to 95°C; fluorescence is measured at every temperature increment. When temperature reaches 95°C, the accumulating fluorescent measurements were plotted against temperature (MxPro™ QPCR Software, Stratagene). A standard curve was then used to derive the initial template quantity. Experiments were performed with triplicates for each data point.

### Immunostaining and imaging

Fibers and cultured cells were stained with anti-Pax7 (IgG1 Developmental Studies Hybridoma Bank (DSHB); 1:2000 dilution); anti-MyoD (IgG1, clone 5.8A, BD Biosciences; 1:400). Antibodies were diluted in blocking solution (1% normal goat serum in TBS) and samples were single or double immunostained as previously described (Shefer et al., 2004). Observations were done with inverted fluorescent microscope (Zeiss, Axiovert 200M, Göttingen, Germany). Images were acquired with an AxiocamMRm monochrome CCD camera. The CCD camera drive and color acquisition were controlled by Axiovision4.4 Imaging System. Composites of digitized images were assembled using Adobe Photoshop software.

### Statistical analysis

Statistical analyses were performed using Statistica 7.1 package. Unless noted otherwise, data was analyzed using multiple analysis of variance (ANOVA), and our statistical significance reporting criteria were $P < 0.05$ and $P < 0.01$. When ANOVA pointed to significant differences, posthoc tests were conducted to establish the contribution of each group to the apparent significance. In cases when data were presented as percentages, data were subjected to an arc-sinus of square root-transformation in order to meet the criteria of the ANOVA method.

## Results

Here we studied the effect of atrophic conditions induced by limb immobilization due to (EF) on satellite cell abundance and myogenic properties as well as the potential of pharmacological and physiological means to counter the EF-induced deleterious effects.

### Pharmacological approach: Tetracycline treatment throughout the external fixation period

The expression levels of Pax7 and MRFs (Myf5, MyoD, and myogenin) served to investigate changes in the satellite cell pool and in myogenesis of their progeny. For this, rats underwent EF of their right hindlimb for short (1 week) or long-terms (3 or 4 weeks) and were treated or untreated with Tet through the EF period. The expression levels of MRFs in the gastrocnemius muscles were analyzed by means of RT-PCR and were compared between EF and No-EF muscles of Tet-treated or untreated rats. In Tet-treated groups several transcription factors were additionally analyzed by qPCR to quantify and compare their levels between muscles.

In untreated rats Myf5 was expressed in muscles of both hindlimbs after 1 week of fixation, and only in the muscles of the No-EF limb after 3-weeks of fixation. In Tet-treated rats, however, Myf5 mRNA was expressed in muscles isolated from either EF or No-EF limbs regardless of the duration of EF (Fig. 2A). These data show that activation and proliferation of quiescent satellite cells was induced in No-EF and EF limbs, probably supporting compensatory growth and myofiber re-growth, respectively only in short term EF. Under Tet treatment, however, these processes are sustained even after prolonged periods of EF (Fig. 2A). To reveal whether Tet exerts differential effects on EF and No-EF limbs, in light of the different processes that occurred in EF and No-EF muscles of the untreated group, Myf5 expression was also analyzed by means of qPCR. Expression levels were significantly higher in muscles from EF compared to No-EF limbs after short and long-term fixation (Fig. 2B, two way ANOVA, $F\ (2,\ 12) = 60\ P < 0.05$).

The effect of Tet on satellite cells was investigated by quantifying the expression levels of Pax7. As shown in Figure 2B, Pax7 expression was lower in muscles from the EF compared with No-EF limb after long-term EF (two way ANOVA, $F(2,\ 12) = 38\ P < 0.05$). The decreased expression of Pax7 and enhanced expression of Myf5 may imply that under Tet-treatment, the long-term EF, inflicted either enhanced differentiation or did not prevent satellite cell loss. Consequent stages of differentiation were assessed based on the expression of MyoD and myogenin. As shown in Figure 2A, MyoD was induced in the Tet-treated group at high levels in the EF-limb after 1 and 3-weeks and declined after 4 weeks of fixation. In contrast, in the untreated group only low levels of MyoD were detected in muscles of the No-EF limb after 3 weeks of EF; comparable low levels of MyoD were detected in the No-EF limbs of the Tet-treated rats. Tet-treatment also induced high levels of myogenin expression in the EF limbs after short-term EF; these levels declined after long-term EF but were still considerably high compared to levels detected in the No-EF limbs (Fig. 2A). The lower levels of myogenin expression detected after long-term EF were comparable to myogenin levels in both hindlimbs of untreated rats. We used qPCR to measure the effect of EF on myogenin expression under Tet-treatment, and revealed that myogenin was expressed 5, 6, and 30 fold more in the EF compared to the No-EF limb (Fig. 2B, two way ANOVA, $F(2,12) = 130\ P < 0.01$). These data indicate that under Tet-treatment rigorous regeneration occurred in muscles of EF limbs after short-term EF, a process that moderates after long-term EF.

The prime effect of Tet on the expression pattern of MRFs was detected after 3-weeks of EF. Specifically, high levels of Myf5 and MyoD were detected in muscles of the EF limb in the

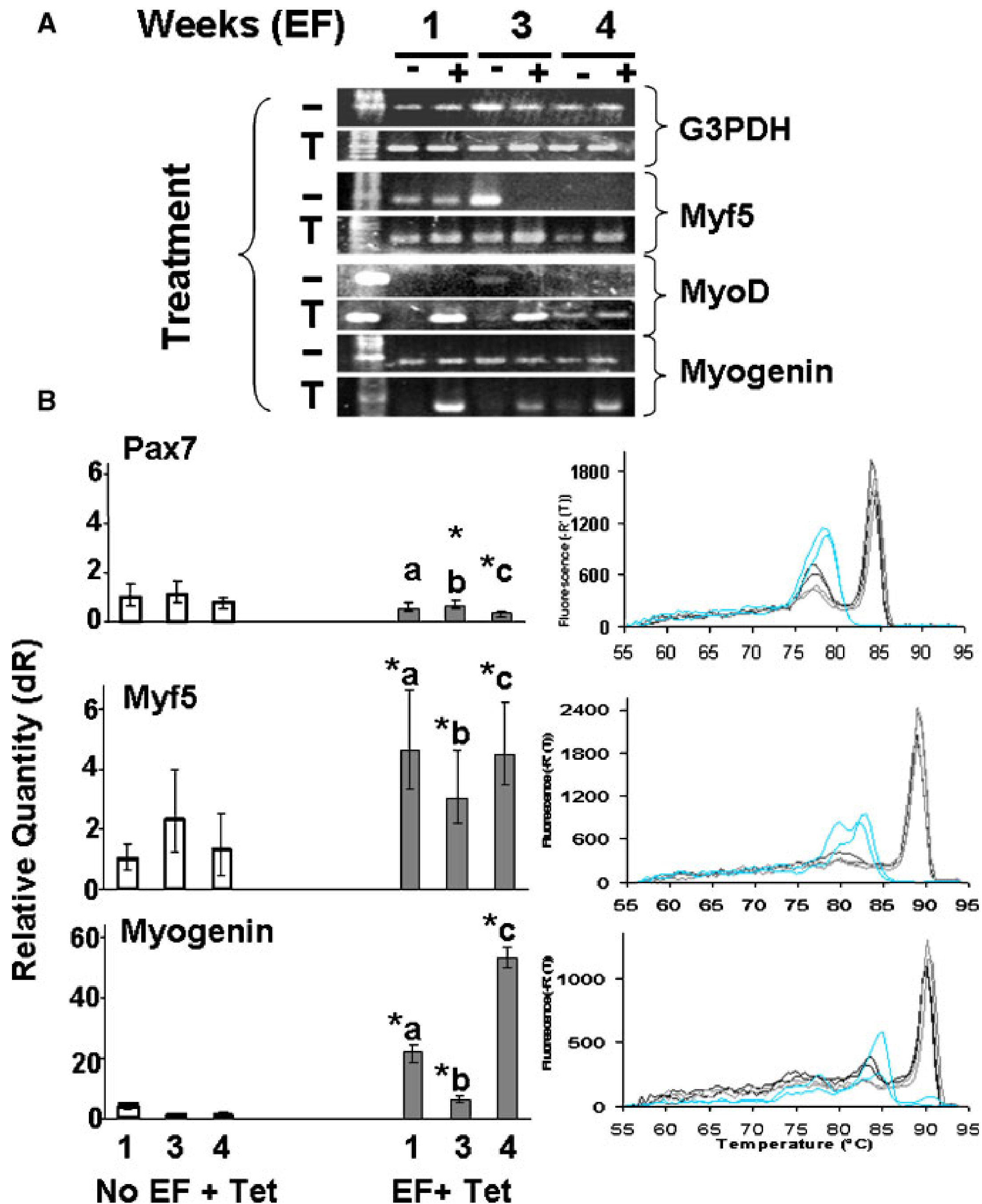


Fig. 2. RT-PCR (A) and qPCR (B) analyses of gene expression in the gastrocnemius muscles of Tet-treated or untreated rats. Muscle regulatory factors from the EF (+) and No-EF (−) limbs of animals treated (T) or untreated (−) with Tet were detected by reverse-transcription PCR (A). Myf5, myogenin, and Pax7 expression was additionally quantified by qPCR for Tet-treated animals in the No-EF (open bars) and EF (gray bars) limbs. Bar histograms and error bars indicate the standard error of the mean of triplicates for each data point and significant differences are marked with asterisks. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Tet-treated group, but not in the untreated group. After 4-weeks of EF, levels of Myf5 and MyoD declined in the Tet-treated group. Data imply that myogenesis can be enhanced after long-term EF by Tet-treatment, with best effect observed after 3-weeks of fixation. In order to compare the effect of the pharmacological (Tet administration) and the physiological intervention (exercise running), a second set of experiments was designed to study the rehabilitating effect of exercise running after 3-weeks of EF.

To investigate the effect of Tet-treatment on muscle mass after 3-weeks of EF, the gastrocnemius muscle was excised and weighted from the following three groups: No-EF No-Tet; EF-Tet; and EF-No-Tet. As depicted in Figure 3A, there was a 6% decline in gastrocnemius muscle weights in all EF groups, regardless to Tet-treatment compared to controls (No-EF No-Tet group; ($P < 0.05$)). There was no difference in EF-muscle weight between tet-treated and untreated groups. These data, obtained immediately after the EF period, imply that

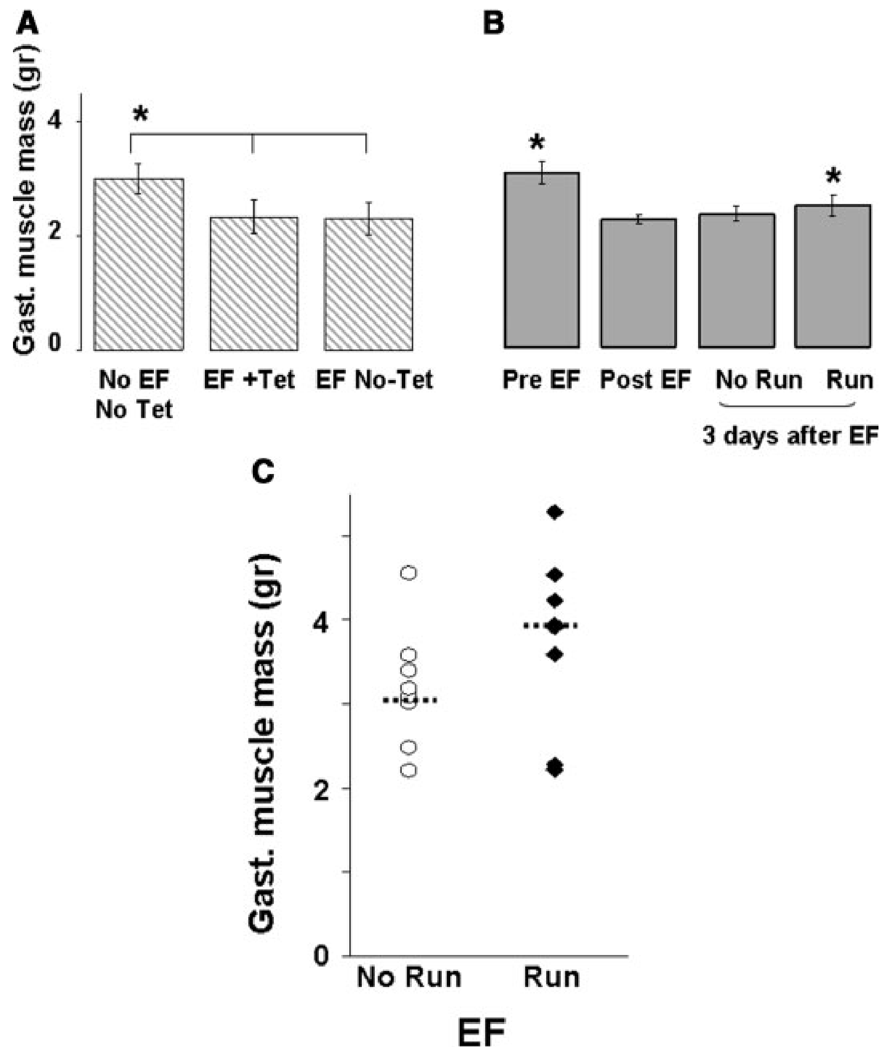


**Fig. 3. Gastrocnemius muscle mass decreased after EF and remained low in groups that were treated or untreated with Tet (A) but was regained in the exercise running groups (B). A: striped bars represent the average wet weight of the gastrocnemius muscle of control and two groups that underwent 3-weeks of EF and were treated or untreated with Tet (n = 5/group). B: Gray bars represent the average total muscle mass that was determined based on peripheral DEXA images taken before and after EF or after exercise running (n = 6/group). Error bars indicate the standard error of the mean for each data point; significant differences are highlighted with asterisks. Individual scores of gastrocnemius mass of the EF-No-Run (open circles) and EF-Run (black circles), determined by DEXA are depicted in part C. Horizontal lines represent the median value of muscle mass in each group. Muscle mass of EF rats that engaged in exercise running was significantly higher compared with the EF sedentary group (C).**

the balance between degeneration processes that occurred during EF were not reversed by Tet (Fig. 2A and B). To explore the influence of exercise running after EF on muscle mass, in experiment II, total gastrocnemius muscle mass of all animals was measured using DEXA at three times: before and after EF and after the completion of the running session. DEXA scans revealed that lower muscle mass measured after 3-weeks of EF remained significantly lower in sedentary rats (no-run group; one way ANOVA, $F\,(3, 76) = 7.0552$, $P < .001$; Fig. 3B). Muscle mass of rats that ran after EF, however, did not differ significantly from the mass measured before hindlimb was externally fixated. Moreover, data on individual gastrocnemius muscle mass as measured after the completion of the running session reveal aggregation of the measures of sedentary rats at the lower range of measured mass, with an average of 3.14 gr; compared with the measures of running rats that aggregated at the higher ranks with an average of 3.9 gr ($Chi^2_1 = 4$, $P < 0.05$; Fig. 3C). These data suggest that after EF, exercise running induced recovery of the muscle mass to levels measured before the EF. Such a recovery was not discernible in sedentary rats.

### Physiological approach: External fixation followed by exercise running

Exercise, including running, is considered as means of muscle rehabilitation. Here we investigated the relationship between exercise and satellite cells by quantifying their numbers along the entire length of myofibers based on Pax7 immunostaining. The number of satellite cells associated with each myofiber was determined after 3 h ex vivo, a time point at which satellite cells are still quiescent and attached to the parent myofiber.

Our data show that the number of satellite cells per myofiber was significantly higher in gastrocnemius muscles of running rats, both in EF and No-EF rats (between-group factor in two

way ANOVA; $F_{1,224} = 15.1$; $P < 0.001$; Fig. 4A, B,B′ and C,C′, respectively). In the EF-Run group the number of satellite cells/myofiber ranged from 5 to 81 and 14 to 90 in the No EF-Run rats (with an average ± SEM of 35 ± 2.13, and 46 ± 5.37, respectively, $P < 0.01$). The range of satellite cells in the sedentary groups was 5–65 in the EF-No Run and 4–42 in the No-EF No-Run group (with an average ± SEM of 20 ± 1.86, and 27 ± 1.02, respectively, $P < 0.01$). In the EF- No Run group the maximum number of satellite cells/myofiber was higher in the contralateral (65) compared to the EF limb (49). This difference was not significant, probably reflecting low levels of compensatory growth processes in the contralateral limb. In general, there was no significant difference in the number of satellite cells per myofiber between the EF and the contralateral No-EF limb (data not shown), contrary to the differential effect of Tet-treatment (Fig. 2). This apparent disagreement probably aroused from determining satellite cell number in rats at the end of 2-weeks of running, compared with sedentary rats. During the 2-weeks of exercise that followed the EF period, rats used both hindlimbs in running, thus inducing systemic and physiological signals that affected the EF and the contralateral limbs to the same extent. In contrast, the expression of Pax7 in

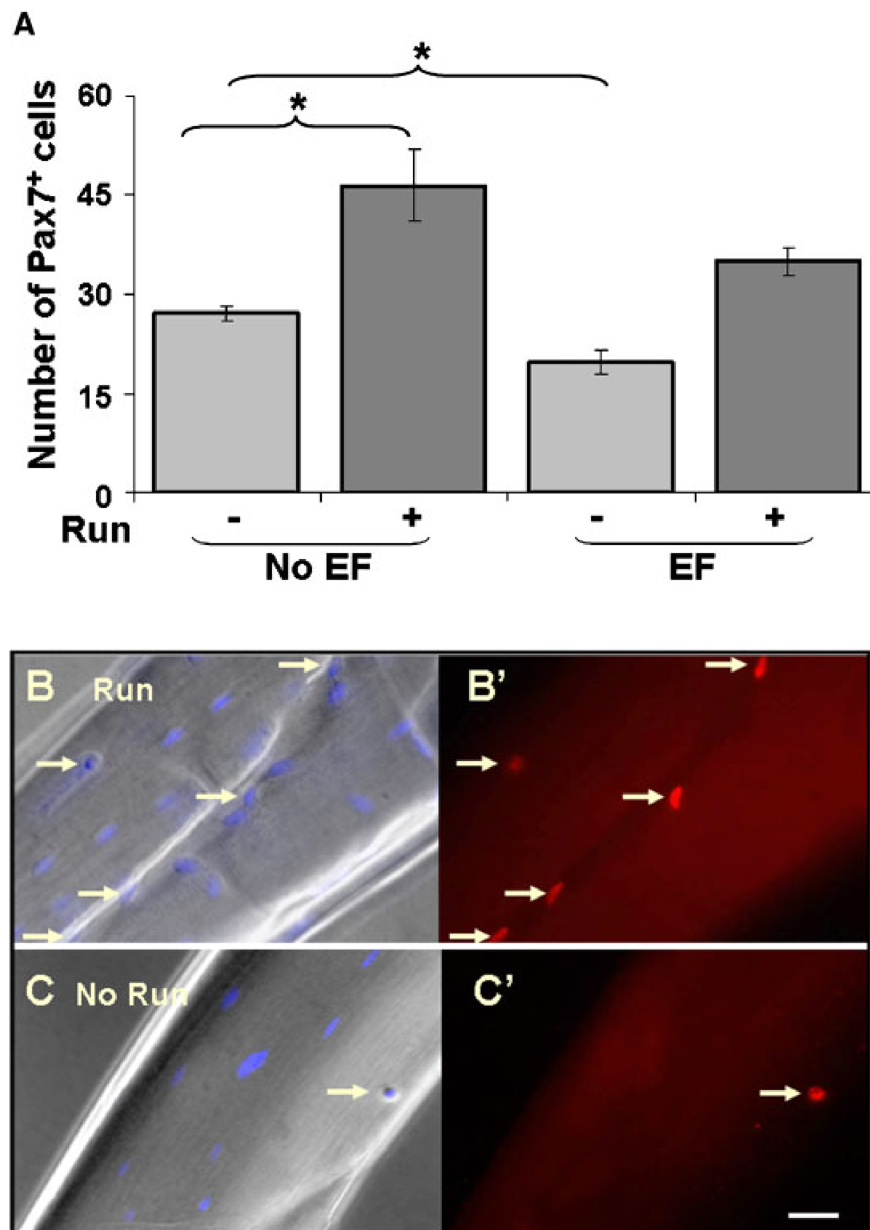


**Fig. 4. Satellite cell numbers in isolated gastrocnemius myofibers of exercise running and sedentary rats. A: The average number of satellite cells/individual myofibers of EF and No-EF rats that did or did not run; at least 25 fibers per hindlimb were isolated for each group and the average number of satellite cells/myofiber is represented by dark grey bars for the No-Run rats and light gray bars for the Run rats. Error bars indicate the standard error of the mean for each data point; significant differences are highlighted with asterisks. Immunofluorescent staining with the anti-Pax7 antibody (B), phase contrast micrographs are merged with DAPI-stained nuclei (B, C). Arrows point to individual satellite cells based on Pax7 expression (B′-C′). Scale bar, 20 µm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]**

the muscle of Tet-treated rats was quantified immediately after EF, reflecting unequal use of hind legs, of which one was fixated and the other was not (Fig. 2B).

The differentiation aptitude after EF and exercise running was based on clonal analysis of satellite cells in vitro. Clonal cultures were established to assess the lineage aptitude of these cells according to the ratio between the lineal identities of the developing clones (Fig. 5). The percent of myogenic clones was lowest (31%) in the sedentary EF group (a mean of 7 clones ±2) and highest (53%) in the exercised No-EF group (a mean of 16 clones ±2). There was a significant difference between the exercised and sedentary No-EF groups (53% vs. 45%, respectively; $F_{1,28} = 6.7$; $P < 0.05$). These data suggest that exercise running contribute to enhanced myogenic differentiation potential of satellite cells.

## Discussion

Muscle atrophy refers to a decrease in skeletal muscle mass, which occurs in a variety of settings and is typically associated with elimination of nuclei from myofibers and with persistent inflammation (Vescovo et al., 2000; Mitchell and Pavlath, 2001; Tidball, 2002; Dupont-Versteegden, 2006; Dupont-Versteegden et al., 2006). Therefore, finding ways to attenuate these processes is crucial for developing therapies for sustaining myofiber maintenance and re-growth during conditions of disuse. Accordingly, in this study we first defined the dynamics of changes in satellite cells and MRFs in response to EF and next assessed the impact of Tet (pharmacological intervention) and of exercise running (physiological intervention). These approaches were taken with the view that maintaining

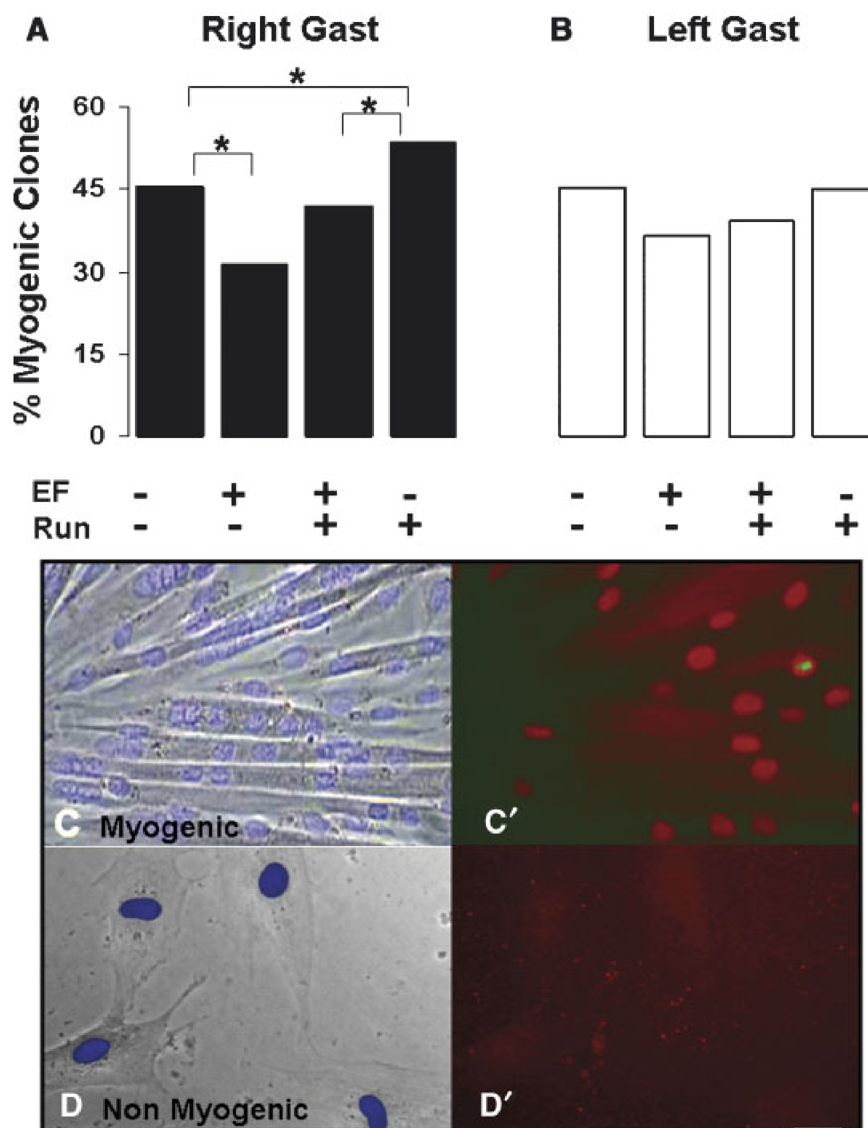


**Fig. 5. Quantification and classification of 2 week-old clones derived from individual gastrocnemius myofibers. Clones were prepared from myofibers isolated from the right and left gastrocnemius muscles (3–5 fibers per hindlimb). Myogenic identity of clones was determined based on myotube formation and positive staining with an anti-MyoD antibody. Each bar depicted in part A,B represents the percent of myogenic clones per group. For example, 47% of the clones that developed in the No-EF No-Run group were myogenic. Immunofluorescent staining with the anti-MyoD antibody (C′, D′); phase contrast micrographs are merged with images of DAPI-stained nuclei (C, D). A myogenic clone (C, C′), and a non-myogenic clone (D, D′). Scale bar, 20 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]**

satellite cell numbers and properties are mandatory for stabilizing muscle loss and/or supporting recovery from atrophy.

Here, we investigated the effect of atrophic conditions induced by hindlimb EF on satellite cell abundance and myogenic properties. We found that EF induces adverse effects at the tissue, cell, and molecular levels. Specifically, there was a muscle mass loss, the expression of MRFs was compromised, satellite cell numbers were reduced, and their fate decision was biased to favor a non-myogenic fate. Treatment with the anti-inflammatory agent Tet induced higher or sustained levels of MRFs but did not play a part in preventing the loss of satellite cells and of muscle mass. On the contrary, when implementing the physiological approach of 2-week exercise running, there was a re-gain of muscle mass, satellite cell numbers, and myogenic differentiation aptitude.

Our results show that Tet treatment had a beneficial effect on muscle cells, as it enhanced the expression levels of MRFs that are essential for proliferation and differentiation, even after prolonged periods of muscle disuse. On the contrary, in rats that were not treated with Tet, ongoing myogenesis was impeded after long-term EF. These data are in accordance with studies showing that proliferation and differentiation of muscle precursor cells, were attenuated, and limited the restoration of muscle mass after immobilization (Chakravarthy et al., 2000; Welle, 2002; Glass, 2003; Dasarathy et al., 2004; Mitchell and Pavlath, 2004; Blazevich and Sharp, 2005; Glass, 2005; Dasarathy et al., 2007). Together, these results prompt us to suggest Tet as a pharmacological means for preventing dysfunction of satellite cell progeny, and therefore aiding muscle maintenance during atrophy. Tet also induced myogenesis in the contralateral limb, based on Myf5, and MyoD expression. This probably represents processes of compensatory growth in response to work overload of the No-EF limb. Overload of the intact limb probably occurred since EF-rats needed to rely more on the No-EF limb in order to maintain symmetrical posture during locomotion. Increased workload on an intact limb was indeed shown to inflict fiber damage and subsequent regeneration (Rosenblatt and Parry, 1993; Oki et al., 1999; Wanek and Snow, 2000; Mitchell and Pavlath, 2004; Krawiec et al., 2005; Liu et al., 2005).Without Tet, early phases of myogenesis, inferred by Myf5 expression, were differentially manifested after long- and short-term periods of EF. Myf5 expression declined after 1 week in the EF limb and after 3 weeks in the No-EF limb. This may be explained by: (i) a more prominent inflammatory response in EF limb that may attenuate have regeneration as inflammatory cytokines have inhibitory effects on myogenesis (Guttridge et al., 2000; Langen et al., 2004; Spate and Schulze, 2004; Langen et al., 2006; Roth et al., 2006); (ii) transition to the subsequent differentiation phase that is accompanied by downregulating Myf5 and upregulating MyoD and myogenin; or (iii) death of satellite cells or of myofibers, as shown by us here and by others to occur during muscle disuse (Mitchell and Pavlath, 2004; Dupont-Versteegden, 2006).

Why would an anti-inflammatory drug exert beneficial effects on myogenesis albeit inflammation is vital for regenerative processes? A possible explanation is that the inflammation response has to end for myogenesis to begin because inflammatory cytokines inhibit positive regulators (such as MyoD and IGFI) and induce negative regulators (TNF$\alpha$ and myostatin) of myogenesis (Carlson et al., 1999; Chakravarthy et al., 2000; Dasarathy et al., 2004; Jackman and Kandarian, 2004; Langen et al., 2004; Mourkioti and Rosenthal, 2005). Therefore, when inflammation persists, as occurs when the atrophic conditions are sustained (Guttridge et al., 2000; Spate and Schulze, 2004; Argiles et al., 2005), Tet may aid in forcing the inflammatory phase to conclude and by that to set the stage for myogenesis to commence. Recently, the effect of non-steroidal anti-inflammatory drugs (NSAIDs) on the activity of satellite cells was investigated (Mackey et al., 2007b). Cell activity was evaluated based on muscle biopsies collected from treated and untreated athletes before and after a race. In accordance with our results, activation and proliferation of cells occurred after exercise running; however unlike Tet, NSAIDs attenuated these responses. This effect of NSAIDs was ascribed to their inhibitory effects on cyclooxygenase (COX), an enzyme that is required for satellite cell activity and for muscle regeneration (Langberg et al., 2003; Bondesen et al., 2006). Tet, on the contrary, was previously shown to up-regulate the expression of COX (Attur et al., 1999), and this may be one of the signaling pathways that transduced its effect on satellite cell myogenesis, shown in this study.

Muscle mass loss after EF was also shown in other models of immobilization, such as hindlimb suspension or cast (Zarzhevsky et al., 1999; Chakravarthy et al., 2000; Hortobagyi et al., 2000; Machida and Booth, 2004; Mitchell and Pavlath, 2004; Degens and Alway, 2006). We also showed that muscle mass was re-gained only in rats that ran for 2 weeks after the secession of EF (and not in EF-No Run or in Tet-treated/untreated rats). In contrast, other studies reported a spontaneous mass re-gain 2 weeks after immobilization (Mitchell and Pavlath, 2001; Bondesen et al., 2006). The apparent discrepancy can be explained by the different recovery rates of slow-twitch muscles, such as soleus, that was investigated by others, compared with fast-twitch muscles, such as gastrocnemius that was investigated here (Witzmann et al., 1982; Gurke et al., 2000; Glenmark et al., 2004; Miyabara et al., 2005). This implies that soleus recovers from atrophy faster than gastrocnemius. Another factor which may account for the discrepancy in results is the age of the tested animals. We investigated rats that were at the plateau phase of growth curve, whereas young growing animals were used in studies that reported spontaneous recovery (9–11 wk old; Jax1, 2000; Mitchell and Pavlath, 2004). Therefore the reported muscle gain after immobilization may have reflected developmental growth (Aoki et al., 2006) rather than mere spontaneous recovery.

Tet-treatment did not induce muscle mass re-gain. The abundance of satellite cells and the capacity of their progeny to undergo myogenesis are the bottleneck of regeneration; results obtained here imply that although Tet enabled myogenesis of satellite cell progeny, it probably failed to stabilize the satellite cell pool (based on MRFs and Pax7 expression levels in the EF and No-EF muscles). Therefore the lesser myoblasts available for supporting myofiber re-growth may have limited muscle re-gain. Alternatively, it is possible that sampling muscle mass immediately after EF removal was not sufficient to draw a definite conclusion about the effect of Tet on the rate of mass re-gain. It may be that the muscle mass of Tet-treated rats could have been re-gained before that of the spontaneously (untreated) recovering rats, but later than the period tested in this study.

To gain insights into the relationships between degenerative and regenerative conditions of skeletal muscles to the pool of satellite cells, the latter were quantified after 3-weeks of EF and after 2-weeks of exercise running. Our data showed that satellite cell numbers declined after EF, but returned to control levels (No-EF No-Run rats) after 2-weeks of exercise running. These results reflect the plasticity of the satellite cell pool in response to degenerating and regenerating stimuli. A decline in muscle precursor cells was previously suggested to occur after a period of immobilization (Mitchell and Pavlath, 2004). Nevertheless the latter results did not mirror the satellite cell pool but rather identified cells that were activated, based on their MyoD expression.

In previous studies with healthy humans and animals, physical activity was shown to correlate with a higher number of satellite

cells (Kadi et al., 2005; Tiidus et al., 2005). Here we further demonstrated that exercise running exerts such beneficial effects after long-term muscle-debilitating conditions (i.e., EF). Compared with human studies, in the present study the effect of exercise on satellite cell properties is advantageous for using adult animals that were chosen randomly from a large population with comparable health conditions and with the same diet. Therefore our results seem more general in comparison to former human studies, where only healthy, non-smoking, and physically fit subjects were chosen to participate (Kadi et al., 2005), not necessarily representing the average adult population. Another advantage of the present study is determining the absolute numbers of satellite cells without any sampling, thus providing a reliable insight into the effect of exercise running on the satellite cell pool. Differently, previous studies evaluated the satellite cell pool based on electron microscopy (Roth et al., 2001), immunostaining of muscle sections (Charifi et al., 2003; Mackey et al., 2007a) or quantification of cells emanating from cultured myofibers (Mitchell and Pavlath, 2004). The latter techniques carry inherent pitfalls that may skew conclusions drawn about the size of the satellite cell pool that was earlier discussed in detail (Shefer et al., 2006).

In addition to measuring the number of satellite cells we also provided an evaluation of their lineage fate decision, showing that degenerating and regenerating conditions exert opposite effects on their propensity to undertake myogenic differentiation. Specifically, EF imposed on satellite cells intrinsic changes that affected their lineage decision, based on the lower propensity of satellite cells to give rise to myogenic clones in EF compared to No-EF groups. A few in vitro studies with myofiber cultures have shown that satellite cells assume a non-myogenic phenotype; we additionally showed, by clonal studies, that Pax7 expressing cells (satellite cells) can divert from myogenesis to mesenchymal alternative differentiation in vitro by a stochastic mechanism (Csete et al., 2001; Shefer et al., 2004). In the present study we introduce novel data showing that the balance between myogenesis and adipogenesis of clones derived from satellite cells correlates with physiological manipulations so that debilitating conditions (EF) reduce myogenic aptitude while exercise running promotes myogenic differentiations. This supports our hypothesis that satellite cells can divert in vivo to a non-myogenic path if the signaling milieu of their niche is changed, for example, due to impaired myogenic signals that are conveyed by damaged muscles, as in disuse atrophy or aging. Additionally, these results lead us to suggest that fault lineage commitment of satellite cells, resulting in progeny that is not able to fuse with myofibers and support re-growth, may contribute to the typical prolonged, sometimes impaired, functional recovery from atrophy (Hortobagyi et al., 2000).

In summary, our data suggest that muscle recovery from atrophy depended, at least partially, on satellite cell numbers and function. Therefore these cells are an important target of physiological and/or pharmacological approaches to prevent or to treat muscle atrophy.

## Acknowledgments

We thank Drs Kariv N. and Eilam D. for invaluable advice and help, and to Mr. Mualem Y. for designing and constructing the treadmill apparatus. The study was supported by Bi-national Science Foundation (BSF) Number 2005312. GS is funded by the Eshkol Fellowship of the Israeli Ministry of Science and Technology, and the Israeli 'Budgeting Committee & Planning' (VATAT) of Tel Aviv University as a post-doc fellow at the laboratory of DB.

## Literature Cited

Aoki MS, Miyabara EH, Soares AG, Salvini TF, Moriscot AS. 2006. Cyclosporin-A does not affect skeletal muscle mass during disuse and recovery. Braz J Med Biol Res 39: 243–251.
Appell HJ. 1990. Muscular-atrophy following immobilization—A review. Sports Med 10:42–58.
Argiles JM, Busquets S, Lopez-Soriano FJ. 2005. The pivotal role of cytokines in muscle wasting during cancer. Int J Biochem Cell Biol 37:2036–2046.
Attur MG, Patel RN, Patel PD, Abramson SB, Amin AR. 1999. Tetracycline up-regulates COX-2 expression and prostaglandin E2 production independent of its effect on nitric oxide. J Immunol 162:3160–3167.
Bar-Shai M, Carmeli E, Reznick AZ. 2005. The role of NF-kappaB in protein breakdown in immobilization, aging, and exercise: From basic processes to promotion of health. Ann N Y Acad Sci 1057:431–447.
Beauchamp JR, Heslop L, Yu DS, Tajbakhsh S, Kelly RG, Wernig A, Buckingham ME, Partridge TA, Zammit PS. 2000. Expression of CD34 and Myf5 defines the majority of quiescent adult skeletal muscle satellite cells. J Cell Biol 151:1221–1234.
Blazevich AJ, Sharp NC. 2005. Understanding muscle architectural adaptation: Macro- and micro-level research. Cells Tissues Organs 181:1–10.
Bondesen BA, Mills ST, Pavlath GK. 2006. The COX-2 pathway regulates growth of atrophied muscle via multiple mechanisms. Am J Physiol Cell Physiol 290:C1651–C1659.
Booth FW, Gollnick PD. 1983. Effects of disuse on the structure and function of skeletal muscle. Med Sci Sports Exerc 15:415–520.
Carlson CJ, Booth FW, Gordon SE. 1999. Skeletal muscle myostatin mRNA expression is fiber-type specific and increases during hindlimb unloading. Am J Physiol 277:R601–R606.
Chakravarthy MV, Davis BS, Booth FW. 2000. IGF-I restores satellite cell proliferative potential in immobilized old skeletal muscle. J Appl Physiol 89:1365–1379.
Charifi N, Kadi F, Feasson L, Denis C. 2003. Effects of endurance training on satellite cell frequency in skeletal muscle of old men. Muscle Nerve 28:87–92.
Collins CA, Olsen I, Zammit PS, Heslop L, Petrie A, Partridge TA, Morgan JE. 2005. Stem cell function, self-renewal, and behavioral heterogeneity of cells from the adult muscle satellite cell niche. Cell 122:289–301.
Conboy IM, Conboy MJ, Smythe GM, Rando TA. 2003. Notch-mediated restoration of regenerative potential to aged muscle. Science 302:1575–1577.
Csete M, Walikonis J, Slawny N, Wei Y, Korsnes S, Doyle JC, Wold B. 2001. Oxygen-mediated regulation of skeletal muscle satellite cell proliferation and adipogenesis in culture. J Cell Physiol 189:189–196.
Dasarathy S, Dodig M, Muc SM, Kalhan SC, McCullough AJ. 2004. Skeletal muscle atrophy is associated with an increased expression of myostatin and impaired satellite cell function in the portacaval anastamosis rat. Am J Physiol Gastrointest Liver Physiol 287:G1124–G1130.
Dasarathy S, Muc S, Hisamuddin K, Edmison JM, Dodig M, McCullough AJ, Kalhan SC. 2007. Altered expression of genes regulating skeletal muscle mass in the portacaval anastamosis rat. Am J Physiol Gastrointest Liver Physiol 292:G1105–G1113.
Day K, Shefer G, Richardson JB, Enikolopov G, Yablonka-Reuveni Z. 2007. Nestin-GFP reporter expression defines the quiescent state of skeletal muscle satellite cells. Dev Biol 304:246–259.
Degens H, Alway SE. 2006. Control of muscle size during disuse, disease, and aging. Int J Sports Med 27:94–99.
Dupont-Versteegden EE. 2006. Apoptosis in skeletal muscle and its relevance to atrophy. World J Gastroenterol 12:7463–7466.
Dupont-Versteegden EE, Strotman BA, Gurley CM, Gaddy D, Knox M, Fluckey JD, Peterson CA. 2006. Nuclear translocation of EndoG at the initiation of disuse muscle atrophy and apoptosis is specific to myonuclei. Am J Physiol Regul Integr Comp Physiol 291:R1730–R1740.
Ferreira RC, Costo MT, Frizzo GG, da Fonseca Filho FF. 2006. Correction of neglected clubfoot using the Ilizarov external fixator. Foot Ankle Int 27:266–273.
Glass DJ. 2003. Molecular mechanisms modulating muscle mass. Trends Mol Med 9:344–350.
Glass DJ. 2005. Skeletal muscle hypertrophy and atrophy signaling pathways. The Int J Biochem Cell Biol 37:1974–1984.
Glenmark B, Nilsson M, Gao H, Gustafsson JA, Dahlman-Wright K, Westerblad H. 2004. Difference in skeletal muscle function in males vs. females: Role of estrogen receptor-beta. Am J Physiol Endocrinol Metab 287:E1125–E1131.
Gurke L, Marx A, Sutter PM, Stierli P, Harder F, Heberer M. 2000. Function of fast- and slow-twitch rat skeletal muscle following ischemia and reperfusion at different intramuscular temperatures. Eur Surg Res 32:135–141.
Guttridge DC, Mayo MW, Madrid LV, Wang CY, Baldwin AS, Jr. 2000. NF-kappaB-induced loss of MyoD messenger RNA: Possible role in muscle decay and cachexia. Science 289:2363–2366.
Hortobagyi T, Dempsey L, Fraser D, Zheng D, Hamilton G, Lambert J, Dohm L. 2000. Changes in muscle strength, muscle fibre size and myofibrillar gene expression after immobilization and retraining in humans. J Physiol 524:293–304.
Jackman RW, Kandarian SC. 2004. The molecular basis of skeletal muscle atrophy. Am J Physiol Cell Physiol 287:C834–C843.
Jax1. 2000. Body weights for selected strains, by age http://www.jax.org/phenome.
Jejurikar SS, Kuzon WM, Jr. 2003. Satellite cell depletion in degenerative skeletal muscle. Apoptosis 8:573–578.
Jespersen J, Kjaer M, Schjerling P. 2006. The possible role of myostatin in skeletal muscle atrophy and cachexia. Scand J Med Sci Sports 16:74–82.
Kadi F, Charifi N, Denis C, Lexell J, Andersen JL, Schjerling P, Olsen S, Kjaer M. 2005. The behaviour of satellite cells in response to exercise: What have we learned from human studies? Pflugers Archiv: Eur J Physiol 451:319–327.
Karakelides H, Sreekumaran Nair K. 2005. Sarcopenia of aging and its metabolic impact. Curr Top Dev Biol 68:123–148.
Kastner S, Elias MC, Rivera AJ, Yablonka-Reuveni Z. 2000. Gene expression patterns of the fibroblast growth factors and their receptors during myogenesis of rat satellite cells. J Histochem Cytochem 48:1079–1096.
Krawiec BJ, Frost RA, Vary TC, Jefferson LS, Lang CH. 2005. Hindlimb casting decreases muscle mass in part by proteasome-dependent proteolysis but independent of protein synthesis. Am J Physiol Endocrinol Metab 289:E969–E980.
Kuang S, Charge SB, Seale P, Huh M, Rudnicki MA. 2006. Distinct roles for Pax7 and Pax3 in adult regenerative myogenesis. J Cell Biol 172:103–113.
LaBianco GJ, Vito GR, Kalish SR. 1996. Use of the Ilizarov external fixator in the treatment of lower extremity deformities. J Am Podiatry Assoc 86:523–531.

Langberg H, Boushel R, Skovgaard D, Risum N, Kjaer M. 2003. Cyclo-oxygenase-2 mediated prostaglandin release regulates blood flow in connective tissue during mechanical loading in humans. J Physiol (Lond) 551:683–689.
Langen RC, Van Der Velden JL, Schols AM, Kelders MC, Wouters EF, Janssen-Heininger YM. 2004. Tumor necrosis factor-alpha inhibits myogenic differentiation through MyoD protein destabilization. FASEB J 18:227–237.
Langen RC, Schols AM, Kelders MC, van der Velden JL, Wouters EF, Janssen-Heininger YM. 2006. Muscle wasting and impaired muscle regeneration in a murine model of chronic pulmonary inflammation. Am J Respir Cell Mol Biol 35:689–696.
Lawler JM, Powers SK, Hammeren J, Martin AD. 1993. Oxygen cost of treadmill running in 24-month-old Fischer-344 rats. Med Sci Sports Exerc 25:1259–1264.
Liu MJ, Li JX, Lee KM, Qin L, Chan KM. 2005. Oxidative stress after muscle damage from immobilization and remobilization occurs locally and systemically. Clin Orthop 434:246–250.
Machida S, Booth FW. 2004. Regrowth of skeletal muscle atrophied from inactivity. Med Sci Sports Exerc 36:52–59.
Mackey AL, Esmarck B, Kadi F, Koskinen SO, Kongsgaard M, Sylvestersen A, Hansen JJ, Larsen G, Kjaer M. 2007a. Enhanced satellite cell proliferation with resistance training in elderly men and women. Scand J Med Sci Sports 17:34–42.
Mackey AL, Kjaer M, Dandanell Jorgensen S, Mikkelsen KH, Holm L, Dossing S, Kadi F, Koskinen SO, Jensen CH, Schroder HD, Langberg H. 2007b. The influence of anti-inflammatory medication on exercise-induced myogenic precursor cell responses in humans. J Appl Physiol 103:425–431.
Mitchell PO, Pavlath GK. 2001. A muscle precursor cell-dependent pathway contributes to muscle growth after atrophy. Am J Physiol Cell Physiol 281:C1706–C1715.
Mitchell PO, Pavlath GK. 2004. Skeletal muscle atrophy leads to loss and dysfunction of muscle precursor cells. Am J Physiol Cell Physiol 287:C1753–C1762.
Miyabara EH, Aoki MS, Moriscot AS. 2005. Cyclosporin A preferentially attenuates skeletal slow-twitch muscle regeneration. Braz J Med Biol Res 38:559–563.
Mourkioti F, Rosenthal N. 2005. IGF-1, inflammation and stem cells: Interactions during muscle regeneration. Trends Immunol 26:535–542.
Oki S, Desaki J, Taguchi Y, Matsuda Y, Shibata T, Okumura H. 1999. Capillary changes with fenestrations in the contralateral soleus muscle of the rat following unilateral limb immobilization. J Orthop Sci 4:28–31.
Pacheco RJ, Saleh M. 2004. The role of external fixation in trauma. Trauma 6:143–160.
Perry RL, Rudnick MA. 2000. Molecular mechanisms regulating myogenic determination and differentiation. Front Biosc 5:D750–767.
Relaix F, Montarras D, Zaffran S, Gayraud-Morel B, Rocancourt D, Tajbakhsh S, Mansouri A, Cumano A, Buckingham ME. 2006. Pax3 and Pax7 have distinct and overlapping functions in adult muscle progenitor cells. J Cell Biol 172:91–102.
Rosenblatt JD, Parry DJ. 1993. Adaptation of rat extensor digitorum longus muscle to gamma irradiation and overload. Pflugers Archiv 423:255–264.
Roth SM, Martel GF, Ivey FM, Lemmer JT, Tracy BL, Metter EJ, Hurley BF, Rogers MA. 2001. Skeletal muscle satellite cell characteristics in young and older men and women after heavy resistance strength training. J Gerontol A Biol Sci Med Sci 56:B240–B247.
Roth SM, Metter EJ, Ling S, Ferrucci L. 2006. Inflammatory factors in age-related muscle wasting. Curr Opin Rheumatol 18:625–630.
Roubenoff R. 2004. Sarcopenic obesity: The confluence of two epidemics. Obes Res 12:887–888.
Seale P, Sabourin LA, Girgis-Gabardo A, Mansouri A, Gruss P, Rudnicki MA. 2000. Pax7 is required for the specification of myogenic satellite cells. Cell 102:777–786.
Shefer G, Yablonka-Reuveni Z. 2005. Isolation and culture of skeletal muscle myofibers as a means to analyze satellite cells. Basic Cell Culture Protocols 290:281–304.
Shefer G, Wleklinski-Lee M, Yablonka-Reuveni Z. 2004. Skeletal muscle satellite cells can spontaneously enter an alternative mesenchymal pathway. J Cell Sci 117:5393–5404.
Shefer G, Van de Mark DP, Richardson JB, Yablonka-Reuveni Z. 2006. Satellite-cell pool size does matter: Defining the myogenic potency of aging skeletal muscle. Dev Biol 294:50–66.
Shur I, Lokiec F, Bleiberg I, Benayahu D. 2001. Differential gene expression of cultured human osteoblasts. J Cell Biochem 83:547–553.
Shur I, Ben-Avraham D, Benayahu D. 2004. Alternatively spliced isoforms of a novel stromal RNA regulating factor. Gene 334:113–121.
Shur I, Zemer-Tov E, Socher R, Benayahu D. 2007. SVEP expression is regulated in estrogen-dependent manner. J Cell Physiol 210:732–739.
Spate U, Schulze PC. 2004. Proinflammatory cytokines and skeletal muscle. Curr Opin Clin Nutr Metab Care 7:265–269.
Thomas DP, Zimmerman SD, Hansen TR, Martin DT, McCormick RJ. 2000. Collagen gene expression in rat left ventricle: Interactive effect of age and exercise training. J Appl Physiol 89:1462–1468.
Tidball JG. 2002. Interactions between muscle and the immune system during modified musculoskeletal loading. Clin Orthop Relat Res 403:S100–S109.
Tiidus PM, Deller M, Liu XL. 2005. Oestrogen influence on myogenic satellite cells following downhill running in male rats: A preliminary study. Acta Physiol Scand 184:67–72.
Vescovo G, Volterrani M, Zennaro R, Sandri M, Ceconi C, Lorusso R, Ferrari R, Ambrosio GB, Dalla Libera L. 2000. Apoptosis in the skeletal muscle of patients with heart failure: Investigation of clinical and biochemical changes. Heart 84:431–437.
Wanek LJ, Snow MH. 2000. Activity-induced fiber regeneration in rat soleus muscle. Anat Rec 258:176–185.
Welle S. 2002. Cellular and molecular basis of age-related sarcopenia. Can J Appl Physiol 27:19–41.
Witzmann FA, Kim DH, Fitts RH. 1982. Recovery time course in contractile function of fast and slow skeletal muscle after hindlimb immobilization. J Appl Physiol 52:677–682.
Yablonka-Reuveni Z, Rivera AJ. 1994. Temporal expression of regulatory and structural muscle proteins during myogenesis of satellite cells on isolated adult rat fibers. Dev Biol 164:588–603.
Zammit PS, Partridge TA, Yablonka-Reuveni Z. 2006. The skeletal muscle satellite cell: The stem cell that came in from the cold. J Histochem Cytochem 54:1177–1191.
Zarzhevsky N, Coleman R, Volpin G, Fuchs D, Stein H, Reznick AZ. 1999. Muscle recovery after immobilisation by external fixation. J Bone Joint Surg Berlin 81:896–901.
Zhang G. 2004. Avoiding the material nonlinearity in an external fixation device. Clin Biomech 19:746–750.