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The activity of satellite cells and myonuclei following 8 weeks of strength training in young men with suppressed testosterone levels

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Abstract

Aim: To investigate how suppression of endogenous testosterone during an 8-week strength training period influences the activity of satellite cells and myonuclei.

Methods: Twenty-two moderately trained young men participated in this randomized, placebo-controlled, and double-blinded intervention study. The participants were randomized to treatment with a GnRH analogue, *goserelin* ($n = 12$), which suppresses testosterone or *placebo* ($n = 10$) for 12 weeks. The strength training period of 8 weeks started after 4 weeks of treatment and included exercises for all major muscles. Biopsies were obtained from the mid-portion of the vastus lateralis muscle.

Results: Testosterone resting level in *goserelin* was 10–20 times lower compared with *placebo,* and the training-induced increase in the level of testosterone was abolished in *goserelin*. Training increased satellite cells number in type II fibres by 20% in *placebo* and by 52% in *goserelin* ($P < 0.01$), whereas the myonuclear number significantly increased by 12% in type II fibres in *placebo* and remained unchanged in *goserelin* ($P < 0.05$). No changes in satellite cells and myonuclei were seen in type I fibres in either group. Data from the microarray analysis indicated that low testosterone affects the bone morphogenetic proteins signalling, which might regulate proliferation vs. differentiation of satellite cells.

Conclusion: Eight weeks of strength training enhances the myonuclear number in type II fibres, and this is largely blocked by the suppression of testosterone. The data indicate that low testosterone levels could reduce the differentiation of satellite cells to myonuclei via the bone morphogenetic proteins signalling pathway, resulting in reduced increases in lean leg mass.

Keywords GnRH analogue, microarray, myonuclei, satellite cells, testosterone, training.

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Strength training-induced muscle hypertrophy is initiated by a number of complex molecular events. We know that activation of satellite cells (SCs) and an increase in myonuclei are two important mechanisms in muscle hypertrophy, where the myonuclear number is increased to satisfy the demand of increased protein synthesis (Kadi & Thornell 2000, Kadi *et al.* 2004). Mechanisms responsible for the molecular events inducing activation, proliferation and differentiation of SCs and enhanced number of myonuclei in relation to strength training are, however, still not clear.

One candidate for SC activation could be the anabolic hormone testosterone. Strength training is a robust physiological stimulator of testosterone and within a few minutes after the onset of training, plasma testosterone starts to increase (Kraemer *et al.* 1990, 1991, Hakkinen & Pakarinen 1993). We know that SCs can be targets for testosterone action through the androgen receptor (Doumit *et al.* 1996, Sinha-Hikim *et al.* 2004, Chen *et al.* 2005). Exogenous testosterone supplementation increases skeletal muscle SCs, and positive associations between testosterone, muscle hypertrophy and the number of SCs have been reported. These findings indicate that testosterone could be an important stimulus for the activation and proliferation of SCs (Sinha-Hikim *et al.* 2003, 2004).

Muscle fibres contain hundreds of myonuclei, and each myonucleus sustains the protein synthesis in a limited cell volume. During the process of muscle fibre enlargement in adult muscles, an elevation of the myonuclear number occurs to satisfy the need for increased protein synthesis (Kadi & Thornell 2000, Kadi *et al.* 2004, Petrella *et al.* 2008). Long-term use of anabolic steroids (e.g. testosterone) has powerful effects on muscle fibre size and myonuclear number (Kadi 2008). However, it remains to be clarified whether SCs and myonuclei are regulated by endogenous testosterone during the process of training-induced skeletal muscle hypertrophy.

To study endogenous testosterone and its role in stimulating and regulating muscle hypertrophy, we performed a strength training study in which testicular production of testosterone was blocked through inhibition of pituitary-secreted luteinizing hormone (LH) by the administration of a GnRH analogue (goserelin). This resulted in resting levels of serum endogenous testosterone, both total and free, remained constant in the placebo group throughout the intervention period, but decreased significantly in the goserelin-treated group to a level 10–20 times lower than normal. Furthermore, the placebo group responded acutely to the strength training sessions with a significantly larger training-induced increase in serum testosterone compared to the goserelin group, whereas the goserelin-treated group responded with a decrease in total and free testosterone (Kvorning *et al.* 2006, 2007). We reported that the suppression of testosterone production in combination with strength training attenuates the response to the training stimulus with a less pronounced increase in lean leg mass (i.e. muscle hypertrophy) compared with strength training combined with placebo (Kvorning *et al.* 2006). In spite of blocked acute testosterone responses as well as very low resting levels of endogenous testosterone in the goserelin-treated group, strength training resulted in a similar mRNA expression of myoD, myogenin, IGF-IEa, IGF-IEb, IGF-IEc, myostatin and androgen receptor as observed in the placebo group, suggesting that endogenous testosterone is not involved in the direct regulation of the expression of these particular signalling mRNAs in the course of strength training-induced muscle hypertrophy (Kvorning *et al.* 2007). The present study used pre-existing data from our previous work, Kvorning *et al.* (2006, 2007), to investigate whether the training-induced SC and myonuclear responses are influenced by the GnRH analogue treatment (i.e. suppression of endogenous testosterone). As SC responses may vary between fibre types, we studied the fibre type-specific changes in SCs and myonuclei.

The aim of the present study was to investigate how suppression of endogenous testosterone during an 8-week strength training period influences the activity of SCs and myonuclei. We hypothesized that the suppression of endogenous testosterone will lead to a less pronounced increase in the number of SCs and therefore less addition of myonuclei compared to a placebo group. In addition, a microarray analysis was performed to test for other potential SC-regulating mechanisms.

Material and methods

Subjects and study design

Details of this study design have been reported elsewhere (Kvorning *et al.* 2006, 2007). The schedule of study procedures are shown in Figure 1. Briefly, 26 subjects volunteered to participate in this randomized, placebo-controlled, and double-blinded intervention study. The subjects participated in leisure sport once or twice a week. Some of the subjects had previously strength trained for shorter periods, but nothing structured, and strength training did not exceed 1 h per week during the last year. The study conformed to the guidelines in the Declaration of Helsinki and was approved by the local Ethical Committee (VF 20040173). All subjects were informed of the risks and purposes of the study before their written consent was obtained. The subjects were carefully matched in

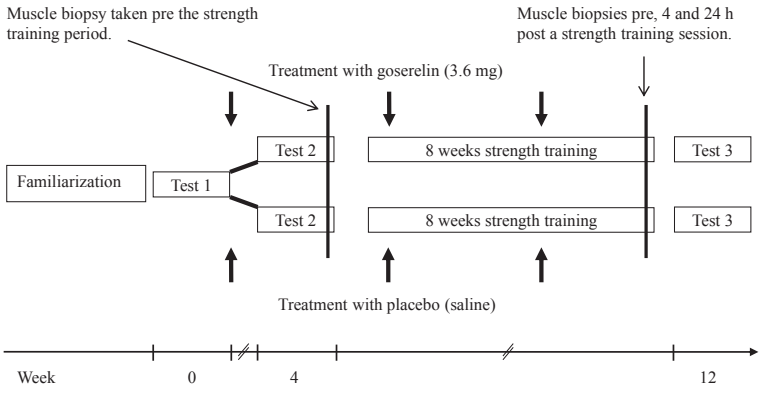

Figure 1 Overview of the study design. After completion of Test 1, the subjects were randomized in to a goserelin group (n = 12) and a placebo group (n = 10). Test 1, 2 and 3 included blood sampling (resting levels and acute hormonal response to a strength training session); muscle biopsies were performed at Test 2 and 3.

pairs with regard to isometric knee extension strength, BMI and age. Within each pair, the subjects were randomized to placebo (saline) or goserelin 3.6 mg (GnRH analogue) injections once every fourth week, three times in total. Clinical examination of the subjects was performed before the experiment, and two subjects were disqualified due to exclusion criteria (metabolic disorders, low testosterone levels, angina pectoris, lower back disorders, prescription medication for heart or lung diseases, or any recent physical trauma). Moreover, two subjects did not complete the study due to an injury unrelated to the study and due to side effects of the goserelin treatment (hot flushes) respectively. Therefore, 22 young men completed the study (Table 1). The subjects and investigators involved in training and testing were blinded regarding the allocation of the subjects, while two investigators (MA and KB) administering the study drugs and monitoring safety parameters were aware of the allocation.

Testing procedures

The subjects underwent three test procedures during the study. Test 1, 2 and 3 included measurements of, isometric knee extension strength, hormonal resting levels, and measurements of acute hormonal responses to a strength training session. These measurements were completed in succession and on a separate day.

Table 1 Anthropometrics of the subjects measured before the strength training period

Anthropometrics	Age (years)	Height (cm)	Body mass (kg)	BMI (kg m^{-2})
Goserelin (n = 12)	25 ± 1	179.5 ± 1.6	80.7 ± 3.7	25.3 ± 1.1
Placebo (n = 10)	23 ± 1	185.0 ± 1.4	83.4 ± 3.9	24.5 ± 1.1

Values are mean ± SE. None of the parameters differed significantly between the groups.

Muscle biopsies were performed on separate days in relation to Test 2 and Test 3 (Fig. 1). The subjects were familiarized with the study procedures approx. 2 weeks before entering Test 1. This included measuring of anthropometrics of the subjects and a careful introduction to the testing procedures and to the strength training exercises. Subsequently, a 10 repetition maximum (RM) load was measured for all exercises in the training programme, to determine the initial training load.

Treatment with goserelin

Goserelin (Zoladex®; AstraZeneca, London, UK) 3.6 mg depot was injected subcutaneously in the abdomen once every fourth week, to reduce and maintain endogenous testosterone concentrations within castrate range. Goserelin prevents the reappearance of LHRH receptors and consequently inhibits the secretion of LH from the pituitary gland and thus testicular production of testosterone (Cockshott 2000). All subjects received three injections in total, either treatment or placebo, starting right after Test 1 (Fig. 1).

Training

A standardized warm-up was performed before training consisting of four sets of squats with 20 repetitions without load and with 1-min rest between sets. Subjects from both groups were trained using the same progressive strength training programme. The programmes were performed three times a week for 8 weeks and consisted of leg press, knee extension, leg curl, bench press, lat pull down, biceps curl and elbow extension. Subjects did four sets of each exercise for the lower body and three sets of each exercise for the upper body. The strength training period consisted of 24 training sessions periodized in three cycles of eight training sessions with changing training loads (6 RM–10 RM). The goserelin group and the placebo group increased training loads to the same extent and

underwent the same training volume. All training sessions were supervised, and both groups carried out the same amount of training sessions (except for one training session), so subjects in the goserelin group completed 23.7 training sessions on average and the placebo group completed 23.6 training sessions on average. All subjects participated in a minimum of 22 training sessions (see Kvorning *et al.* 2006, for details).

Muscle biopsies

In a resting condition, muscle biopsies (~100 mg) from the middle portion of the *vastus lateralis* were obtained by using the Bergström needle technique (Bergström 1962). Incisions were made through the skin and muscle fascia following the administration of local anaesthesia (2–3 mL of 1% lidocaine). Pre- and post-training biopsies were taken from the same region and depth of the muscle. The tissue was immediately freed from blood and visible connective tissue, rapidly frozen in liquid N_2 and stored at −80 °C. Biopsies were obtained at four time points to measure SC and myonuclei response to strength training. The first biopsy was taken in the right leg (Test 2) and served as a pre-training period biopsy (Pre). In connection with the second last strength training session (Test 3), three biopsies were taken. One biopsy was taken in the right leg before the start of the training session and served both as a post-training period biopsy and a pre-training session biopsy (Post/Pre). This biopsy was taken 48 h after the previous strength training session. The subjects then completed the exercises, and another biopsy was taken in the left leg 4 h after completion of the strength training session (Post4h). The final biopsy was taken in the right leg 24 h after the pre-training biopsy (Post24h). Time points for all subjects were standardized and equal from day to day. The subjects had been fasting from 24:00 hours the day before and had refrained from strenuous physical activity for 48 h. Two hours prior to all biopsies, subjects were served a standardized meal. Subjects were divided into three groups (e.g. light, medium and heavy body mass group) receiving different sizes of meals, containing in total 8.01 ± 0.14 kcal kg^{-1}, 0.45 ± 0.01 g of protein per kg, 1.31 ± 0.03 g of carbohydrate per kg and 0.10 ± 0.00 g of fat per kg.

RNA purification

The RNA samples from Kvorning *et al.* (2007) were retrieved from the freezer and subjected to DNase treatment with the TURBO-DNA-free kit from Ambion (Life Technologies, Paisley, UK) according to manufacturer's protocol. After DNase treatment, the RNA samples were precipitated with 1/10 vol. 3 M sodium acetate and 2.5 vol. 96% ethanol, washed with 70% ethanol and dissolved in RNase-free water. The RNA quality was reassessed by denaturing gel electrophoresis. Some samples were partially degraded and therefore excluded from further analyses. In consequence, the number of biopsies used was the following: placebo group, Pre ($n = 7$), Post/Pre ($n = 6$), Post4h ($n = 7$) and Post24h ($n = 6$) and for the goserelin group, Pre ($n = 8$), Post/Pre ($n = 8$), Post4h ($n = 9$) and Post24h ($n = 7$).

Microarray

We theorized that suppressed testosterone levels could have several potential effects on mRNA levels in this study. The lack of testosterone may (i) constitutively increase/decrease the level of specific mRNA, (ii) influence the increase/decrease of mRNA due to the training period or (iii) influence the acute increase/decrease of mRNA after the training session. The 4 h post-samples (Post4h) will capture all three possibilities (though, the acute effect only 4 h after training), and therefore, these samples were screened for mRNA differences between the goserelin group and the placebo group by subjecting them to microarray analysis.

Ten samples from the Post4h time point were analysed on Affymetrix U133 Plus 2.0 microarrays by a microarray core facility, the RH Microarray Center (Rigshospital, Copenhagen). Briefly, total RNA was converted into double-stranded cDNA with the Superscript Choice System (Invitrogen, Carlsbad, CA, USA) using an oligo-dT primer containing a T7 RNA polymerase promoter (Genset, Evry, France). The cDNA was used as a template for an *in vitro* transcription reaction to synthesize biotin-labelled antisense cRNA (BioArray High Yield RNA Transcript Labeling Kit; Enzo Diagnostics, Farmingdale, NY, USA). After fragmentation at 94 °C for 35 min in fragmentation buffer (40 mM Tris, 30 mM magnesium acetate, 10 mM potassium acetate), the labelled cRNA was hybridized for 16 h to Affymetrix HG-U133 Plus 2.0 (Affymetrix, Santa Clara, CA, USA). After hybridization, the array was washed and stained with streptavidin phycoerythrin solution using the Fluidics Station 450 (Affymetrix). Finally, the arrays were scanned using the GeneChip Scanner 3000 (Affymetrix).

The probe data (CEL files) were analysed with the Partek Genomic Suite v6.6 (Partek, MO, USA) using the RMA normalization method (Irizarry *et al.* 2003). One array (goserelin group) deviated highly from the other nine in principal component analysis and was therefore excluded from the analysis. Statistical comparison of the two groups was performed and probe set with false discovery rate (FDR) of <5% selected. As this resulted in only a single probe set, a less stringent

test was also performed with raw *P*-values <5% combined with a fold change (both ways) above 1.5.

The data have been uploaded to the NCBI Gene Expression Omnibus and can be found at http://www.ncbi.nlm.nih.gov/geo/ with accession number GSE43437.

Real-time RT-PCR

Real-time RT-PCR was performed as described in Kvorning *et al.* (2007), except new cDNA was synthesized based on the DNase-treated RNA. Briefly, 500 ng DNase-treated total RNA was converted to cDNA with OmniScript reverse transcriptase (Qiagen, CA, USA) and poly-dT. cDNA corresponding to 1.25 ng total RNA was used in each SYBR Green-based reaction (Quantitect from Qiagen) with 100 nM of each primer (Table 2) in an MX3005P real-time PCR machine (Stratagene, CA, USA). Ct values were related to a standard curve made with synthetic oligonucleotides (Ultramers, IDT, Leuven, Belgium) containing the PCR target sequence. Specificity was confirmed by the melting curve analysis. GAPDH mRNA was used for normalization and RPLP0 mRNA for validating that GAPDH mRNA did not differ between the goserelin and placebo group (Fig. 5).

Fibre type-specific CD56$^+$SC content

Fibre type-specific *CD56$^+$SC* content was analysed using immunohistochemistry as previously described (Mackey *et al.* 2009). SC were visualized on muscle cross sections using a monoclonal mouse anti-CD56 antibody (1 : 100, cat. no. 347740; Becton Dickinson, San Jose, CA, USA), using a protocol described in Mackey *et al.* (2009). Briefly, sections were incubated for 2 h at 37 °C with the primary antibody, followed by a biotinylated goat anti-mouse secondary antibody (1 : 200, cat. no. BA-9200; Vector Laboratories, Burlingame, CA, USA) and then the Vectastain ABC reagent (cat. no. PK-6100, Vector Laboratories). For the visualization of primary antibody binding, the DAB substrate kit for peroxidase (cat. no. SK-4100, Vector Laboratories) were used. Sections were then rinsed, counterstained with Mayer's haematoxylin, cleared and mounted using Mountex. Myonuclei were blue, and satellite cells were stained brown (Fig. 2). At least 150 muscle fibres per muscle biopsy were used in the analysis. Consecutive cross sections were incubated with the monoclonal antibodies A4.951 and N2.261 (Developmental Studies Hybridoma Bank, University of Iowa) for determination of fibre-type composition (Kadi *et al.* 1998). The number of CD56$^+$SCs/fibre in type I and type II fibres was evaluated on whole cross sections, which corresponded to an average of 450 fibres. The number of myonuclei/fibre was measured on an average of 25 fibres of each fibre type as previously determined by Mackey *et al.* (2009).

Statistics

A two-way repeated-measures ANOVA was used to test for statistical significance of differences in SC and myonuclei. The test included (1) two time points, *before* and *after* the intervention, and (2) intervention, *goserelin* and *placebo*.

All mRNA data from RT-qPCR were log-transformed before statistical analysis. Unpaired *t*-test at Post4h was performed for comparison with array data (Table 3). Otherwise the RT-qPCR data were analysed with two-way repeated-measures ANOVA (Time*Treatment) with Student–Newman–Keuls post

Table 2 Primers for RT-PCR

Name	GenBank Acc.	Sense primer	Antisense primer
ACBP/DBI	NM_020548.6	ACCAAGCCATCGGATGAGGAGA	CCTTGCCCGTGAAGTCCAACA
ARNTL/BMAL1	NM_001178.4	GCCTCCCCCTGATGCCTCTT	TGATAGTAGGCCACTGGAAGGAATGT
CHURC1	NM_145165.3	CCTGCTGAGGAAATCGTTTGGTG	ACTCAAGGAAGGGACAATTCAGAGG
COL4A3	NM_000091.4	ATCGTGGTCCACCAGGCTCA	CAGGGTGGCCCATAGACCCTTT
ACTN3	NM_001104.1	CCGAGACTGACACGACTGAGCAA	CAGCTCCTCGGGGGTGATGTAG
FHL2	NM_001450.3	TGCCTGAACTGCTTCTGTGACTTG	TCGTTATGCCACTGCCGTTCCT
Gremlin1	NM_013372.6	GTCAGGGGAAACAAAATCTTGACCCA	AGATTCAGGCACTGACTCAGGAAGAC
IRX3	NM_024336.2	GCTGTAGTGCCTTGGAAGTGGAGA	GGATGAGGAGAGAGCCGATAAGACC
MED30	NM_080651.2	TGCAATGAAAACTGTGGTGGGA	TCTTCACTAGCAAAACGAGGTGGG
MEGF10	NM_032446.2	CCACATCCCCTCACGTCAACAA	GGCCTCTCTTCCCAGGGTTCA
MFAP5	NM_003480.2	GTGCATCGGCCGGTTAAACAA	TCGTGTTCCTTACAGACAAGACGAGA
NARG2	NM_024611.4	CCGAAATCACTGGATACCACAACA	TTGCTGAGCAGGCAAGCAACT
NR1D2	NM_005126.4	ATTTAGTGAGAAGCTAAATGCCCTCCA	TCCACAGAGTTGACGTTTTCTATTCCA
RPS16	NM_001020.4	GCCCCTGGAGATGATTGAGCC	GCTCCTTGCCGAGAAGCAGAAC
GAPDH	NM_002046.4	CCTCCTGCACCACCAACTGCTT	GAGGGGCCATCCACAGTCTTCT

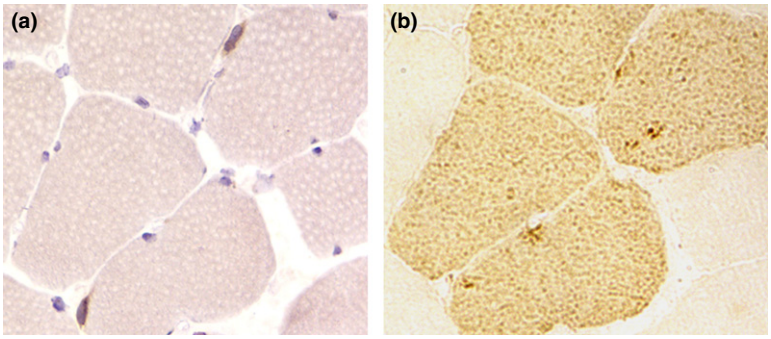

Figure 2 Fibre type-specific identification of satellite cells using the antibodies against CD56 (a) and A4.951 (b). Satellite cells are blue nuclei surrounded by a brown rim (a). In (b), type I fibres are stained and type II fibres are unstained.

hoc test and presented as Geometric Mean ± back-transformed SE (Fig. 5).

All other data are presented as mean ± SE. A significance level of $P < 0.05$ was chosen, with trends ($P < 0.1$) indicated in parenthesis.

Results

Satellite cells

The strength training period increased *CD56⁺SC* content in type II fibres in the placebo group from 12.2 to 14.6% ($P < 0.01$) and in the goserelin group from 10.3 to 15.7% ($P < 0.01$) (Fig. 3). The number of *CD56⁺SCs/fibre* in type I fibres was unaffected by the strength training period in both groups. Furthermore, no acute effect in satellite cell activity was seen; thus, the number of satellite cells remained unchanged in muscle biopsies taken before, 4 and 24 h following the second last strength training session.

Myonuclei

Myonuclear numbers in type I and type II fibres are shown in Figure 4. No change in the myonuclear number of type I fibres was seen in any of the groups. Strength training significantly increased the myonuclear number from 2.10 ± 0.13/fibre to 2.35 ± 0.13/fibre in type II fibres in the placebo group ($P < 0.05$), whereas no changes were observed in the goserelin group.

Microarray

Using stringent criteria of FDR less than 5%, only a single mRNA, CHURC1, is shown to be differently expressed in the goserelin-treated subjects (226736_at in Table 3). This difference was confirmed with qRT-PCR against the 3′UTR of the CHURC1 mRNA (Table 3).

However, due to the vast number of mRNA analysed at the same time, smaller changes may well be lost as type II errors (false negatives). To reduce this problem, the microarray data were reanalysed with less stringent criteria often used in microarray analyses (Guo *et al.* 2006). Using unadjusted $P < 0.05$ and fold change (FC) >1.5, a total of 36 significant hits were found (Table 3). Of course a number of those findings may be false positive, but because only a subset of the subjects was applied on microarrays, qRT-PCR on all the subjects can be used for confirmation of the findings. Fifteen of the hits were selected for confirmation. Only four, CHURC1, Gremlin1, Myostatin and IRX3, turned out to be confirmed by the PCR at Post4h. However, when including all time points, it turns out that also FHL2 and ACBP/DBI were influenced in the goserelin group (Fig. 5). Additionally, ARNTL did show a trend towards an effect of the suppressed testosterone. That is, of the 15 selected hits, about half of them could be confirmed by PCR.

Discussion

In this study, we report that subjects, after 8 weeks of strength training with suppressed endogenous testosterone levels, increase the amount of SCs to a similar extent as subjects with normal testosterone levels, but without any effect on the number of myonuclei. Whereas, strength training with normal testosterone levels increases SC content and myonuclear number by 20 and 12% respectively. These results are in accordance with earlier observations (Roth *et al.* 2001, Kadi *et al.* 2004, Dreyer *et al.* 2006, Olsen *et al.* 2006, Petrella *et al.* 2008).

Previous studies indicate that testosterone regulates SC activation, proliferation and differentiation into myonuclei by increasing the number of PCNA⁺SCs (proliferating cell nuclear antigen-positive SCs) (Sinha-Hikim *et al.* 2002, 2006). These findings support a connection between testosterone and SCs, and thus, the role of SCs as mediators of the myotrophic action of testosterone on human skeletal muscle (Kadi 2008). As testosterone may be able to regulate SCs, we speculated that the suppression of testosterone production, would lead to a smaller increase in the number of SCs following strength training, a phenomenon that could explain

Table 3 Microarray and RT-qPCR comparison of the goserelin and placebo groups at Post4h. Goserelin group, $n = 9$; placebo group, $n = 7$

Probeset ID	Gene title	Gene symbol	FC* array	*P*-value array	FC* PCR	*P*-value PCR
232628_at	–	–	1.61	0.015		
236114_at	–	–	1.51	0.037		
222348_at	–	–	1.95	0.041		
206891_at	Actinin, alpha 3	ACTN3	−1.76	0.044	−1.30	0.683
209824_s_at	Aryl hydrocarbon receptor nuclear translocator-like (BMAL1)	ARNTL	−1.51	0.032	−1.11	0.725
238465_at	Chromosome 5 open reading frame 35	C5orf35	−1.74	0.007		
226736_at	Churchill domain containing 1	CHURC1	5.21	4.10^{-7} †	1.45	0.033
223210_at	Churchill domain containing 1	CHURC1	1.52	0.006	1.45‡	0.033‡
222073_at	Collagen, type IV, alpha 3 (Goodpasture antigen)	COL4A3	1.60	0.038	−1.14	0.577
228391_at	Cytochrome P450, family 4, subfamily V, polypeptide 2	CYP4V2	−2.34	0.032		
209389_x_at	Diazepam binding inhibitor (GABA receptor modulator, acyl-CoA binding protein, ACBP)	DBI	−1.53	0.021	−1.16	0.332
202949_s_at	Four and a half LIM domains 2	FHL2	−1.57	0.039	−1.14	0.550
218469_at	Gremlin 1	GREM1	−2.79	0.005	−3.62	0.007
218468_s_at	Gremlin 1	GREM1	−1.63	0.013	−3.62‡	0.007‡
229638_at	Iroquois homeobox 3	IRX3	−2.45	0.023	−2.95	0.001
244623_at	Potassium voltage-gated channel, KQT-like subfamily, member 5	KCNQ5	1.53	0.006		
244572_at	Kyphoscoliosis peptidase	KY	2.09	0.019		
244741_s_at	Hypothetical LOC100128252	LOC100128252	−1.98	0.024		
231013_at	Hypothetical LOC100505608	LOC100505608	−1.69	0.038		
1560646_at	Hypothetical LOC121952	LOC121952	−1.98	0.048		
1560750_at	Hypothetical LOC151121	LOC151121	−2.14	0.042		
229007_at	FSHD region gene 1 pseudogene	LOC283788	−1.65	0.030		
225857_s_at	Hypothetical LOC388796	LOC388796	2.26	0.012		
65588_at	Hypothetical LOC388796	LOC388796	2.05	0.032		
242770_at	Similar to FRG1 protein (FSHD region gene 1 protein)	LOC642236	−1.82	0.038		
1569110_x_at	Programmed cell death 6 pseudogene	LOC728613	−1.78	0.010		
227787_s_at	Mediator complex subunit 30	MED30	−1.55	0.009	1.05	0.798
236517_at	Multiple EGF-like-domains 10	MEGF10	1.92	0.002	1.41	0.278
232523_at	Multiple EGF-like-domains 10	MEGF10	1.98	0.009	1.41‡	0.278‡
213765_at	Microfibrillar-associated protein 5	MFAP5	−1.75	0.023	1.13	0.771
209758_s_at	Microfibrillar-associated protein 5	MFAP5	−1.65	0.044	1.13‡	0.771‡
207145_at	Myostatin	MSTN	−1.55	0.038	−1.50§	0.023§
228960_at	NMDA receptor regulated 2	NARG2	−1.96	0.026	−1.10	0.555
209750_at	Nuclear receptor subfamily 1, group D, member 2	NR1D2	1.62	0.032	1.17	0.386
201258_at	Ribosomal protein S16	RPS16	−1.70	0.020	−1.16	0.378
216913_s_at	Ribosomal RNA processing 12 homolog (*Saccharomyces cerevisiae*)	RRP12	−1.56	0.028		
234018_s_at	Sel-1 suppressor of lin-12-like 2 (*Caenophaptitis elegans*)	SEL1L2	−1.55	0.010		
226650_at	Zinc finger, AN1-type domain 2A	ZFAND2A	1.68	0.013		

*FC, fold change between goserelin and placebo at Post4h.
†FDR < 0.05.
‡Same PCR data as above.
§Data from Kvorning *et al.* 2007.

the reduced training-induced increase in lean leg mass in the goserelin group as reported earlier (Kvorning *et al.* 2006). However, the present study shows that 8 weeks of strength training increase the amount of SCs independently of the testosterone levels. On the other hand, we demonstrate that the significant increase

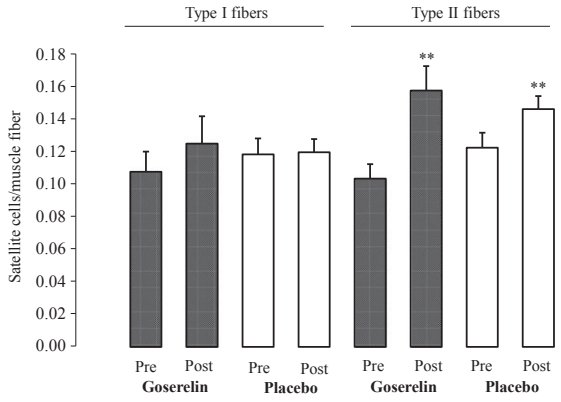

Figure 3 Satellite cells per muscle fibre before and after the 8-week strength training period. Goserelin group, $n = 11–12$; placebo group, $n = 9$. Values are mean ± SE. **Significantly different from pre 8 weeks of strength training ($P < 0.01$).

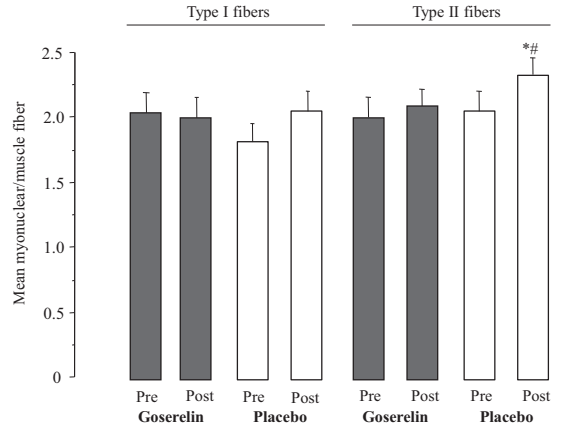

Figure 4 Myonuclear number per muscle fibre, before and after the 8-week strength training period. Goserelin group, $n = 11–12$; placebo group, $n = 9$. Values are mean ± SE. *Significantly different from pre 8 weeks of strength training ($P < 0.05$). #Significant increase compared to goserelin ($P < 0.05$).

in SCs in the goserelin group did not lead to a significant addition of myonuclei, which is seen, as expected, in the placebo group, where a significant addition of myonuclei in type II fibres occurs in parallel with a significant increase in lean leg mass (Kvorning *et al.* 2006). These data support the importance of myonuclear addition in muscle hypertrophy (Allen *et al.* 1995, Kadi & Thornell 2000, Kadi *et al.* 2004). Interestingly, Petrella *et al.* argue that if the demand for myonuclear addition was the only driving force determining the expansion rate of the SCs, the doubling of SCs in subjects considered as extreme responders in terms of muscle mass gained after a strength training period would not be expected. In other words, the degree of SC content expansion would be expected to keep pace with the level of myonuclear addition but not to exceed it (Petrella *et al.* 2008). Furthermore, Petrella *et al.* (2008) reported that the rate of myonuclear addition lagged behind the rate of muscle hypertrophy. If we relate this to our study, it can be postulated that SC response is exaggerated, whereas the process of myonuclear addition is regulated by the hypertrophic need (i.e. muscle mass driven). Mackey *et al.* (2011) further support this by showing that resistance training induces enhancement of the SC pool without muscle hypertrophy in young men. Furthermore, it seems that the increases in SC are related to the intensity of the exercise stimuli and increases in SC do not automatically increase myonuclear addition (Kurosaka *et al.* 2012, Smith & Merry 2012).

We have earlier reported from the present study that MyoD, myogenin, IGF-IEa, IGF-IEb, IGF-IEc and myostatin showed similar expression independently of the testosterone level (Kvorning *et al.* 2007). These signalling genes are known to be involved, or at least partly involved, in the regulation of SCs (Adams 1998, Chen *et al.* 2005), suggesting that SCs would be activated quite similar in both groups independently of the level of testosterone and because of a significant increase in lean leg mass in the placebo group and the goserelin group although significantly larger in the placebo group (Kvorning *et al.* 2006, 2007). On the other hand, when taking the relatively short training period (8 weeks) into consideration, it could be argued that any possible difference in the amount of SCs has not been able to express itself in such a short period. Thus, it could be speculated that if the strength training period was longer in duration, then the number of SCs measured may have turned out significantly different between the placebo and goserelin group, because of a larger demand for myonuclear addition (i.e. to satisfy the need for increased protein synthesis) in placebo vs. the goserelin group.

Only few earlier studies have investigated the acute effect of a single training bout on SC. Studies on eccentric exercise have showed an increase in the number of SCs in response to a single training session, when biopsies were taken 0, 2, 4, 6 and 8 days after training (Crameri *et al.* 2004), but also when taken 24 h after training (Dreyer *et al.* 2006). These results are in contrast to our findings, but the fact that both studies used training protocols characterized by strenuous eccentric muscle actions may explain the difference in SC activity. Furthermore, our acute biopsies (Post4h and Post24h) were taken in relation to the second last strength training session in the strength training period, and it could be speculated that a possible response in SC activity may be blunted. Thus, when interpreting data on gene expression from a single training session without a prior training period, it is important to acknowledge that this response is

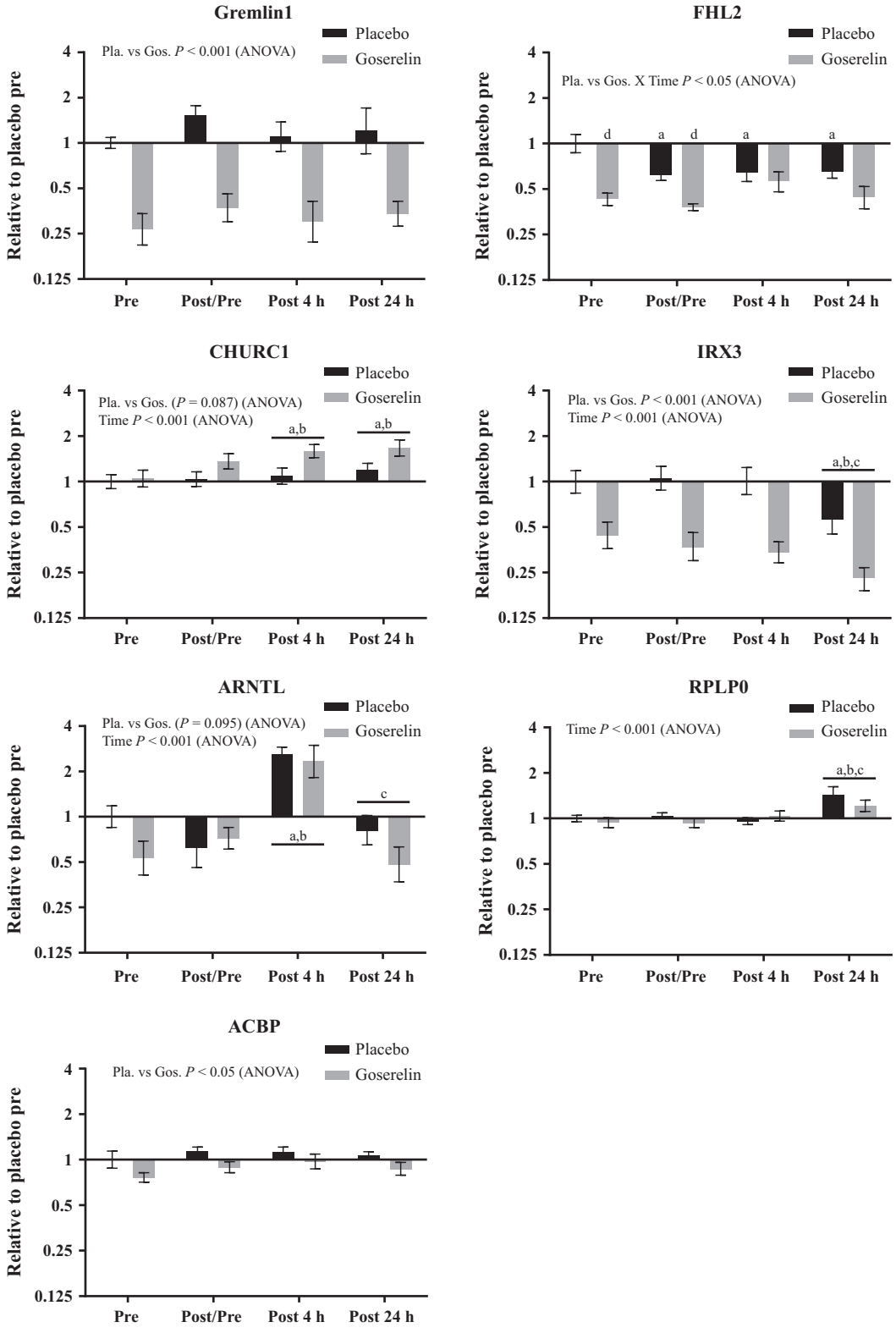

Figure 5 RT-qPCR mRNA data. mRNA levels shown relative to mean of Pre-samples for the placebo group. Placebo (n = 7, 6, 7, 6) and goserelin (n = 8, 8, 9, 7). Values are presented as geometric Mean ± back-transformed SE. Significant ANOVA results are shown in text (trends in parentheses). Post hoc comparisons ($P < 0.05$) vs. Pre, Pre/Post and Post4h are indicated with the letters a, b and c, respectively, and goserelin vs. placebo at specific time point with the letter d.

likely to differ markedly from the gene expression measured on a single training session with a prior training period (Cameron-Smith 2002). Thus, the Post4h biopsies, taken at the end of the 8-week training period, were deliberately chosen to get the 'true training response' rather than measuring a response, which is related to the unaccustomed insult to the muscle (Mackey *et al.* 2009).

A microarray analysis was performed to screen for other potential SC-regulating mechanisms and if other relevant genes are regulated by testosterone. As the number of arrays was a limiting factor, we chose to directly compare the placebo and goserelin group at the 4-h time point (Post4h). It should also be mentioned that we did not compare to Pre-values; hence, exclusively differential effects between placebo and goserelin will be captured, not common exercise effects. Because the 4-h time point is also 52 h after the previous training bout, we should be able to capture both acute changes as well as long-term accumulated changes from the strength training period with differential response in the goserelin group vs. placebo (see Material and methods). However, even then very few changes in mRNA were found, suggesting that the testosterone effect is rather specific. Still, it should be kept in mind that this is a screening and changes might be lost due to small changes (<1.5 fold) which was not picked up by the analysis, acute changes by the training session occurring between the 4-h and 52-h time points or changes during the early weeks of training which have disappeared after the 8 weeks. Furthermore, targets not confirmed by qRT-PCR in Table 3 are likely due to false positive in array, but could also be due to differences in target sequences in array vs. PCR due to unexpected alternative splicing. Attempts to isolate overrepresented pathways using the gene list in Table 3 and 'Gene set analysis' in the Partek software did not provide any significant pathways (data not shown).

Of those changed by goserelin treatment, all except CHURC1 were reduced by suppression of testosterone. This appears to be the case already before training, and ARNTL, ACBP, Gremlin1 and IRX3 seem to keep this lower level throughout the training period. In the case of FHL2, the downregulation of FHL2 induced by strength training is blunted by the lack of testosterone. However, as goserelin also reduces the FHL2 level, the end-result is that even though the suppression of testosterone blunted the training session response, the level of FHL2 mRNA mimics the training session response anyway. CHURC1 is the only mRNA that is induced by the lack of testosterone.

Unfortunately, the few goserelin-dependent mRNA does not provide any clear indication of how suppression of testosterone influence muscle hypertrophy. However, it is worth noting that CHURC1 has been shown to interfere with bone morphogenetic proteins (BMP) signalling by inducing a SMAD interaction protein, ZEB2 (Sheng *et al.* 2003). Furthermore, Gremlin1 is a BMP antagonist (Glister *et al.* 2005), and myostatin uses the same signalling pathway as that of the BMP (Rebbapragada *et al.* 2003). Also, both FHL2 and IRX3 have recently been shown to interfere with the BMP signalling (Neuman *et al.* 2009, Gaborit *et al.* 2012). These findings suggest that BMP signalling is somehow involved in testosterone-dependent muscle hypertrophy, although further experiments are warranted. Because BMP signalling has recently been shown to regulate proliferation vs. differentiation of SCs (Friedrichs *et al.* 2011), this could offer an explanation for the goserelin effect, that is blockage of the SC fusion to myotubes via the BMP pathway.

In conclusion, an 8-week strength training period significantly increased the number of SCs in type II fibres in the placebo and in the goserelin group independently of endogenous testosterone levels. But we demonstrate a significant addition of myonuclei in type II fibres in parallel with significant larger gains in lean leg mass in the placebo group compared to the goserelin group. Testosterone does not seem to be a main regulator of SCs and myonuclei. On the other hand, testosterone may influence the accretion of myonuclei to muscle fibres tentatively via the BMP signalling pathway.

Conflict of interest

This investigation does not involve any financial or personal relationships or other conditions that could be viewed as presenting a potential conflict of interest.

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