Horm Mol Biol Clin Invest 2010;1(2):73–79 © 2009 by Walter de Gruyter • Berlin • New York. DOI 10.1515/HMBCI.2010.007

# Monitoring gene expression in muscle tissue of *macaca fascicularis* under the influence of testosterone and SARM

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## Abstract

The focus of this study was to evaluate data on the gene expression profiles induced by testosterone and a selective androgen receptor modulator (SARM, TAP Pharmaceutical Products Inc., Lake Forest, IL, USA) in androgen sensitive muscle tissue to obtain a better understanding on the molecular mechanisms of action and to identify biomarkers for SARM function in primate organs. A total of 24 male cyomolgus monkeys were divided into four groups: testosterone group, SARM1 group, SARM10 group, and control group, each consisting of six animals. The testosterone group was treated i.m. with 3.0 mg/kg Testostoviron®-depot-250 (Schering, Berlin, Germany) every 2 weeks, the SARM1 and SARM10 groups with 1 mg/kg or 10 mg/kg SARM LGD2941 daily, and the control group was not treated. Muscle biopsies from *musculus quadriceps* and *musculus triceps* were collected at three time points: baseline time point before SARM application (control), on day 16, and on day 90 of treatment. A total of 30 candidate genes were selected according to their functionality by screening the actual literature and were composed to the following functional groups: cell cycle, endocrine factors, energy metabolism, muscle fiber proteins, muscle specific transcription factors, protein metabolism, and satellite cell biology. Biomarkers were identified as genes regulated from baseline in any of the three treatment groups at day 16 or day 90 using analysis of variance with baseline defined as the contrast group. Out of 23 tested candidate genes, 3 were significantly regulated in *m. quadriceps* after 90 days treatment; in *m. triceps* no significant differences were identified. Cathepsin L, calpain 3, and insulin like growth factor binding protein 3 could be identified as first biomarkers, and first physiological differences between control and treatment samples were determined. Both testosterone and SARM LGD2941 appear to have similar effects after 90 days treatment, and thus a longer-term therapy with these substances can be recommended.

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Received June 2, 2009; accepted July 20, 2009; previously published online September 17, 2009

**Keywords:** candidate gene approach; expression profiling; qRT-PCR; selective androgen receptor modulator; testosterone.

## Introduction

Owing to demographic changes in our population, sarcopenia and frailty have become major topics in the healthcare profession. Approximately 30% of those over 60 years of age are affected. Sarcopenia, the loss of skeletal muscle mass and strength, in both males and females results in a high incidence of accidental falls and can compromise quality of life. Chronic and age-related afflictions, such as osteoporosis, are linked to sarcopenia. The decrease in the production of hormones, such as testosterone, estradiol, growth hormone, insulin like growth factor 1, and the general decline in muscle protein turnover, as well as neuromuscular alterations, are the major reasons for the appearance of frailty. These result in weakness, impaired mobility, and poor endurance, factors that lower independence and reduce quality of life (1–4).

Muscles are affected by lower endogenous anabolic hormone levels and muscle protein turnover. Studies have shown that muscle strength and muscle cross-sectional area decreases and hypotrophy arises. Other findings, in particular testosterone deficiency, are similar to those associated with aging. These include loss of energy, depressed mood, decreased libido, erectile dysfunction, decreased muscle mass and strength, increased fat mass, frailty, osteopenia, and osteoporosis (5–7). Androgen replacement therapies have been applied as therapies for some of these symptoms, not only for men but also for women (8–11). Particularly for women, the therapeutic uses of androgens do not only have beneficial effects because numerous side effects exist (12–16).

A promising alternative for androgen replacement therapies is the discovery of selective androgen receptor modulators (SARMs). SARMs have the ability to mimic the central and peripheral androgenic and anabolic affects of testosterone without having the negative side effects of the natural hormone (17). Further positive properties of investigated SARMs, such as oral activity, increase in muscle mass and strength, and increase in fat-free mass, are important factors for its use as medication (18–20).

Applying anabolic agents, changes in the biochemical pathways are notified in the organs and tissues. Expression of specific enzymes, receptors, and cytokines can be activated or suppressed on the cellular mRNA expression level.

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Using appropriate specific and sensitive quantification methods, such as the quantitative real-time RT-PCR, even minute expression changes are measurable as a difference in the respective mRNA.

The aim of this study was to show how a SARM in comparison to testosterone can act in muscle tissues via mRNA expression of different candidate genes. Sufficient plurality of regulated candidate genes could then serve as biomarkers to show molecular and myogenic effects of the treatments.

## Experimental section

### Animal experiment

The animal husbandry and tissue collection were done by Covance Laboratories GmbH (Münster, Germany). Groups of six male cyomolgus monkeys (*macaca fascicularis*) were either injected i.m. with 3.0 mg/kg Testostoviron®-depot-250 (Bayer Schering, Berlin, Germany) biweekly (Testo) or orally treated with 1 mg/kg (SARM1) or 10 mg/kg (SARM10) LGD2941 (TAP Pharmaceutics, Lake Forest, IL, USA) every day. The control group did not receive treatment and consisted of six animals. All monkeys were 5–6 years old and had an average body weight of 6 kg.

### Sample collection

Muscle biopsies of *musculus quadriceps* and *musculus triceps* were taken at three time points. Control samples were taken without prior treatment. Further samples were taken at day 16 and day 90 of treatment. Biopsy samples were placed in cryotubes before freezing in liquid nitrogen and stored at −80°C until RNA extraction.

### Total RNA extraction

In total, 50 mg of muscle biopsies at baseline time point (control) and 100 mg at day 16 and day 90 time points were collected. Frozen tissue was homogenized in guanidinium thiocyanate buffer to destroy RNase activity. For the extraction of the RNA, a TriFast clean protocol was used (PeqLab Biotechnologies, Erlangen, Germany). The principal of this protocol was phenol/chloroform extraction for total RNA. To quantify the amount of total RNA extracted, optical density was measured with a photometer (Eppendorf Biophotometer, Hamburg, Germany) for each sample. All RNA stem solutions were diluted to 100 ng/μL for Bioanalyzer analysis and to 1 ng/μL for PCR analysis.

### Analysis of RNA quality

RNA integrity and quality control was done by capillary electrophoresis in a Bioanalyzer 2100 (Agilent Technology, Palo Alto, CA, USA). Eukaryotic total RNA nano assay algorithm was taken for sample analysis and the RNA integrity number (RIN) was used to indicate RNA quality (21, 22). For optimal RNA quality biopsy, storage in cryotubes is recommended.

### Selection of candidate genes

Candidate genes, factors, and enzymes were selected if assumed to play an important role in the myogenic pathways of the analyzed tissues. The following 30 candidate genes (TGs) were chosen by screening the literature. *Muscle fiber proteins*: myosin (MYHC-2×) and α-actin (ACTA1); *muscle specific transcription factors*: myogenin and myogenic differentiation factor (MyoD); *protein metabolism group*: cathepsin B (CTSB), cathepsin L (CTSL), calpain 3 (CAPN3), and tyrosine aminotransferase (TAT); *factors of cell cycle regulation*: cyclin-dependent kinase 2 (CDK2), Cyclin A, Cyclin D1, and myostatin (GDF8); *endo- and paracrine group*: androgen receptor (AR), estrogen receptors (ERα, ERβ), growth hormone receptor (GHR), glucocorticoid receptor (GR), insulin like growth factor 1 (IGF1), insulin like growth factor receptor 1 (IGF1R), insulin receptor (IR, transcript1), insulin like growth factor binding proteins (IGFBP3, IGFBP4); *energy metabolism group*: hexokinase (HK), lactate dehydrogenase (LDH), creatine kinase (CK), glucose transporter 4 (GLUT4) and glycogen phosphorylase (PYGM); *satellite cell group*: myogenic factors (Myf5, Myf6) and m-cadherin (CDH15).

As *reference genes* (RGs), β-actin (ACTB), glycerinaldehyde-3-phosphate-dehydrogenase (GAPDH) cyclophilin A (PPIA), tyrosine3/trypCthan5-monooxygenase activation protein (YWHAZ), ubiquitin C (UBC), and 18S RNA primers were tested.

### Primer design and primer testing

Primers were designed using published nucleic acid sequences of the human genome GenBank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi). PPIA and ACTB were taken from the Human Endogenous Control Gene Panel Kit (TATAA Biocenter, Göteborg, Sweden). Primer design and optimization was done using primer design program of MWG (MWG Biotech, Ebersberg, Germany) with regard to primer dimer formation, self-priming formation, and primer annealing temperature (60°C). Newly designed primers were ordered and synthesized at MWG Biotech and Invitrogen (Karlsruhe, Germany). Primer testing was performed on four muscle samples and a negative control (RNAse free water) for each primer set.

Specificity of the primer was controlled by melt curve analysis and agarose gel electrophoresis.

### One-step quantitative RT-PCR

Quantitative real-time RT-PCR was performed using a SuperScript III Platinum SYBR Green One-Step qPCR Kit (Invitrogen, Carlsbad, CA, USA) by a standard protocol, recommended by the manufacturer.

Crossing points (CTs) and melting curves were acquired by using the ''comparative quantitation'' and ''melting curve'' program of the Rotor-Gene 3000 analysis software (V.1.7.2007). CT is the equivalent nomenclature of Cycle of threshold used in Rotor-Gene software.

Only genes with clear melting curves were taken for further data analysis. Samples with irregular melting peaks were excluded from the calculation.

### Data analysis and statistics

Statistical descriptions of the expression data as well as statistical tests were produced with SAS v. 9.1.3 for Windows (Microsoft, USA). The raw data was the CT values obtained from each qPCR sample. Each qPCR sample was associated with a biopsy sample, whereas from each experimental animal two biopsy samples were analyzed. Because the amplification efficiency was not known, the assumption of identical amplification efficiency 100% was made, allowing for a more simple quantification model.

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The CT values of each gene were averaged by the arithmetic mean for each biopsy sample. The obtained mean CT values were then translated to normalized expression quantities using all three reference genes in the form of a normalization index. The normalization index was calculated as an arithmetic mean of the CT values of the three reference genes:

$$\text{reference index} = \text{mean}\,(CT_{PPIA}, CT_{ACTB}, CT_{YWHAZ}) \quad (1)$$

Then, an expression of every target gene was calculated relatively to the expression of the housekeeping gene as:

$$\text{normalized expression} = 2^{\text{reference index}}/2^{\text{CT target gene}} \quad (2)$$

The 2 represents the 100% amplification efficiency. The normalized expressions of the time points 16 and 90 days were then divided with the normalized expressions of the baseline generating the expression ratio R as:

$$R_{\text{time point/baseline}} = \text{normalized expression}_{\text{time point}}/\text{normalized expression}_{\text{baselinee}} \quad (3)$$

The expression ratio R was then analyzed statistically. The box-whisker plot was constructed to facilitate visual screening of regulated genes (Figures 1–4).

The objective of the statistical analysis was to disclose genes with significant regulation between the control group and any of the treated groups. It was not intended to perform all treatment-to-treatment tests for all genes to avoid statistical type I error (false positive difference). Hence, analysis of variance (ANOVA) was calculated on the $\log_2$ transformed R values employing the SAS procedure GLM with contrast sentence defining the control group as the contrast group for all treatment groups, and thus adjusting the overall test confidence level to the number of relevant comparisons only.

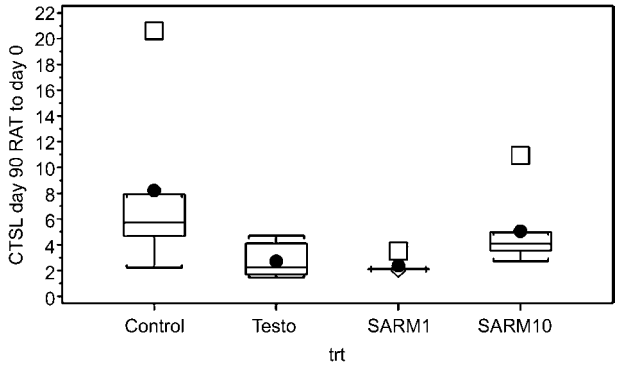


**Figure 1** Significant downregulation of CTSL between control and treated samples. The box-whisker plot shows the expression ratio R between the normalized expression of the time point 90 days and baseline. The horizontal line inside the box indicates the median and the box indicates the interquartile range. The filled symbol indicates the mean. The whiskers indicate the maximum and minimum and the square indicates the outliers. The diamond indicates weak outliers. The calculation of the outliers is based on the box-whisker rule which states that an outlier is a point which falls more than 1.5 times (weak outlier) or 3 times (strong outlier) the interquartile range above the third quartile or below the first quartile.

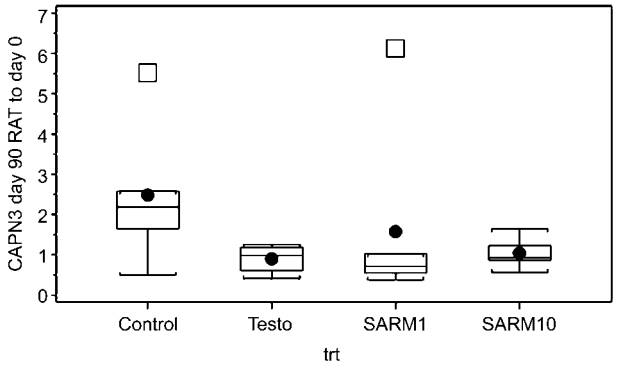


**Figure 2** Significant downregulation for CAPN3 between control and treated samples. The box-whisker plot shows the expression ratio R between the normalized expression of the time point 90 days and baseline. The horizontal line inside the box indicates the median and the box indicates the interquartile range. The filled symbol indicates the mean. The whiskers indicate the maximum and minimum and the square indicates the outliers. The diamond indicates weak outliers. The calculation of the outliers is based on the box-whisker rule which states that an outlier is a point which falls more than 1.5 times (weak outlier) or 3 times (strong outlier) the interquartile range above the third quartile or below the first quartile.

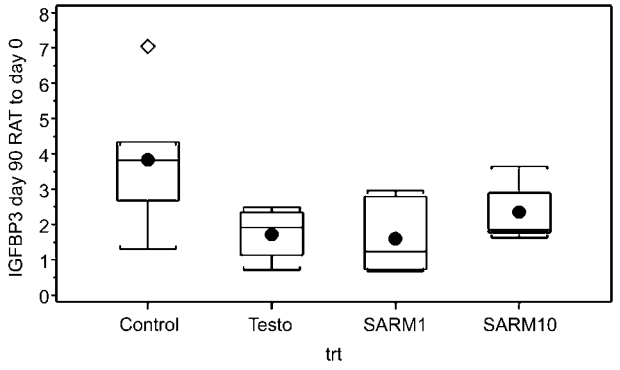


**Figure 3** Significant downregulation for IGFBP3 between control and treated samples. The box-whisker plot shows the expression ratio R between the normalized expression of the time point 90 days and baseline. The horizontal line inside the box indicates the median and the box indicates the interquartile range. The filled symbol indicates the mean. The whiskers indicate the maximum and minimum and the square indicates the outliers. The diamond indicates weak outliers. The calculation of the outliers is based on the box-whisker rule which states that an outlier is a point which falls more than 1.5 times (weak outlier) or 3 times (strong outlier) the interquartile range above the third quartile or below the first quartile.

Further adjustment of the overall confidence level with regard to number of investigated genes was not performed. Hence, this study is to be considered as purely explorative, whereas significant findings here indicate candidate biomarkers. Tests generating significant ($p < 0.05$) results were reviewed based on descriptive parameters of the compared groups and visually by means of the box-whisker plots to disclose possible outliers. As comparable trends were observed between the three treatment groups, no further tests were employed.

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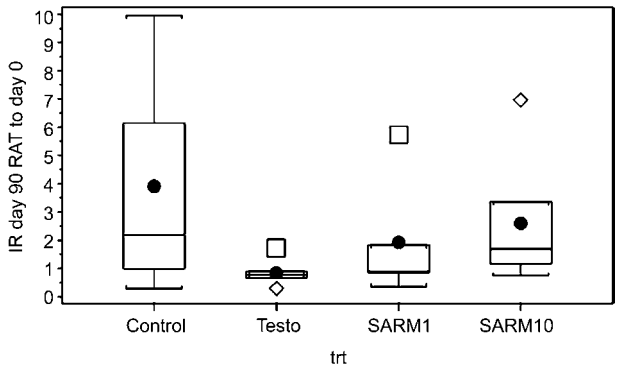


**Figure 4** IR in the control group shows a high statistical spread which is not observed in the treatment groups, especially in the Testo group. The box-whisker plot shows the expression ratio R between the normalized expression of the time point 90 days and baseline. The horizontal line inside the box indicates the median and the box indicates the interquartile range. The filled symbol indicates the mean. The whiskers indicate the maximum and minimum and the square indicates the outliers. The diamond indicates weak outliers. The calculation of the outliers is based on the box-whisker rule which states that an outlier is a point which falls more than 1.5 times (weak outlier) or 3 times (strong outlier) the interquartile range above the third quartile or below the first quartile.

## Results and discussion

### RNA quality of muscle tissue

The mean RIN value of control samples was 6.5 for the *m. quadriceps* samples and 6.9 for the *m. triceps* samples, representing a total RNA of average quality. The *m. quadriceps* samples of day 16 had a mean RIN of 7.1, and the *m. triceps* of 7.6. At day 90, *m. quadriceps* samples showed a mean RIN of 6.9, and *m. triceps* samples of 7.6.

Control samples showed a higher 5S RNA peak for all samples which might indicate partly degraded total RNA. Because control biopsy samples were directly frozen in liquid nitrogen, it is supposed that large RNA was partly damaged but the smaller 5S RNA remained intact. For samples placed in cryotubes before freezing in liquid nitrogen, large RNA was more intact.

The RNA quality of all samples was sufficient to obtain satisfactory results in qRT-PCR.

### Primer pairs and primer testing

Based on the human genome, primer pairs for 30 TGs and 6 RGs were developed, 23 TGs and 3 RGs were successfully running in qRT-PCR (Table 1), showing specific melt peaks and specific bands on agarose gel electrophoresis. Until now, nearly no gene sequences from *macaca fascicularis* exist and because these sequences appear to be different from human, some primer pairs did not anneal. Only a few gene sequences of *macaca fascicularis* were available in public gene banks, and thus the gene sequences of *homo sapiens* and *macaca mulatta* were used for primer pair design. Despite this problem, 23 candidate genes could be successfully amplified.

### qRT-PCR results and data analysis

The calculation of the expression ratios [Eq. (1)] produced non-normally distributed data with frequent extreme values. Some of the extreme values can be outliers and were indicated in the box-whisker plot as squares outside the beyond interquartile range (box). Nonetheless, no exclusion of extreme values/outliers was performed.

In *m. quadriceps*, significant differences between control and treatment were identified after 90 days treatment, including CTSL ($p = 0.0138$), CAPN3 ($p = 0.0625$), and IGFBP3 ($p = 0.0280$) (Figures 1–3).

There were no significant regulated genes found in *m. triceps*. Possible effect of outliers was investigated and was not excluded. Only the three significant regulated genes at the time point 90 days in *m. quadriceps* are hence discussed further. Surprisingly, the expression ratio R deviated from the value 1 in the control group, thus showing endogenously induced upregulation throughout the growth period of the animals. This conclusion is supported by the fact that no pronounced regulation was found at the time point 16 days.

The control group also showed high variability compared with the treatment groups, as indicated by the box-whisker plot. This reflects the natural variability of the non-induced expression in each studied subject. In contrast to the control group, in the treated groups which showed lower R ratios smaller variability was also observed, possibly supporting evidence for an induced suppressing effect on an individual's expression.

In *m. quadriceps*, the control samples showed an expression with a high statistical spread in the significantly regulated candidate genes what mirrors natural differences between gene expressions in the individuals. This spread was lowered in the treatment groups, showing a suppression of the individual expression differences induced by the treatment. This effect was not only observed in the significantly regulated genes, also IR in *m. quadriceps* showed a larger spread in the control group than in the treatment groups (Figure 4). This difference in spread was strongly revealed in the testosterone group, indicating an inhibitory effect of testosterone on IR expression.

To identify physiological differences between the control group and the treatment groups, only the significantly regulated genes IGFBP3, CTSL, and CAPN3 were discussed.

In skeletal muscle cell lines, four IGFBPs were released: IGFBP2, -4, -5, and -6. IGFBP3 was mainly acting as a major carrier of the IGFs in circulation. Many studies have also shown an inhibitory effect of IGFBP3 on IGF1 and IGF2. Owing to its higher affinity to IGF1, it can also prevent IGF1–IGF1-R interactions (23–26). In this study, IGFBP3 was downregulated in the treatment samples, which could show a switch-off effect of the inhibiting IGFBP3. Even if IGF1 was not significantly upregulated, a blockage of IGFBP3 could increase its functionality in the organism.

In the protein metabolism of the muscle, CTSL is induced early in the catabolic states and an increase of CTSL expression also characterizes muscle wasting. Anabolic steroids, such as testosterone, increase protein retention and decrease

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**Table 1** List of primer pairs used for PCR analysis in muscle tissue.

| Group | Primer | Sequence 5′-3′ | bp | Accession no. |
|---|---|---|---|---|
| Muscle fiber proteins | ACTA1 for | CAT GGT CGG TAT GGG TCA GAA | 21 | NM001100 |
| | ACTA1 rev | CGC GAA GCT CGT TGT AGA AG | 20 | |
| | MYHC-2× for | GCA CAT CCA GAG CAG AGA AGA A | 22 | NM017534 |
| | MYHC-2× rev | GAC AGT GAC ACA GAA GAG ACC T | 22 | |
| Transcription factors | Myogenin for | ATC ATC TGC TCA CGG CTG AC | 20 | NM002479 |
| | Myogenin rev | TTT CAT CTG GGA AGG CCA CAG A | 22 | |
| Protein metabolism | CTSB for | ACAATTCCTACAGCGTCTCC | 20 | L16510 |
| | CTSB rev | CACCACTTCTGATTCGATTCC | 21 | |
| | CTSL for | ACATCCCTAAGCAGGAGAAGGC | 22 | M20496 |
| | CTSL rev | CCAGCACACCATGATCCATGTC | 22 | |
| | CAPN3 for | TCCAGTTCGTCTGGAAGAGACC | 22 | AY902237 |
| | CAPN3 rev | TGTACGTTGGCAGGCAGTCATC | 22 | |
| Cell cycle regulation | Cyclin D for | AAC AAG CTC AAG TGG AAC CTG G | 22 | NM053056 |
| | Cyclin D rev | CAT GGA GGG CGG ATT GGA AAT | 21 | |
| | Myostatin for | AAC TTG ACA TGA ACC CAG GCA C | 22 | NM005259 |
| | Myostatin rev | ACG GAT TCA GCC CAT CTT CTC | 21 | |
| Endo-paracrine factors | AR for | TTG TCC ATC TTG TCG TCT TCG G | 22 | L29496 |
| | AR rev | TGT CCA GCA CAC ACT ACA CC | 20 | |
| | ERβ for | ATG CTC ACT TCT GCG CTG TC | 20 | NM001437 |
| | ERβ rev | CAC ACT TCA CCA TTC CCA CTT C | 22 | |
| | GHR α for | ATC CAC CCA TTG CCC TCA AC | 20 | NM00163 |
| | GHR α rev | ATC TCA CAC GCA CTT CAT ATT CC | 23 | |
| | IGF-1R for | CAT TTC ACC TCC ACC ACC AC | 20 | NM000875 |
| | IGF-1R rev | AGG CAT CCT GCC CAT CAT AC | 20 | |
| | IGFBP3 for | CAC AGA TAC CCA GAA CTT CTC C | 22 | M35878 |
| | IGFBP3 rev | CCA TAC TTA TCC ACA CAC CAG C | 22 | |
| | IGFBP4 for | CAA CTT CCA CCC CAA GCA GT | 20 | NM001552 |
| | IGFBP4 rev | CTG GTG GCA GTC CAG CTC | 18 | |
| | IR for | TCC AGA CAG ATG CCA CCA AC | 20 | NM000208 |
| | IR rev | ATC CAG CTC GAA CAG CTC AC | 20 | |
| | IGF1 for | TGG ATG CTC TTC AGT TCG TGT G | 22 | ENSMMUT03165 |
| | IGF1 rev | TAG AGG GAG TGC AGG AAA CAA G | 22 | |
| | GR for | TTC TGC GTC TTC ACC CTC AC | 20 | AH002750 |
| | GR rev | CTG TCT CTC CCA TAT ACA GTC C | 24 | |
| Energy metabolism | HK for | CGC ATC TGC TTG CCT ACT TC | 20 | NM000189 |
| | HK rev | AAC TCT CCG TGT TCT GTC CC | 20 | |
| | LDH for | TTC AGC CCG ATT CCG TTA CC | 20 | NM005566 |
| | LDH rev | CAC CTC ATA AGC ACT CTC AAC C | 22 | |
| | CK for | GAC ATC GTC TAC AGT GAA GCC | 21 | NM001824 |
| | CK rev | AGT TCA AGC TGA ATT TAC AAG CCT G | 25 | |
| | PYGM for | ACA TCA ACC CCA ACT CAC TCA C | 22 | AF066859 |
| | PYGM rev | GCC TTC CCT CCA ATC ATC AC | 20 | |
| Satellite cell biology | Myf5 for | TGA GAG AGC AGG TGG AGA AC | 20 | NM005593 |
| | Myf5 rev | GGC AAC TGG AGA GAG AGA AG | 20 | |
| | Myf6 for | GCC AAG TGT TTC CGA TCA TTC C | 22 | NM002469 |
| | Myf6 rev | ACT TCT CCA CCA CTT CCT CC | 20 | |
| Reference genes | β-actin for | TATAA Biocenter | | |
| | β-actin rev | TATAA Biocenter | | |
| | PPIA for | TATAA Biocenter | | |
| | PPIA rev | TATAA Biocenter | | |
| | YWHAZ for | GCA ACC AAC ACA TCC TAT CAG AC | 23 | NM145690 |
| | YWHAZ rev | TTC TCC TGC TTC AGC TTC GTC | | |

protein catabolism (27). In this study, the significant down-regulation of CTSL in treatment confirms this protein retention effect.

CAPN3 is a member of the three distinct calpains expressed in the muscle. The ubiquitous calpains 1 and 2 and CAPN3 are members of the $Ca^{2+}$-activated cysteine proteases, but CAPN3 is known to show inverse regulations compared with the ubiquitous calpains. Many studies have proven that a decrease of CAPN3 is directly related to muscle wasting (9, 28). No studies exist that show an effect of

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testosterone on calpain expression. In this study, CAPN3 was significantly downregulated by treatment compared with control. Such downregulation of CAPN3 could also be observed in conditions related to disuse and denervation of the muscle, an age-related phenomenon (29). In this study, an increase in CAPN3 was expected but could not be shown. Further studies are needed to identify the mechanism which caused CAPN3 downregulation under testosterone treatment.

No significant regulation for both the androgen and the glucocorticoid receptor could be detected. This was not expected but shows that there does not appear to be any regulation at the receptor level.

## Conclusion

In this study, both the anabolic steroid testosterone and the SARM LGD2941 showed effects on the gene expression in the muscle tissues. Significant influences of the treatments appeared after 90 days only, indicating that the differences between the control group and the treated groups is rather based on suppression of long-term natural development of the studied factors. A general long-term suppression effect of the treatments on the natural expression spread could be identified.

First influences of the treatments on the protein metabolism and the muscle growth could be observed via the significant expression of CTSL, CAPN3, and IGFBP3. The reason for predominant absence of significantly regulated target genes could be a too low dosage of the treatments.

Nonetheless, CTSL, CAPN3, and IGFBP3 can be identified as first biomarkers for a gene expression pattern of anabolic agents, but further studies are needed to identify more significantly regulated genes and to show physiological influences of the treatment.

## Acknowledgements

We are indebted to TAP Pharmaceuticals Inc., Lake Forest, IL, USA who initiated and supported this study.

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