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Expression profiling of skeletal muscle in young bulls treated with steroidal growth promoters

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Carraro L, Ferraresso S, Cardazzo B, Romualdi C, Montesissa C, Gottardo F, Patarnello T, Castagnaro M, Bargelloni L. Expression profiling of skeletal muscle in young bulls treated with steroidal growth promoters. *Physiol Genomics* 38: 138–148, 2009. First published April 21, 2009; doi:10.1152/physiolgenomics.00014.2009.— Dexamethasone (Dex), alone or in association with estrogens, is often illegally administered per os at very low dosage as a growth promoter in beef cattle, with effects that are opposite to the muscle wasting and atrophy induced by repeated administration at therapeutic dosages. In vitro and in vivo studies have investigated the catabolic effects of Dex at therapeutic doses on skeletal muscle, demonstrating an increase in the expression of GDF8 (myostatin) gene, a well-known negative regulator of skeletal muscle mass, in a dose-dependent way. This suggested a direct role of myostatin in Dex-induced muscle wasting. In the present study, an oligonucleotide microarray platform was used to compare expression profiles of beef cattle muscle in animals treated with either Dex or Dex plus 17-β estradiol (Estr) administered at subtherapeutic dosage, against untreated controls. Data analysis demonstrates that the expression profiles were strongly affected by Dex treatment with hundreds of genes upregulated with relevant fold-change, whereas seven genes were downregulated including the myostatin gene. On the contrary, the number of differentially regulated genes was lower in response to the addition of Estr to the Dex treatment. Differentially regulated genes were analyzed to describe the effects of these treatments on muscle physiology, highlighting the importance of specific pathways (e.g., Wnt or cytokine signaling) and cellular processes (e.g., cell shape and motility). Finally, the observed differences in the expression profile will allow the development of indirect bio-markers to detect illegal Dex treatments in beef cattle using quantitative RT-PCR.

microarray; dexamethasone; estradiol; myostatin

GROWTH IS A FUNDAMENTAL BIOLOGICAL process in all living organisms, and the mechanisms that regulate such a process constitute one of the most important topics in animal physiology. Body size and, more specifically, muscle development are obviously of great interest also for farm animal breeders. Innumerable efforts have been devoted to selective breeding for fast-growing and/or large-size animals or to the improvement of husbandry conditions to optimize growth rates. The administration of certain bioactive substances (e.g., hormones) has also been demonstrated to increase body size and/or muscle mass through various mechanisms. However, while partially accepted in other countries, the use of natural and synthetic hormones as growth promoters in meat-producing animals is prohibited in the European Union (12, 13). Despite the ban, their use in beef cattle is still practiced to increase the productivity and to reduce farming costs (14, 40).

Recent surveys revealed that dexamethasone (Dex), a synthetic glucocorticoid, is often present at detectable concentrations in the liver of slaughtered animals (Ref. 79 and C. Montesissa, unpublished results). Dex and other synthetic glucocorticoids (GCs) are used therapeutically for a wide variety of conditions that require immune and/or inflammatory modulation; therefore, the presence of such substances in farmed animals is illegal, but not necessarily fraudulent. Detection of these substances after slaughtering might occur in consequence of inappropriate use (e.g., exceeding doses, insufficient withdrawal time). However, synthetic GCs are detected too frequently not to raise the suspicion that they are used to achieve a larger body size and/or to improve meat quality and yield. The use of GCs as growth promoters might seem counterintuitive. Pharmacological doses of Dex has been shown to induce loss of body weight through mechanisms that involve increased secretion of leptin (29). GCs at therapeutic dosage have also a wide range of adverse effects on skeletal muscle, i.e., muscle wasting and atrophy (18, 67). These effects on muscle have been investigated in vitro (38) and in vivo (39), demonstrating an increase in the expression of the myostatin gene after Dex administration in a dose-dependent way. Myostatin, a member of transforming growth factor-beta family (TGF-β) is a negative regulator of skeletal muscle mass (see Refs. 49 and 61 for review). Recently, Gilson and colleagues (19) further demonstrated a direct relationship between GC-induced muscle atrophy and myostatin expression, showing that muscle wasting could not be observed after Dex administration in myostatin-null mice.

On the other hand, GCs are the only drug reported to have a positive effect on muscle degenerative disorders. For instance, intermittent treatments with low-dosage GCs have been shown to improve the condition of patients affected by Duchenne muscular dystrophy (17). The mechanism by which patients benefit from GC treatment is not fully understood, although it is thought that the clinical benefits arise in part from the anti-inflammatory and immunosuppressive effects of these drugs (73). Additional effects of GCs might be related to the increased expression of utrophin (43), possibly through activation of the calcineurin/NF-AT pathway (68).

As in the case of the positive effects on muscle degenerative disorders, low dosage and limited duration of treatment might be the key to explain the use of GCs as growth promoters. Basal levels of GCs are known to stimulate appetite (15, 64).

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Synthetic GCs such as Dex more specifically increase carbohydrates consumption. Increased food intake and stimulated carbohydrate metabolism might therefore explain, at least partially, why the use of low doses of GCs, alone or in association with anabolic steroids, have long been reported to improve growth or slaughter performance in farm animals (1, 28). Confirming these reports, a recent study (23) evaluated the effects on productive traits, animal behavior, and meat quality after oral administration of Dex at low daily dose (0.75 mg per day) in finishing bulls. A substantial increase in growth rate was observed in the initial phase of treatment, with significantly higher average daily gain. However, at the end of the experiment (7 wk) no significant differences in body and carcass size were observed in treated animals compared with controls (23).

We report here a novel animal experiment, where low-dosage Dex was administered either alone or in association with estrogens, and gene transcription profiles of skeletal muscle in treated animals against controls were obtained using a cattle-specific oligo-DNA microarray.

The purpose of the present study was twofold. First, to evaluate, at the mRNA level, the effects of low-dosage GCs directly on skeletal muscle in cattle, and their potential interactions with other steroid hormones. Second, to identify a set of differentially expressed genes in treated animals compared with controls. Gene expression might then be used as an indirect biological marker (57, 74) to screen for illicit use of corticosteroids in samples collected at slaughterhouses.

MATERIAL AND METHODS

Animals. Eighteen mixed-breed (Charolaise × Limousine) bulls 18 mo old, ~450 kg mean body weight, were randomly allocated in three groups: six were untreated (controls), six were administered with Dex via feed 0.75 mg/head daily for 43 days (group Dex), the last six animals were administered via feed for 43 days with Dex (0.75 mg/head) and intramuscularly three times with 17β-estradiol (Estr), 20 mg/head, (group Dex+Estr) after 7 days, 21 days, and 35 days from the start of the experiment. The animals were housed in ventilated stables, and the experiment was carried out according to the European Union animal welfare legislation. A single animal in the Dex group was excluded from all the analyses because of severe health problems experienced during the experiment. At the end of the experiment, all the animals were weighed and slaughtered 1 day after. At the slaughterhouse, tissue samples (2–300 mg) from a specific anterior limb muscle (Biceps brachii) were collected in 2 ml of RNAlater solution (Ambion-Applied Biosystems, Austin, TX) and stored at −20°C until extraction. Three additional control animals, matching in sex and age, collected in a previous experiment were included to increase sample size and to control for biological variation. The above experimental design builds upon a previous experiment where a similar Dex daily dose was forced-fed to treated animals for 7 wk (23). In the present study, the duration of treatment was shortened, and the mode of hormone administration modified by adding the compound directly to feed rather than force-feeding the animals with a capsule containing the hormone, in the attempt to more closely simulate the supposed illegal use of Dex in animal production.

The experimental plan was designed according to the guidelines of Italian law (DL 116/92) and European legislation (11 and subsequent amendments) for care and use of experimental animals, and the study was approved by the Italian Ministry of Health ethical committee.

RNA Extraction. RNA was extracted using the RNAeasy Mini kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. RNA quality was preliminarily checked by gel electrophoresis on a 1% agarose gel containing SYBR Safe DNA Gel stain 10,000× (Invitrogen, Carlsbad, CA). RNA concentration was also determined using a UV-Vis spectrophotometer, NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE). RNA integrity and quality were then estimated on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). RNA integrity number (RIN) index was calculated for each sample using the Agilent 2100 Expert software. RIN provides a numerical assessment of the integrity of RNA that facilitates the standardization of interpretation of RNA quality. To reduce experimental biases in microarray analysis due to poor RNA quality, only RNAs with RIN number >7.0 were further processed.

RNA amplification, labeling, and array hybridization. For each sample, 500 ng of total RNA were linearly amplified and labeled with Cy3-dCTP following the Agilent One-Color Microarray-Based Gene Expression Analysis protocol. A mixture of 10 different viral polyadenylated RNAs (Agilent Spike-In Mix) was added to each RNA sample before amplification and labeling, to monitor the microarray analysis workflow. Labeled cRNA was purified with the Qiagen RNAeasy Mini Kit, and sample concentration and specific activity (pmol Cy3/μg cRNA) were measured in a NanoDrop ND-1000 spectrophotometer. A total of 1,650 ng of labeled cRNA was prepared for fragmentation adding 11 μl of 10× blocking agent and 2.2 μl of 25× fragmentation buffer, heated at 60°C for 30 min, and finally diluted by addition with 55 μl of 2× GE hybridization buffer. A volume of 100 μl of hybridization solution was then dispensed in the gasket slide and assembled to the microarray slide (each slide containing four arrays). Bovine-specific oligo-arrays (Agilent Bovine-Four-Plex G2519F) were used. Slides were incubated for 17 h at 65°C in an Agilent hybridization oven, subsequently removed from the hybridization chamber, quickly submerged in GE *wash buffer 1* to disassembly the slides, and then washed in GE *wash buffer 1* for ~1 min followed by one additional wash in prewarmed (37°C) GE *wash buffer 2*. After washing them and removing any excess of washing solution, we immediately scanned the arrays on a Agilent G2565BA DNA microarray scanner, at a resolution of 5 μm, modifying default settings to scan the same slide twice at two different sensitivity levels (XDR Hi 100% and XDR Lo 10%). The two linked images generated were analyzed together, and data were extracted and background subtracted using the standard procedures contained in the Agilent Feature Extraction Software 9.5.1. Raw and normalized data from microarray experiments described in the present study have been submitted to the Gene Expression Omnibus, under series accession number GSE12179 (data are scheduled for public access by 15 July 2009).

Normalization of microarray data and statistical analysis. The Feature Extraction software returns a series of spot quality measures that enable to evaluate goodness and reliability of spot intensity estimates. Among these measures, the Feature Extraction Software 9.5.1 flag "glsFound" (set to 1 if the spot has an intensity value significantly different from the local background, 0 otherwise) was used to filter out not-found probes. From now on those probes with FeatureExtraction flag equal to 0 will be noted as "missing." To make more robust and unbiased the statistical analysis, probes with a high proportion of missing values were removed from the dataset. The proportion of missing values used as threshold in the filtering process was decided according to the experimental set-up. Only probes with at least four positive flags in the controls group or three in the treated groups (D or D+E) were considered. Finally, spike-in control intensities (Spike-In Viral RNAs) were used to identify the best normalization procedure for each dataset. After normalization, spike intensities are expected to be uniform across the experiments of a given dataset. Based on the comparison of spike-in probe signal between arrays after normalization, quantile approach was found to outperform averaging with median fluorescence value, which is the method suggested by Agilent for one-color array experiments (data not shown). Each spike-in RNA has a different known concentration following a dilution series and there are 32 replicate probes for each

spike-in RNA on the array. For this reason, comparison of normalized, averaged spike-in signals across different experimental replicates provides a strong indication of the robustness of normalization process. Filtering, normalization, and correlation analysis were performed using R statistical software freely available at http://www.r-project.org.

Identification of differentially expresses genes. A two-class SAM test (76) was performed to identify differentially expressed genes between treatment groups, with a false discovery rate (FDR) ranged between 0.01 and 0.05.

Gene Ontology clustering. The Gene Ontology (GO) classification of up- and downregulated genes after both treatments was performed using the Functional Annotation tool available in the DAVID Database (http://david.abcc.ncifcrf.gov/). For analysis of the KEGG Pathway Database the following parameters were used: gene count 5; ease 1. For the GO Biological Process Database, settings were BP_4, with gene count 10, ease 0.1 for D treatment, and gene count 2, ease 0.1 for DE treatment.

Quantitative RT-PCR. Eighteen target genes were selected for real-time RT-PCR analysis. The selected genes were: 11 genes overexpressed with fold change (FC) >5 in D treatment, plus one gene, Myostatin or GDF-8, significantly underexpressed in the D treatment with the largest FC, five genes overexpressed in both treatments, and one gene overexpressed only in the D+E treatment. A list of the selected genes is presented in Supplementary Table S1.[1] For each selected target gene and for the reference gene (RPS9), a quantitative (q)RT-PCR assay was designed using the Universal Probe Library (UPL) system (Roche Diagnostics, Mannheim, Germany; www.universalprobelibrary.com). Gene-specific primers that encompass one intron and the most appropriate universal probe were defined for each transcript with the ProbeFinder software (https://www.roche-applied-science.com/sis/rtpcr/upl/acenter.jsp?id=030000). To design intron-spanning probes, putative intron-exon boundaries were inferred from the Genome Browser Database (http://genome.ucsc.edu/cgi-bin/hgGateway?hgsid=105081852&clade=vertebrate&org=Cow&db=0). A complete list of primers and probe is presented in Supplementary Table S1.

One microgram of total RNA for each sample was reverse transcribed to cDNA using Superscript II (Invitrogen). An aliquot (2.5 μl) of diluted (1:50) cDNA template was amplified in a final volume of 10 μl, containing 5 μl of FastStart TaqMan Probe Master 2× (Roche Diagnostics, Milan, Italy), 0.25 μl of each gene-specific primer (10 μM), and 0.1 μl of UPL probe (100 μM). The amplification protocol consisted of an initial step of 2 min at 50°C and 10 min at 95°C, followed by 45 cycles of 10 s at 95°C and 30 s at 60°C. All experiments were carried out in a LightCycler 480 (Roche Diagnostics). To evaluate the efficiency of each assay, standard curves were constructed amplifying twofold serial dilutions of the same cDNA, which was used as calibrator. For each sample, the Cp (crossing point) was used to determine the relative amount of target gene; each measurement was made in duplicate and normalized to the reference gene RPS9, which was also measured in duplicate. The correlation index between real-time RT-PCR and microarray FC data was calculated for each gene using Spearman rank correlation test implemented in SPSS ver. 12.0. Probability values <0.05 were considered significant.

Expression values obtained with qRT-PCR were also analyzed using the method of nearest shrunken centroids, as implemented in the PAM program (72), available online at http://www-stat.stanford.edu/~tibs/PAM. The program first performs a discriminant analysis to choose the set of genes that provide the greatest accuracy of class prediction (the smallest misclassification error), then it tests the accuracy of predicting unknown samples through cross-validation (10% of samples are randomly extracted and classified based on the discriminant function calculated on the remaining cases).

RESULTS

At the end of the experiment, average animal weights were 556 kg (controls), 580 kg (Dex+Estr), and 600 kg (Dex). In the comparison between the Dex group and controls a marginally significant difference was observed (*t*-test, $P = 0.042$). Average daily gain was higher for both Dex (1.54 kg per day) and Dex+Estr (1.84 kg per day) groups compared with controls (1.33 kg per day). The effects of treatments on productive traits and especially on meat quality are presented in detail elsewhere (Ref. 5 and Gottardo F., unpublished observations). In brief, none of the various production/quality parameters examined (e.g., color, lightness, fat content, crude protein, water content) were significantly different, although the latter two parameters were higher in meat from treated animals.

Total RNA was extracted from muscle samples of all 17 animals from the experiment plus three additional untreated controls and successfully processed as described in MATERIAL AND METHODS. A total of 5,703 probes were excluded because of the excess of missing values. For the remaining 38,704 probes, intensity values were normalized by a quantile normalization procedure. As the same probe for each unique transcript is synthesized on the array at two distinct positions, for the majority of transcripts two fluorescence values were available. In this case, the average value between the two probe intensities was used. Processed signals for 21,475 unique transcripts were analyzed with SAM to identify differentially expressed genes between treated and control animals.

The comparison of Dex treatment with controls showed a large number of upregulated genes, even when we applied a relatively stringent FDR (0.01, Δ1.7) in SAM. A detailed list of all 835 upregulated transcripts is reported in Supplementary Table S2. A small number of genes (7) was found to be significantly downregulated (Supplementary Table S3). Among the upregulated genes, 410 ones had an FC >2, and 27 genes resulted to have an FC >5. The highest FC (67-fold) was observed for the LOC196541 gene. Among the downregulated genes, only one showed a FC <0.5, the myostatin (MSTN) gene.

In the comparison between controls and animals treated with the same dose of Dex but in combination with Estr (group Dex+Estr), the analysis with SAM evidenced a much shorter list of differentially expressed genes, despite a less stringent FDR (0.05, Δ1.1) was applied. A total of 77 genes resulted to be upregulated (Supplementary Table S4) and 11 downregulated (Supplementary Table S 5), with maximum FC, respectively, of 3.4 and 0.55. Comparing genes that are differentially expressed in treated animals against controls revealed a partial overlap with 35 upregulated transcripts shared between the Dex and Dex+Estr groups (Supplementary Tables S2–S6). Analysis of FC values for these genes revealed a highly significant correlation (Pearson $R = 0.94$, $P < 0.00001$). The correlation curve slope, 1.13 (Fig. 4), as well as visual inspection of FC estimates (Supplementary Table S6) indicate that highly similar gene expression levels for the two treatment groups (Dex and Dex-Estr).

To better describe the functional modifications of the transcriptome in hormone-treated skeletal muscle, up- and downregulated genes were clustered according to GO or KEGG

[1] The online version of this article contains supplemental material.

definitions. Among the 835 differentially expressed transcripts in the Dex group, 609 had a unique gene identity that could be assigned to either a KEGG pathway or a GO_BP entry. For the Dex+Estr group, genes that could be included in the analysis were 75.

Twenty KEGG pathways containing five or more genes were obtained from the analysis of upregulated genes in the Dex group (Table 1). Although the identified pathways reflect a variety of cellular functions, the majority of them could be roughly grouped into three broader clusters. The first group concerns cell shape, cell motility, and cell-cell communication (regulation of actin cytoskeleton, focal adhesion, tight junction, cell communication, and axon guidance). The second one comprises signaling pathways (cytokine-cytokine receptor interaction, Wnt signaling pathway, MAPK signaling pathway, JAK-STAT signaling pathway, adipocytokine signaling pathway, TGF-β signaling pathway). The third one centers on nucleic acids or protein metabolism (purine or pyrimidine metabolism, ribosome, ubiquitin-mediated proteolysis). The GO Biological Process definitions for differentially regulated genes upon Dex treatment (Table 2) partially reflect what is already described for KEGG pathways. The most numerous group is "biopolymer metabolism," which includes DNA, RNA, and protein metabolism and partially overlaps with the third set of KEGG pathways. Likewise, the GO entries "cell motility" and "cell morphogenesis" largely coincides with the first cluster of KEGG pathways, while the significant entries "negative and positive regulation of cellular physiological processes" account for the different signaling pathways that have been identified across the significant genes. Other GO entries reveal additional cell processes that are modified after hormonal treatment, including anabolic mechanisms (biogenesis of cell organelles) as well as catabolic ones.

As mentioned above, the number of differentially expressed genes was lower in response to the combined administration of GCs and estrogens than to the treatment with Dex alone. Not surprisingly, no significant KEGG pathways were observed among the differentially regulated transcripts, and only four GO biological processes were identified. Of these, two are shared with the Dex-only treatment (Table 3).

An additional approach to reduce the complexity of long lists of significant genes is to focus on those transcripts that show the most relevant increase/decrease in expression levels, although this does not always imply the highest biological relevance. Setting a FC threshold at >5, we can identify 27 genes (Table 4). Of these, two are uncharacterized transcripts and seven encode a protein with poorly known or unknown function (LOC196541, RIBC1, LOC615236, ZNF474, KLHL3, Ells1, LOC521737). Among the 18 transcripts that code for known proteins, R-Spo3 and Dkk3, although not included in the original KEGG group, represent a further link with Wnt signaling pathway. Other genes refer to the large cluster of cellular processes described above, which centers on cell shape and motility regulation, such as cytoskeleton-binding proteins (FRMD5 with two transcript variants, and EPB41L4B, both containing a FERM domain), or cell-adhesion and migration-related proteins (PDLIM2, AMIGO2). The remaining proteins have various functions, some with a well-recognized role in skeletal muscle (e.g., MCAT2/SLC16A7, a lactate cotransporter expressed especially in glycolytic fibers; KCNQ1, a potassium channel that is associated with myoblast proliferation; ANKRD2, a sarcomeric protein involved in muscle hypertrophy and myoblast differentiation).

To cross-validate microarray data, but also to identify a set of potential molecular markers, the expression of which could be linked to a specific treatment (Dex or Dex+Estr), a list of target genes was defined as described in MATERIAL AND METHODS. Expression levels of selected target genes were estimated with an independent method (qRT-PCR). The results of qRT-PCR analysis are presented in Table 5, in a comparison with the corresponding microarray FC data. For each gene/transcript, a positive correlation of expression values in individual samples was observed. In all cases the correlation coefficient was also statistically significant except for VASH2, where a marginal

Table 1. *KEGG pathway analysis for Dex upregulated genes*

KEGG Pathway	Gene Count	Genes
Regulation of actin cytoskeleton*	14	ARPC1A, BRAF, FGD1, PELO, ACTC, CFL2, GRLF1, DIAPH1, ITGA7, ARHGEF12, GNA12, ROCK2, GNA13, PFN2
Cytokine-cytokine receptor interaction	11	IFNAR2, TNFRSF12A, TNFSF8, CSF3R, IL4, BLR1, TGFB2, CCR9, IL28RA, TNFRSF19, IL13RA1
Focal adhesion*	10	CAST, BRAF, LAMA4, PTEN, PELO, ACTC, DIAPH1, ITGA7, GRLF1, ROCK2
Wnt signaling pathway*	10	PPP2R2C, TBL1XR1, PPP2CB, PPP2R1A, SFRP5, PPP3CC, FZD9, VANGL1, TBL1X, ROCK2
Purine metabolism	9	NT5C2, AMPD3, PDE7B, POLR2I, GUCY1B2, POLE4, NT5M, AK3L1, IMPDH2
MAPK signaling pathway*	8	PLA2G12A, BRAF, CASP3, MAP3K14, PPP3CC, CASP4, TGFB2, GNA12
Tight junction	8	PPP2R2C, TJAP1, PPP2CB, SPTAN1, PPP2R1A, PTEN, ACTC, EXOC3
Axon guidance*	8	ABLIM1, PLXNA2, RGS3, PPP3CC, DPYSL2, ARHGEF12, CFL2, ROCK2
Long-term depression	7	PPP2R2C, PPP2CB, PPP2R1A, PLA2G12A, GUCY1B2, GNA12, GNA13
Pyruvate metabolism	6	ACSS1, HAGHL, ACSS2, GRHPR, ME3, LDHB
Ribosome*	6	RPS5, RPL38, RPS27, RPL35, RPL36AL, RPS19
JAK-STAT signaling pathway	6	IFNAR2, SOCS4, CSF3R, IL4, IL28RA, IL13RA1
Pyrimidine metabolism	5	NT5C2, CTPS2, POLR2I, POLE4, NT5M
Cell comunication	5	GJA5, KRT18, LAMA4, ACTC, KRT8
Glycerophosholipid metabolism	5	PLA2G12A, CDS2, GNPAT, HSD3B7, PAFAH2
Adipocytokine signaling pathway	5	ACSL3, ADIPOR1, PPARA, SLC2A1, CPT1B
Glycolisis/Gluconeogenesis	5	ACSS1, ACSS2, HK1, LDHB, PFKL
TGF-β signaling pathway	5	PPP2CB, TFDP1, ID2, TGFB2, ROCK2
Ubiquitin-mediated proteolysis	5	ANAPC7, UBE2E2, WWP2, CDC34, UBE1
Arginine and proline metabolism	5	PYCR2, P4HA1, ARG2, AMD1, P4HA2

*KEGG Pathways present in the list of Cassar-Malek et al. (9) (see DISCUSSION).

Table 2. *GO Biological Process analysis of Dex upregulated genes*

GO Biological Process BP_4	Gene Count	*P* Value
Biopolymer metabolism	106	0.023
Cell motility	14	0.030
Negative regulation of cellular physiological process	30	0.023
Cellular morphogenesis	18	0.005
Programmed cell death	26	0.036
Cellular catabolism	26	0.022
Lipid metabolism	28	0.043
Positive regulation of cellular physiological process	23	0.022
Organic acid metabolism	27	0.006
Organelle organization and biogenesis	39	0.010

GO, Gene Ontology. *P* value: modified Fisher exact *P* value calculated by David software.

probability was obtained ($P = 0.06$). Correlating average FC values calculated on data from the two methods (DNA microarray and qRT-PCR) showed a very high correlation coefficient (Spearman's $\rho = 0.878$ $P < 0.00001$), although the slope of the linear regression line was 2.6 (Fig. 1), indicating that qRT-PCR FC values are on average twice as larger than those estimated on array data. If the three highest values are excluded, the regression line slope becomes 1.1, pointing to a one-to-one correspondence between measures from the two techniques. The potential underestimation of expression values for highly expressed genes using microarrays likely reflects the well-known phenomenon of "fold-change compression," which is due to various technical limitations, including limited dynamic range, signal saturation, and cross-hybridization of microarrays (82). Overall, however, qRT-PCR confirms the reliability of results obtained with microarray analysis.

Assigning unknown individual samples to a specific group (hormonally treated or untreated) based on qRT-PCR expression data represents the next step toward the implementation of a molecular screening method for illegal hormone administration. To assess such possibility, a preliminary analysis on the available sample set was carried out using a specific discriminant analysis developed for expression data (PAM, Ref. 72). This approach allows the definition of a minimal set of genes that best classify the samples. The nearest shrunken centroid analysis implemented in the PAM program was effective in discriminating between Dex-treated samples and untreated controls with a 100% accuracy (training $error_{DEX/C} = 0.0$), even when only four genes are selected. This minimal gene set (LOC196541, AMIGO2, CRYM, CNIH3) was then used in the cross-validation procedure yielding a probability of correct identification close to one for all samples (Fig. 2). In the case of Dex+Estr-treated animals, all the available markers (6) needed to be used to achieve the smallest training error (0.0), and the cross-validation based on the complete gene set indicates that sample classification is less reliable (Fig. 3), with probabilities of correct assignment as low as 0.55–0.6 for some samples (e.g., untreated samples 3 and 6) or misclassified individuals (Dex+Estr sample 5).

DISCUSSION

In the present study, the administration of low-dosage Dex, either alone or in association with Estr, was found to positively affect final animal live weight. Although the observed differences in growth performance compared with controls are marginal, the obtained results confirm the potential of GCs as growth promoters. In fact, even a small increase in daily gain might be extremely attractive for animal breeders (e.g., Ref. 52), as it directly translates into lower farming costs and reduced finishing time, especially if one considers that meat quality parameters are not significantly affected. The main goal of this work, however, was to investigate the effects of low-dosage GCs on skeletal muscle in beef cattle and to identify potential biomarkers for the illegal use of such compounds. In this respect, three major points can be made. First, very relevant effects on muscle transcriptional profile are observed in GC-treated animals. Second, the addition of estrogens to GCs appears to significantly dampen these effects. Third, a substantial set of potential biomarkers has been identified and cross-validated with an independent method.

The first point is easily appreciated by just looking at the list of differentially regulated genes in Dex-treated animals. Over 800 transcripts are significantly upregulated, more than half with an FC >2. Interpreting long lists of differentially expressed transcripts is usually the most difficult task in DNA microarray studies. Despite all the known limitations, clustering significant genes in functional categories offers a powerful means to extract more general patterns from array data.

Regulation of the actin cytoskeleton is the most represented KEGG pathway among overexpressed genes in Dex-treated animals (Table 1). Remodeling of actin cytoskeleton has an important role during myogenesis and muscle regeneration. RhoA, a RhoGTPase that orchestrates actin filaments rearrangement, has been demonstrated to promote myogenesis (83, 6). Two known RhoA downstream effectors, diaphanous 1 (DIAPH1) and the muscle-specific Rho-associated, coiled-coil containing protein kinase 2 (ROCK2) (50), and one RhoA activator, leukemia-associated Rho guanine nucleotide exchange factor (LARG/ARHGEF12) (3), were found to be significantly upregulated after Dex administration (Table 1). The guanine nucleotide binding proteins GNA12 and GNA13 act downstream to LARG to induce hypertrophic responses (70). Increased expression was also observed for other transcripts encoding proteins that are supposed to promote muscle development/regeneration/hypertrophy. V-raf murine sarcoma viral oncogene homolog B1 (BRAF) was reported to act on the ROCK2/LIMK/Cofilin pathway (54) and to support cardiac muscle hypertrophy (45). Like LARG/ARHGEF12, FYVE RhoGEF and PH domain containing 1 (FGD1) is a RhoGTPase activator, which acts on Cdc42. The latter protein appears also to be involved in myogenesis, albeit with a less clear role compared with RhoA (6, 7). ARPC1A is the 1A subunit of the actin-related protein 2/3 complex, which has a critical role in

Table 3. *GO Biological Process analysis of Dex+Estr differentially regulated genes*

GO Biological Process BP_4	Gene Count	*P* Value
Regulation of transport	3	0.02
Biopolymer metabolism	23	0.0003
Negative regulation of cellular physiological process	8	0.009
Mitotic cell cycle	4	0.042

Dex, dexamethasone; Estr, 17β-estradiol. *P* value: modified Fisher exact *P* value calculated by David software.

Table 4. *List of upregulated genes with FC >5*

Accession Number	Gene Name	FC
XM_597666	hypothetical protein LOC196541 (LOC196541)	67.0
XM_581413	crystallin, mu (CRYM), transcript variant 1	14.0
XM_866893	FERM domain containing 5 (FRMD5)	10.2
XM_597805	dynein axonemal, heavy polypeptide 9 (DNAH9)	8.90
XM_614487	R-spondin 3 homolog (RSPO3)	7.51
XM_608720	LOC530255	7.3
NM_001017942	arginase, type II (ARG2)	6.9
XM_613938	RAB40B, member RAS oncogene family (RAB40B)	6.8
XM_870103	monocarboxylate transporter 2 (MCT 2) (LOC617782)	6.6
XM_590498	RIB43A domain with coiled-coils 1 (RIBC1)	6.6
XM_866992	cornichon homolog 3 (LOC615236)	6.5
BM104989	unidentified transcripts on BTA24 position 33768876-33769819	6.2
NM_001037490	zinc finger protein 474 (ZNF474)	6.2
XM_866023	chromosome 11 open reading frame 52 (C11orf52)	6.1
XM_869134	stearoyl-CoA desaturase 4 (LOC616975)	5.9
XM_866121	solute carrier family 16, member 7 (monocarboxylic acid transporter 2) (SLC16A7)	5.9
BM287143	erythrocyte membrane protein band 4.1 like 4B (EPB41L4B), transcript variant 2	5.7
BI683243	Kelch-like 3 (Drosophila) (KLHL3)	5.4
CB167599	hypothetical protein Ells1 (Ells1)	5.4
XM_600004	ring finger protein 30 (predicted) (LOC521737)	5.3
AV592965	potassium voltage-gated channel, KQT-like subfamily, member 1 (KCNQ1)	5.3
NM_001034430	PDZ and LIM domain 2 (mystique) (PDLIM2)	5.2
XM_876445	ankyrin repeat domain 2 (stretch responsive muscle) (ANKRD2)	5.2
XM_592100	adhesion molecule with Ig-like domain 2 (AMIGO2)	5.0
XM_583102	Dickkopf homolog 3 (DKK3)	5.0
AW445540	unidentified transcripts on BTA21 position 34601033-34599866	5.0
NM_174257	calbindin 3, (vitamin D-dependent calcium binding protein) (CALB3)	5.0

FC, fold change.

myoblast fusion during either muscle development or regeneration (59). The muscle-specific form of cofilin (CFL2) (71) and the integrin α-chain 7 (ITGA7) (8) are also positively associated with regeneration of skeletal muscle. For instance, overexpression of ITGA7 promotes muscle cell proliferation, adhesion, and resistance to apoptosis (37).

Table 5. *Comparison of FC values detected by qRT-PCR and microarray for selected target genes*

	Dex Treatment		Dex + Estr Treatment		
Gene Symbol	FC RT-qPCR	FC array	FC RT-qPCR	FC array	Correlation Coefficient
LOC196541	173.9	67	ND	NS	0.96‡
RSPO3	8.6	7.5	ND	NS	0.94‡
ARG2	4.8	6.9	ND	NS	0.76†
SLC16A7	9.4	5.9	ND	NS	0.89†
EPB41L4B	5.1	5.7	ND	NS	0.94‡
ANKRD2	5.2	5.3	ND	NS	0.90‡
SCD5	2.8	5.9	ND	NS	0.92‡
AMIGO2	6.9	5.1	ND	NS	0.96‡
CRYM	35.4	14	ND	NS	0.97‡
CNIH3	34.4	6.6	ND	NS	0.97‡
DNAH9	6.1	8.9	ND	NS	0.94‡
MSTN	−8.1	−3.2	ND	NS	0.77†
ME3	3	3.2	3.1	3.5	0.61†
GATA2	2.3	2.2	2.9	2.0	0.57†
TLE2	2.2	2.0	1.8	2.4	0.72‡
GAB2	2.2	1.7	2.4	1.9	0.49*
INSIG1	3.6	3.5	7.9	4	0.78‡
VASH2	ND	NS	2.8	3.1	0.49

FC is calculated as ratio of Dex (or Dex+Estr) group vs. control group, using mean signal intensity across biological replicates of each group. qRT-PCR, quantitative RT-PCR; ND, not determined; NS, not significant. *$P <$ 0.05, †$P < 0.01$, ‡$P < 0.001$.

"Cytokine-cytokine receptor" is the second most represented KEGG pathway (Table 1). The list of cytokines expressed in the skeletal muscle is rapidly increasing (46). These cytokines are thought to exert autocrine, paracrine, or endocrine effects. Among the upregulated cytokine or cytokine-receptor genes in Dex-treated animals, interleukin 4 (IL-4) and one subunit of the IL-4 receptor (IL13RA1) are noteworthy. IL-4 is a Th2 cytokine, which is involved in various immune functions. A central role in muscle growth, however, has emerged for this molecule, which acts as the molecular signal that promotes myoblast fusion and myotube growth (26). Expression of IL-4 is specifically upregulated by one isoform of NF-AT transcription factors (NF-ATc2), which is, in turn, activated through the

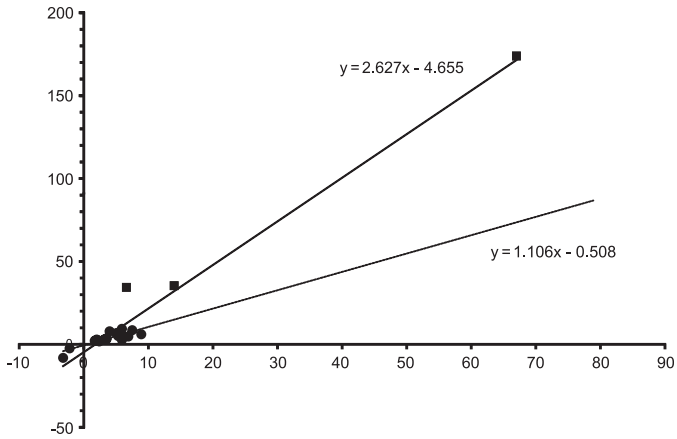

Fig. 1. Plot of fold change (FC) values based on microarray (*x*-axis) and quantitative (q) RT-PCR data (*y*-axis). Linear regression line (■) across all gene data or excluding 3 genes (dotted).

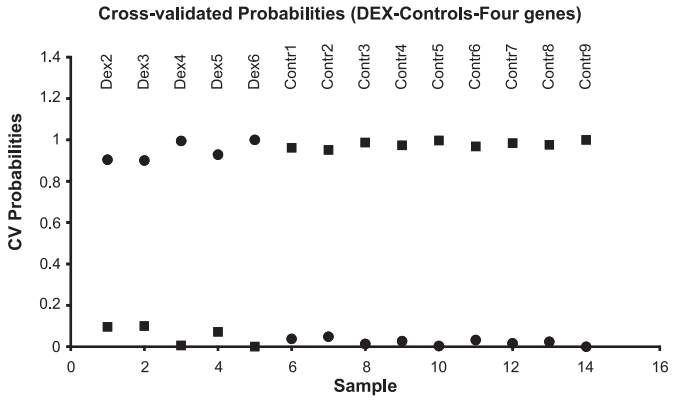

Fig. 2. Plot of cross-validated probabilities for sample classification. On *x*-axis individual samples: 1–5 dexamethasone (Dex)-treated animals, 6–14 controls. On *y*-axis the probability of being classified as Dex-treated (●) or control (■).

calcineurin pathway (65). Another cytokine receptor that was upregulated after GC treatment, tumor necrosis factor receptor superfamily, member 12A (TNFRSF12A, also known as FN14 or TWEAKR) has been demonstrated to be crucial in myoblast proliferation. Fn14-deficient primary myoblasts displayed significantly reduced proliferative capacity and altered myotube formation (21). Other cytokine receptors such as interferon (alpha, beta, and omega) receptor 2 (IFNAR2), colony stimulating factor 3 receptor (CSF3R GM-SFR), and interleukin 28 receptor alpha (IL28RA) insist on the JAK1-STAT1-STAT3 pathway, which has been shown to induce myoblast proliferation (69).

With 10 differentially regulated genes, the Wnt signaling pathway is the second-most represented signaling pathway, or even the most important one, if two additional Wnt-related proteins, RSPO3 and DKK3, which were not included in the original KEGG group, are taken into account. Wnt signaling can be divided into three main branches, the canonical, the planar-cell-polarity (PCP or noncanonical) and the calcium-dependent pathway. The latter, which is centered on Ca^{2+}-activated calcineurin, has a well established role in muscle regeneration (63). The γ-isoform of the calcineurin catalytic subunit (PPP3CC) was found to be upregulated after Dex treatment (Table 1). The noncanonical or planar cell polarity (PCP) pathway has been recently shown to provide a directional cue for the elongation of early muscle fibers (24). Two genes involved in the PCP pathway, Vang-like 1 (VANGL1/Strabismus2), and ROCK2, are overexpressed in Dex-treated muscle samples. Last, the canonical pathway has been demonstrated to be fundamental in muscle development (10), while its role in muscle regeneration appears to be more controversial (53, 4, 51). A recent study, however, has demonstrated that activated β-catenin, the downstream effector of canonical Wnt signaling is associated with proliferation of muscle satellite cells during regeneration (48). Several overexpressed genes after GC treatment encode proteins involved in the canonical Wnt pathway (Table 1). Of these, some have a clear activating function. TBLXR1 and TBLX1 represent alternative transcripts of the transducin (β)-like 1X-linked protein that is needed to recruit β-catenin to Wnt target-gene promoters (36). Frizzled 9 (FZD9) is a Wnt receptor, and FZD9 overexpression leads to accumulation of β-catenin (30). Protein phosphatase 2 (PPP2), of which two regulatory subunit isoforms (PP2R2C, PP2R1A) and one catalytic subunit isoform (PPP2CB) are upregulated, has an activating role in Wnt signaling (56). RSPO3 is a secreted protein that amplifies Wnt signaling through inhibition of Dickkopf (DKK) proteins (31). On the other side, one member (DKK3) of this small family of secreted proteins that typically antagonize Wnt/β-catenin signaling is upregulated as well. A second overexpressed gene encoding a protein antagonist of Wnt signaling is SFRP5 (secreted frizzled-related protein 5).

Other signaling pathways as well as other KEGG pathways involved in cell shape and motility are represented in Table 3. Several genes included in these pathways have been already examined above, because of partial overlapping between pathways. While a detailed analysis of all listed genes would require too long a discussion, it seems quite clear that the overall pattern points toward increased cell growth and proliferation, although notable exceptions (e.g., TGF-β2, PTEN, Wnt antagonists) are observed. Indirect support to this interpretation comes from the analysis of the third group (cell metabolism) of pathways that characterize the expression profile of Dex-treated muscle. Key enzymes for some metabolic pathways are found to be upregulated. Inosine monophosphate dehydrogenase 2 (IMPDH2) is the rate-limiting enzyme in the de novo synthesis of guanine nucleotides, while available levels of ribosomal protein S29 (RPS29) seem to control ribosome assembly (27). The same might be true for other ribosomal proteins (RPS5, RPL38, RPS27, RPL35, RPL36AL).

Other unrelated genes that are found to be significantly upregulated in Dex-treated animals suggest a potential growth-promoting action for GCs at very low dosage. For instance, elevated expression of Delta-like 1 (DLK1) (Supplementary Table S2) is involved in the hypertrophic callipyge phenotype in the sheep, while experimental Dlk1 overexpression in the skeletal muscle induces hypertrophy in the mouse (Ref. 81 and references therein). The most convincing evidence for a pro-growth transcriptional signature comes, however, from the much shorter list of downregulated genes (Supplementary Table S3). Myostatin, a key regulatory gene that negatively controls muscle growth, was found to be significantly down-

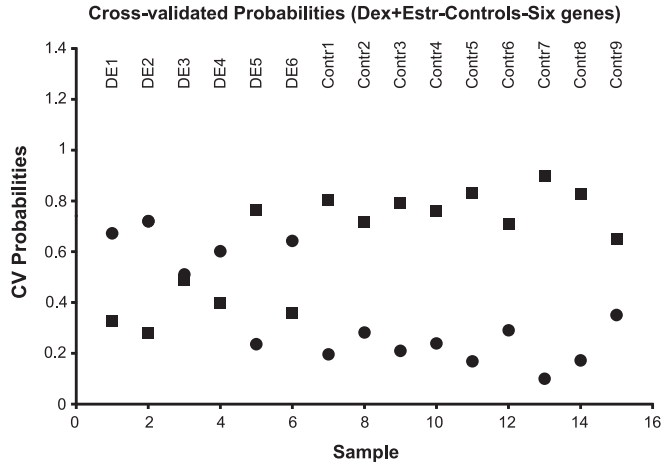

Fig. 3. Plot of cross-validated (CV) probabilities for sample classification. On *x*-axis individual samples: 1–6 Dex+Estr-treated animals, 7–15 controls. On *y*-axis the probability of being classified as Dex+Estr-treated (●) or control (■). Estr, 17-β estradiol.

regulated (−3.2) after GC treatment, based on microarray data. This evidence is even stronger when myostatin mRNA is measured with qRT-PCR (FC = −8.1). Natural or induced null mutants at the myostatin locus show a nearly double muscle mass compared with wild-type animals (reviewed in Ref. 61). The obvious prospect of manipulating myostatin expression/activity in patients and/or farm animals has prompted a large number of studies investigating myostatin regulation as well as its functions. Myostatin appears to be regulated at different levels, from transcription to posttranslational maturation and activity of the mature peptide (61). To what extent might the observed reduction of myostatin mRNA levels be linked to other changes in the transcriptional profile of Dex-treated muscle? An indirect answer to such a question comes from the analysis of relevant KEGG pathways identified in myostatin null mutants (double-muscled, DM) (9) or genetic variants associated with low myostatin expression (62) in the bovine species. Despite important differences in the methodology, the list of KEGG pathways in Table 3 (this study) contains several items in common with those characterizing differentially expressed genes in the muscle expression profiles between normal and DM cattle (9). These common pathways are "regulation of actin cytoskeleton," "focal adhesion," "Wnt signaling pathway," "MAPK signaling pathway," "axon guidance," and "ribosome." This evidence suggests that such common pathways might be directly or indirectly regulated by myostatin, which is downregulated in Dex-treated bulls and absent in DM animals. In a different study (62), Holstein bulls carrying a homozygous genotype "CC" at a polymorphic site in the 5′-flanking region of myostatin gene were analyzed. The "cc" genotype is associated with 50–40% reduction of myostatin mRNA. Expression profiles of "CC" animals revealed 32 differentially expressed genes. The most represented pathways were Wnt signaling, integrin signaling, chemokine and cytokine signaling, and regulation of cytoskeleton.

A second, more direct evidence for the potential effects of lower myostatin expression after Dex treatment comes from a paper by Marshall and colleagues (41). A novel gene, Mighty, was found to be significantly upregulated in the skeletal muscle of myostatin-KO mice. Analysis of the Mighty gene promoter suggested that myostatin represses Mighty expression at the transcriptional level (41). In vitro, Mighty overexpression enhanced cell differentiation with the expression of myogenic factors, while in vivo it increased muscle fiber size. Quite remarkably, the unidentified transcript XM_880207-LOC615039, which is found in the list of significantly upregulated genes after GC treatment (Supplementary Table S2), actually encodes the bovine homolog of Mighty. The product of the Mighty locus has been recently analyzed in a different context and named Akirin1 (22). In the mouse, Akirin1/Mighty KO has no apparent phenotype, but its paralog Akirin2 is required for embryonic development and defense to Gram-bacterial pathogens, like its fruit fly single homolog Akirin (22). In *Drosophila* Akirin (also known as bhringi) has also been demonstrated to be a potential modulator of Wnt signaling (16), while the phenotype of certain Akirin/bhringi mutants reveal a role for this gene during muscle development (47). All this evidence points toward a potential link, through Mighty/Akirin1, between myostatin and Wnt signaling, a link that is evident also from expression profiling analyses in cattle (Refs. 9, 62, and this study).

The relationship between administration of Dex and downregulation of myostatin expression, however, is quite unexpected. As already mentioned, Dex increases myostatin expression in vitro and in vivo (38, 39), although at the lowest dose (60 μg/kg/day Dex, intraperitoneal injection) only a 1.7-fold change in myostatin mRNA levels was observed, while maximal response was achieved at 600–1,200 μg/kg/day (FC = 5.5–6.3) (39). Comparatively, the administered dose in the present work was <1.5 μg/kg/day with a mode of administration that is likely much less efficient. It might be possible that at very low dosage GCs preferentially activate other cellular pathways. For instance, low doses of Dex have been shown to stimulate proliferation of primary myoblasts (25), possibly through enhancement of IGF-1 mediated effects (20). Indeed, post hoc analysis of IGF-1 receptor gene (IGF-1R) expression on array data shows a significant upregulation in Dex-treated animals ($P < 0.02$), while the receptor for a IGF-related peptide, relaxin, is listed among SAM-selected overexpressed genes (Supplementary Table S2). Relaxin has been demonstrated to enhance muscle regeneration (35).

While GC-induced repression of myostatin requires further investigation, it should be noted that in at least two different fish species myostatin has been already reported to be downregulated either under stress-inducing conditions (overcrowding, prolonged fasting) (80, 60) or after experimental administration of cortisol (60).

Overall, low-dosage Dex seems to activate several signaling pathways potentially leading to muscle hypertrophy, and to significantly repress a key negative regulator of muscle mass, myostatin. The majority of these pathways appears to be involved especially in satellite cell recruitment and differentiation (e.g., canonical and noncanonical Wnt pathways, actin cytoskeleton regulation), while there is limited evidence for activation of cell growth.

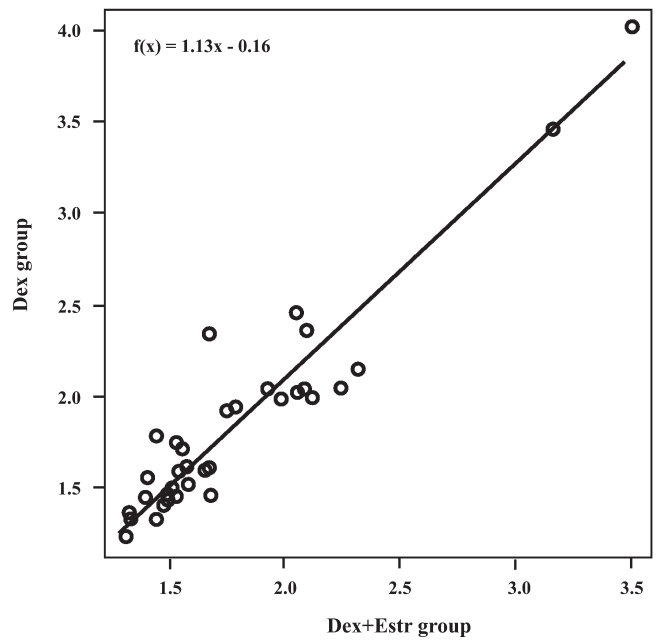

Fig. 4. Plot of FC values of the 35 upregulated genes shared between Dex (*y*-axis) and Dex+Estr (*x*-axis) groups. Linear regression line and the corresponding slope are also shown.

The second major point of the present work is the marked reduction of the transcriptional effects when estrogens are combined with the same dose of Dex. Estrogens are known to influence skeletal muscle since both α- and β-estrogen receptors have been found in muscle cells. Estrogen replacement in immature ovariectomized rats was shown to reduce body weight, muscle growth, and muscle fiber size (42). More specifically, 1-wk estrogen replacement significantly reduced expression of IGF-1 mRNA and increased protein levels of myostatin (75). Therefore, IGF-1 and myostatin pathways might represent a first level of interaction between Dex and Estr, with the two hormones having opposite effects on such signaling pathways. GCs and estrogens have also been reported to act in opposition in the regulation of several physiological processes, both in vitro (e.g., 84, 66) and in vivo (e.g., 33, 58). Cross talk between GCs and estrogens appears to occur at several different levels. For instance, negative transcriptional interference between estrogen- and GC-receptors was demonstrated at the AP1 response element (77). In addition, estrogens seem to induce proteasomal degradation of the GC receptor (32), while GCs suppress positive autoregulation of estrogen-receptor expression by estrogens (34). The observed antagonist action of Dex and Estr at the transcriptional level is therefore in agreement with previous studies. To our knowledge, however, this is the first evidence of such interaction in vivo in the skeletal muscle. On the other hand, there is a number of genes (Supplementary Table S6) that appear to be regulated by Dex without significant influence by Estr, as nearly identical expression values were observed between the two treatments (Fig. 4). Analysis of gene function did not reveal one or more specific pathways since the commonly regulated transcripts encode proteins with various roles in cell biology (e.g., transcription factors, kinases, metabolic enzymes, RNA binding, phosphatases). However, the two genes showing the highest FC [insulin induced gene 1 (NSIG1) and malic enzyme 3 (ME3)] are both positively regulated by insulin (44). Glucocorticosteroids are known to increase blood glucose levels and consequently to cause hyperinsulinemia. At low doses GCs still induce a significant rise of insulinemia even if glucose level is normal (55). This points to a possible explanation for estrogen-independent upregulation of some genes, which might occur indirectly as a consequence of GC stimulation of β-cell function, increased insulinemia, activation of insulin-dependent signaling, and upregulation of insulin-sensitive genes in the muscle cell.

The third and final point concerns the potential for differentially expressed genes to be used as biomarkers for revealing illegal hormonal treatments in beef cattle. Indirect biological markers may offer a valid alternative to traditional screening methods (RIA, ELISA, HPLC) to detect the presence of hormone residues (2, 78). In fact, they have comparable costs, higher throughput, and potentially higher sensitivity compared with immunological methods, which often perform poorly even in the presence of minimal chemical modifications of the active molecule(s). Furthermore, indirect markers rely on the biological effects of hormonal treatments. These effects may persist and be revealed when the active compound has been catabolized and it is no longer detectable. An additional advantage of gene expression markers is the possibility to test muscle samples collected several days after slaughtering, working on animal carcass or even single meat cuts. Quite surprisingly, preliminary evidence from analysis of RNA stability and qRT-PCR repeatability on skeletal muscle samples stored at 4°C for a variable time (1–10 days) suggests that reliable measures of gene expression can be obtained up to 10 days after slaughtering (S. Ferraresso, unpublished data). On the other hand, in vivo analysis of blood and/or urine samples searching either directly the hormone residue or indirectly its metabolites is certainly more efficient to prevent illegal use of growth promoters in the farm.

Based on the results of the present study, the administration of Dex alone could be reliably identified by means of just four marker genes, with high confidence, and gene expression markers might therefore provide a rapid and relatively inexpensive tool for routine screening of muscle samples collected after slaughtering. However, the association of Dex and estrogens will need a higher number of biomarkers to be detected, and some samples might be misclassified. Although Dex alone seems more than sufficient for increasing growth rates, dishonest breeders may use estrogens just to "mask" the presence of Dex stimulation from gene expression analysis, but at increased risk of being exposed by other screening methods. The two marker sets developed in the present study are currently being tested on a larger group of tissue samples that have been directly collected at the slaughterhouse. For these samples, liquid-chromatography and gas-chromatography data on hormone residues as well as histological analysis of target organs (thymus, testes, prostate) are being produced. This will allow full validation of the use of molecular markers for routine screening.

In conclusion, gene expression is not the "magic bullet" to detect every illicit treatment, but it might well represent a powerful tool to complement the existing arsenal of weapons against the illegal use of growth promoting hormones, especially when working on samples collected after slaughtering. In our opinion, only a combination of direct and indirect methods and different analytical techniques will prove effective toward prevention and repression of the illegal use of growth promoters.

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REFERENCES

1. **Adams NR, Sanders MR.** Improved feed intake and body weight change in sheep treated with dexamethasone at entry into pens or feedlots. *Austr Vet J* 69: 209–213, 1992.
2. **Balizs G, Hewitt A.** Determination of veterinary drug residues by liquid chromatography and tandem mass spectrometry. *Anal Chim Acta* 492: 105–131, 2003.
3. **Becknell B, Shen T, Maghraby E, Taya S, Kaibuchi K, Caligiuri MA, Marcucci G.** Characterization of leukemia-associated Rho guanine nucleotide exchange factor (LARG) expression during murine development. *Cell Tissue Res* 314: 361–366, 2003.
4. **Brack AS, Conboy MJ, Roy S, Lee M, Kuo CJ, Keller C, Rando TA.** Increased Wnt signaling during aging alters muscle stem cell fate and increases fibrosis. *Science* 317: 807–810, 2007.

5. **Brscic M, Miotello S, Mazzenga A, Cozzi G, Gottardo F.** Growth performance and behaviour of finishing beef cattle illegally treated with growth promoters. *Ital J Anim Sci* 6: 403–405, 2007.
6. **Bryan BA, Li D, Wu X, Liu M.** The Rho family of small GTPases: crucial regulators of skeletal myogenesis. *Cell Mol Life Sci* 62: 1547–1555, 2005.
7. **Bryan BA, Mitchell DC, Zhao L, Ma W, Stafford LJ, Teng BB, Liu M.** Modulation of muscle regeneration, myogenesis, and adipogenesis by the Rho family guanine nucleotide exchange factor GEFT. *Mol Cell Biol* 25: 11089–11101, 2005.
8. **Burkin DJ, Wallace GQ, Milner DJ, Chaney EJ, Mulligan JA, Kaufman SJ.** Transgenic expression of $\alpha_7\beta_1$ integrin maintains muscle integrity, increases regenerative capacity, promotes hypertrophy, and reduces cardiomyopathy in dystrophic mice. *Am J Pathol* 166: 253–263, 2005.
9. **Cassar-Malek I, Passelaigue F, Bernard C, Léger J, Hocquette JF.** Target genes of myostatin loss-of-function in muscles of late bovine fetuses. *BMC Genomics* 8: 63, 2007.
10. **Cossu G, Borello U.** Wnt signaling and the activation of myogenesis in mammals. *EMBO J* 18: 6867–6872, 1999.
11. **Council Directive 86/609/EEC.** *Off J Eur Commun* L358, 1986.
12. **Council Directive 96/22/EC.** *Off J Eur Commun* L125: 3, 1996.
13. **Council Directive 96/23/EC.** *Off J Eur Commun* L125: 10, 1996.
14. **Courtheyn D, Le Bizec B, Brambilla G, De Brabander HF, Cobbaert E, Van de Wiele M, Vercammen J, De Wasch K.** Recent developments in the use and abuse of growth promoters. *Anal Chim Acta* 473: 71–82, 2002.
15. **Dallman M, Strack A, Akana S, Bradbury M, Hanson E, Scribner K, Smith M.** Feast and famine: critical role of glucocorticoids with insulin in daily energy flow. *Front Neuroendocrinol* 14: 303–347, 1993.
16. **DasGupta R, Kaykas A, Moon RT, Perrimon N.** Functional genomic analysis of the Wnt-wingless signaling pathway. *Science* 308: 826–833, 2005.
17. **Dubowitz V, Kinali M, Main M, Mercuri E, Muntoni F.** Remission of clinical signs in early Duchenne muscular dystrophy on intermittent low-dosage prednisolone therapy. *Eur J Paediatr Neurol* 6: 153–159, 2002.
18. **Frauman A.** An overview of the adverse reaction to adrenal corticosteroids. *Adverse Drug React Toxicol Rev* 15: 203–206, 1996.
19. **Gilson H, Schakman O, Combaret L, Lause P, Grobet L, Attaix D, Ketelslegers JM, Thissen JP.** Myostatin gene deletion prevents glucocorticoid-induced muscle atrophy. *Endocrinology* 148: 452–460, 2007.
20. **Giorgino F, Smith RJ.** Dexamethasone enhances insulin-like growth factor-I effects on skeletal muscle cell proliferation. Role of specific intracellular signaling pathways. *J Clin Invest* 96: 1473–1483, 1995.
21. **Girgenrath M, Weng S, Kostek CA, Browning B, Wang M, Brown SA, Winkles JA, Michaelson JS, Allaire N, Schneider P, Scott ML, Hsu YM, Yagita H, Flavell RA, Miller JB, Burkly LC, Zheng TS.** TWEAK, via its receptor Fn14, is a novel regulator of mesenchymal progenitor cells and skeletal muscle regeneration. *EMBO J* 25: 5826–5839, 2006.
22. **Goto A, Matsushita K, Gesellchen V, El Chamy L, Kuttenkeuler D, Takeuchi O, Hoffmann JA, Akira S, Boutros M, Reichhart JM.** Akirins are highly conserved nuclear proteins required for NF-kappaB-dependent gene expression in drosophila and mice. *Nat Immunol* 9: 97–104, 2008.
23. **Gottardo F, Brscic M, Pozza G, Ossensi C, Contiero B, Marin A, Cozzi G.** Administration of dexamethasone per os in finishing bulls. I. Effects on productive traits, meat quality and cattle behavior as indicator of welfare. *Animal* 2: 1073–1079, 2008.
24. **Gros J, Serralbo O, Marcelle C.** WNT11 acts as a directional cue to organize the elongation of early muscle fibres. *Nature* 457: 589–593, 2009.
25. **Guerriero V Jr, Florini JR.** Dexamethasone effects on myoblast proliferation and differentiation. *Endocrinology* 106: 1198–1202, 1980.
26. **Horsley V, Jansen KM, Mills ST, Pavlath GK.** IL-4 acts as a myoblast recruitment factor during mammalian muscle growth. *Cell* 113: 483–494, 2003.
27. **Idol RA, Robledo S, Du HY, Crimmins DL, Wilson DB, Ladenson JH, Bessler M, Mason PJ.** Cells depleted for RPS19, a protein associated with Diamond Blackfan Anemia, show defects in 18S ribosomal RNA synthesis and small ribosomal subunit production. *Blood Cells Mol Dis* 39: 35–43, 2007.
28. **Istasse L, De Haan V, Van Eenaeme C, Buts B, Baldwin P, Gielen M, Demeyer D, Bienfait JM.** Effects of dexamethasone injections on performances in a pair of monozygotic cattle twins. *J Anim Physiol Anim Nutr* 62: 150–158, 1989.
29. **Jahng JW, Kim NY, Ryu V, Yoo SB, Kim BT, Kang DW, Lee JH.** Dexamethasone reduces food intake, weight gain and the hypothalamic 5-HT concentration and increases plasma leptin in rats. *Eur J Pharmacol* 581: 64–70, 2008.
30. **Karasawa T, Yokokura H, Kitajewski J, Lombroso PJ.** Frizzled-9 is activated by Wnt-2 and functions in Wnt/beta -catenin signaling. *J Biol Chem* 277: 37479–37486, 2002.
31. **Kim KA, Wagle M, Tran K, Zhan X, Dixon MA, Liu S, Gros D, Korver W, Yonkovich S, Tomasevic N, Binnerts M, Abo A.** R-Spondin family members regulate the Wnt pathway by a common mechanism. *Mol Biol Cell* 19: 2588–2596, 2008.
32. **Kinyamu HK, Archer TK.** Estrogen receptor-dependent proteasomal degradation of the glucocorticoid receptor is coupled to an increase in mdm2 protein expression. *Mol Cell Biol* 23: 5867–5881, 2003.
33. **Lethimonier C, Flouriot G, Valotaire Y, Kah O, Ducouret B.** Transcriptional interference between glucocorticoid receptor and estradiol receptor mediates the inhibitory effect of cortisol on fish vitellogenesis. *Biol Reprod* 62: 1763–1771, 2000.
34. **Lethimonier C, Flouriot G, Kah O, Ducouret B.** The glucocorticoid receptor represses the positive autoregulation of the trout estrogen receptor gene by preventing the enhancer effect of a C/EBPbeta-like protein. *Endocrinology* 143: 2961–2974, 2002.
35. **Li Y, Negishi S, Sakamoto M, Usas A, Huard J.** The use of relaxin improves healing in injured muscle. *Ann NY Acad Sci* 1041: 395–397, 2005.
36. **Li J, Wang CY.** TBL1-TBLR1 and beta-catenin recruit each other to Wnt target-gene promoter for transcription activation and oncogenesis. *Nat Cell Biol* 10: 160–169, 2008.
37. **Liu J, Burkin DJ, Kaufman SJ.** Increasing $\alpha_7\beta_1$-integrin promotes muscle cell proliferation, adhesion, and resistance to apoptosis without changing gene expression. *Am J Physiol Cell Physiol* 294: C627–C640, 2008.
38. **Ma K, Mallidis C, Artaza J, Taylor W, Gonzalez-Cadavid N, Bhasin S.** Characterization of 5′-regulatory region of human myostatin gene: regulation by dexamethasone in vitro. *Am J Physiol Endocrinol Metab* 281: E1128–E1136, 2001.
39. **Ma K, Mallidis C, Bhasin S, Mahabadi V, Artaza J, Gonzalez-Cadavid N, Arias J, Salehian B.** Glucocorticoid-induced skeletal muscle atrophy is associated with upregulation of myostatin gene expression. *Am J Physiol Endocrinol Metab* 285: E363–E371, 2003.
40. **Marin A, Pozza G, Gottardo F, Moro L, Stefani AL, Cozzi G, Brscic M, Andrighetto I, Ravarotto L.** Administration of dexamethasone per os in finishing bulls. II. Effects on blood parameters used as indicators of animal welfare. *Animal* 2:7: 1080–1086, 2008.
41. **Marshall A, Salerno MS, Thomas M, Davies T, Berry C, Dyer K, Bracegirdle J, Watson T, Dziadek M, Kambadur R, Bower R, Sharma M.** Mighty is a novel promyogenic factor in skeletal myogenesis. *Exp Cell Res* 314: 1013–1029, 2008.
42. **McCormick KM, Burns KL, Piccone CM, Gosselin LE, Brazeau GA.** Effects of ovariectomy and estrogen on skeletal muscle function in growing rats. *J Muscle Res Cell Motil* 25: 21–27, 2004.
43. **Miura P, Andrews M, Holcik M, Jasmin BJ.** IRES-mediated translation of utrophin A is enhanced by glucocorticoid treatment in skeletal muscle cells. *PLoS ONE* 3: e2309, 2008.
44. **Mounier C, Posner BI, Can J.** Transcriptional regulation by insulin: from the receptor to the gene. *Physiol Pharmacol* 84: 713–724, 2006.
45. **Muslin AJ.** Role of raf proteins in cardiac hypertrophy and cardiomyocyte survival. *Trends Cardiovasc Med* 15: 225–229, 2005.
46. **Nielsen S, Pedersen BK.** Skeletal muscle as an immunogenic organ. *Curr Opin Pharmacol* 8: 346–351, 2008.
47. **Nowak SJ, Gonzalez K, Baylies MK.** Bhringi, a highly conserved regulator of Twist transcription factor activity. *Dros Res Conf* 48: 337, 2007.
48. **Otto A, Schmidt C, Luke G, Allen S, Valasek P, Muntoni F, Lawrence-Watt D, Patel K.** Canonical Wnt signalling induces satellite-cell proliferation during adult skeletal muscle regeneration. *J Cell Sci* 121: 2939–2950, 2008.
49. **Patel K, Amthor H.** The function of myostatin and strategies of myostatin blockade-new hope for therapies aimed at promoting growth of skeletal muscle. *Neuromuscul Disord* 15: 117–126, 2005.
50. **Pelosi M, Marampon F, Zani BM, Prudente S, Perlas E, Caputo V, Cianetti L, Berno V, Narumiya S, Kang SW, Musarò A, Rosenthal N.**

ROCK2 and its alternatively spliced isoform ROCK2m positively control the maturation of the myogenic program. *Mol Cell Biol* 27: 6163–6176, 2007.

51. **Perez-Ruiz A, Ono Y, Gnocchi VF, Zammit PS.** beta-Catenin promotes self-renewal of skeletal-muscle satellite cells. *J Cell Sci* 121: 1373–1382, 2008.
52. **Perrett T, Wildman BK, Jim GK, Vogstad AR, Fenton RK, Hannon SJ, Schunicht OC, Abutarbush SM, Booker CW.** Evaluation of the efficacy and cost-effectiveness of melengestrol acetate in feedlot heifer calves in western Canada. *Vet Ther* 9: 223–240, 2008.
53. **Polesskaya A, Seale P, Rudnicki MA.** Wnt signaling induces the myogenic specification of resident CD45+ adult stem cells during muscle regeneration. *Cell* 113: 841–852, 2003.
54. **Pritchard CA, Hayes L, Wojnowski L, Zimmer A, Marais RM, Norman JC.** B-Raf acts via the ROCKII/LIMK/cofilin pathway to maintain actin stress fibers in fibroblasts. *Mol Cell Biol* 24: 5937–5952, 2004.
55. **Rafacho A, Cestari TM, Taboga SR, Boschero AC, Bosqueiro JR.** High doses of dexamethasone induce increased β-cell proliferation in pancreatic rat islets. *Am J Physiol Endocrinol Metab* 296: E681–E689, 2009.
56. **Ratcliffe MJ, Itoh K, Sokol SY.** A positive role for the PP2A catalytic subunit in Wnt signal transduction. *J Biol Chem* 275: 35680–35683, 2000.
57. **Reiter M, Walf VM, Christians A, Pfaffl MW, Meyer HH.** Modification of mRNA expression after treatment with anabolic agents and the usefulness for gene expression-biomarkers. *Anal Chim Acta* 586: 73–81, 2007.
58. **Rhen T, Cidlowski JA.** Estrogens and glucocorticoids have opposing effects on the amount and latent activity of complement proteins in the rat uterus. *Biol Reprod* 74: 265–274, 2006.
59. **Richardson BE, Beckett K, Nowak SJ, Baylies MK.** SCAR/WAVE and Arp2/3 are crucial for cytoskeletal remodeling at the site of myoblast fusion. *Development* 134: 4357–4367, 2007.
60. **Rodgers BD, Weber GM, Kelley KM, Levine MA.** Prolonged fasting and cortisol reduce myostatin mRNA levels in tilapia larvae; short-term fasting elevates. *Am J Physiol Regul Integr Comp Physiol* 284: R1277–R1286, 2003.
61. **Rodgers BD, Garikipati DK.** Clinical, agricultural, and evolutionary biology of myostatin: a comparative review. *Endocr Rev* 29: 513–534, 2008.
62. **Sadkowski T, Jank M, Zwierzchowski L, Siadkowska E, Oprzadek J, Motyl T.** Gene expression profiling in skeletal muscle of Holstein-Friesian bulls with single-nucleotide polymorphism in the myostatin gene 5′-flanking region. *J Appl Genet* 49: 237–250, 2008.
63. **Sakuma K, Nishikawa J, Nakao R, Watanabe K, Totsuka T, Nakano H, Sano M, Yasuhara M.** Calcineurin is a potent regulator for skeletal muscle regeneration by association with NFATc1 and GATA-2. *Acta Neuropathol (Berl)* 105: 271–280, 2003.
64. **Sapolsky RM, Romero LM, Munck AU.** How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocr Rev* 21: 55–89, 2000.
65. **Schulz RA, Yutzey KE.** Calcineurin signaling and NFAT activation in cardiovascular and skeletal muscle development. *Dev Biol* 266: 1–16, 2004.
66. **Shuto T, Kukita T, Hirata M, Jimi E, Koga T.** Dexamethasone stimulates osteoclast-like cell formation by inhibiting granulocyte-macrophage colony-stimulating factor production in mouse bone marrow cultures. *Endocrinology* 134: 1121–1126, 1994.
67. **Solomon AM, Bouloux PM.** Modifying muscle mass - the endocrine perspective. *J Endocrinol* 191: 349–360, 2006.
68. **St-Pierre SJ, Chakkalakal JV, Kolodziejczyk SM, Knudson JC, Jasmin BJ, Megeney LA.** Glucocorticoid treatment alleviates dystrophic myofiber pathology by activation of the calcineurin/NF-AT pathway. *FASEB J* 18: 1937–1939, 2004.
69. **Sun L, Ma K, Wang H, Xiao F, Gao Y, Zhang W, Wang K, Gao X, Ip N, Wu Z.** JAK1-STAT1-STAT3, a key pathway promoting proliferation and preventing premature differentiation of myoblasts. *J Cell Biol* 179: 129–138, 2007.
70. **Suzuki N, Nakamura S, Mano H, Kozasa T.** Galpha 12 activates Rho GTPase through tyrosine-phosphorylated leukemia-associated RhoGEF. *Proc Natl Acad Sci USA* 100: 733–738, 2003.
71. **Thirion C, Stucka R, Mendel B, Gruhler A, Jaksch M, Nowak KJ, Binz N, Laing Lochmüller H NG.** Characterization of human muscle type cofilin (CFL2) in normal and regenerating muscle. *Eur J Biochem* 268: 3473–3482, 2001.
72. **Tibshirani R, Hastie T, Narasimhan B, Chu G.** Diagnosis of multiple cancer types by shrunken centroids of gene expression. *Proc Natl Acad Sci USA* 99: 6567–6572, 2002.
73. **Tidball JG, Wehling-Henricks M.** Evolving therapeutic strategies for Duchenne muscular dystrophy: targeting downstream events. *Pediatr Res* 56: 831–841, 2004.
74. **Toffolatti L, Rosa Gastaldo L, Patarnello T, Romualdi C, Merlanti R, Montesissa C, Poppi L, Castagnaro M, Bargelloni L.** Expression analysis of androgen-responsive genes in the prostate of veal calves treated with anabolic hormones. *Domest Anim Endocrinol* 30: 38–55, 2006.
75. **Tsai WJ, McCormick KM, Brazeau DA, Brazeau GA.** Estrogen effects on skeletal muscle insulin-like growth factor 1 and myostatin in ovariectomized rats. *Exp Biol Med* 232: 1314–1325, 2007.
76. **Tusher VG, Tibshirani R, Chu G.** Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci USA* 98: 5116–5121, 2001.
77. **Uht RM, Anderson CM, Webb P, Kushner PJ.** Transcriptional activities of estrogen and glucocorticoid receptors are functionally integrated at the AP-1 response element. *Endocrinology* 138: 2900–2908, 1997.
78. **Van Poucke C, Van Peteghem C.** Development and validation of a multi-analyte method for the detection of anabolic steroids in bovine urine with liquid chromatography-tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci* 772: 211–217, 2002.
79. **Vascellari M, Pozza G, Poppi L, Capello K, Angeletti R, Ravarotto L, Andrighetto I, Mutinelli F.** Evaluation of indirect biomarkers for detecting corticosteroids used as illegal growth promoters in beef cattle. *Vet Rec* 163: 147–151, 2008.
80. **Vianello S, Brazzoduro L, Dalla Valle L, Belvedere P, Colombo L.** Myostatin expression during development and chronic stress in zebrafish (*Danio rerio*). *J Endocrinol* 176: 47–59, 2003.
81. **Vuocolo T, Byrne K, White J, McWilliam S, Reverter A, Cockett NE, Tellam RL.** Identification of a gene network contributing to hypertrophy in callipyge skeletal muscle. *Physiol Genomics* 28: 253–272, 2007.
82. **Wang Y, Barbacioru C, Hyland F, Xiao W, Hunkapiller KL, Blake J, Chan F, Gonzalez C, Zhang L, Samaha RR.** Large scale real-time PCR validation on gene expression measurements from two commercial long-oligonucleotide microarrays. *BMC Genomics* 7: 59, 2006.
83. **Wei L, Zhou W, Croissant JD, Johansen FE, Prywes R, Balasubramanyam A, Schwartz RJ.** RhoA signaling via serum response factor plays an obligatory role in myogenic differentiation. *J Biol Chem* 273: 30287–30294, 1998.
84. **Zhou F, Bouillard B, Pharaboz-Joly MO, Andre J.** Non-classical antiestrogenic actions of dexamethasone in variant MCF-7 human breast cancer cells in culture. *Mol Cell Endocrinol* 66: 189–197, 1989.