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Transcriptional Profiling of Testosterone-Regulated Genes in the Skeletal Muscle of Human Immunodeficiency Virus-Infected Men Experiencing Weight Loss

Monty Montano, John N. Flanagan, Lan Jiang, Paola Sebastiani, Matthew Rarick, Nathan K. LeBrasseur, Carl A. Morris, Ravi Jasuja, and Shalender Bhasin

Sections of Infectious Diseases (M.M., M.R.) and Endocrinology, Diabetes, and Nutrition (J.N.F., L.J., N.K.L., C.A.M., R.J., S.B.), Center for HIV-1/AIDS Care and Research (M.M., M.R.), School of Medicine, and Department of Biostatistics (P.S.), School of Public Health, Boston University, Boston, Massachusetts 02118

Context: HIV-associated wasting and weight loss remain clinically significant concerns even in the era of potent antiretroviral therapy. Although androgen treatment increases muscle mass, the cell-intrinsic mechanisms engaged remain poorly understood.

Objective: This study was an unbiased approach to identify expression profiles associated with testosterone treatment using genome-wide microarray analysis of skeletal muscle biopsies.

Design, Setting, and Participants: Forty-four HIV-positive men with weight loss were randomized to receive either 300 mg testosterone enanthate or placebo injections im weekly for 16 wk. Muscle biopsies were obtained at baseline and on treatment d 14. A subset of specimens was chosen for microarray analysis, with changes in selected genes confirmed by real-time PCR, Western blot analysis, and *in vitro* culture of muscle precursor cells.

Results: Significantly greater gains in body mass (+2.05 and −1.07 kg, respectively; $P = 0.003$) and lean body mass by dual-energy x-ray absorptiometry (2.93 *vs.* 0.35 kg, respectively; $P = 0.003$) were observed in subjects treated with testosterone compared with placebo. Microarray analysis revealed up-regulation in genes involved in myogenesis and muscle protein synthesis, immune regulation, metabolic pathways, and chromatin remodeling. Representative genes were confirmed by real-time PCR and protein expression studies. In an independent analysis, gene networks that differentiate healthy young men from older men with sarcopenia had substantial overlap with those activated by testosterone treatment.

Conclusions: These data provide new insights into the mechanisms of androgen action and have implications for both development of muscle biomarkers and anabolic therapies for wasting and sarcopenia. **(*J Clin Endocrinol Metab* 92: 2793–2802, 2007)**

HIV-ASSOCIATED MUSCLE WASTING and weight loss remain pervasive clinical concerns (1–3), despite the availability of highly active antiretroviral therapy (1, 4, 5). In developing countries where highly active antiretroviral therapy is not broadly available, muscle protein wasting and weight loss persist as life-threatening problems. The pathogenesis for HIV-associated muscle wasting is poorly understood and likely multifactorial. Similarly, as men and women in the general population grow older, their muscle mass decreases, leading to increased risk of disability, dependency, falls, and fractures. The possibility that sarcopenia may share patterns of gene activity in common with HIV-associated muscle wasting has not been evaluated previously.

Our previous studies suggest that testosterone supplementation increases skeletal muscle mass (6–8), potentially by regulating the differentiation of resident, multipotent mesenchymal stem cells (9), thereby promoting myogenesis and potentially inhibiting adipogenesis (10). However, the molecular mechanisms engaged in testosterone-induced muscle hypertrophy are not well defined and were the subject of this investigation. To elucidate the mechanisms by which testosterone mediates its anabolic effects on the muscle in patients with HIV-associated weight loss, we used microarray analysis to determine the gene networks regulated by testosterone treatment. In this study, we hypothesized that androgen treatment in HIV-infected men would increase muscle mass in association with changes in myogenic gene expression.

Aging is associated with loss of skeletal muscle mass and a progressive decline in circulating testosterone concentrations in adult males (11, 12). Undoubtedly, aging-associated loss of muscle mass is multifactorial in its etiology, but decline in testosterone concentrations has been postulated to contribute to the age-related decline in skeletal muscle mass. Bioavailable testosterone levels are associated with skeletal muscle mass and muscle strength. We, therefore, surmised that some of the differences in im gene expression between young and older men would be similar to those induced by testosterone in HIV-infected men with weight loss. Accordingly, we contrasted the differences in gene expression profiles of older and young men with those in HIV-infected men with weight loss before and after testosterone therapy.

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Abbreviations: AR, Androgen receptor; DHT, dihydrotestosterone; ECM, extracellular matrix; GEO, Gene Expression Omnibus; mTOR, mammalian target of rapamycin; SkMC, skeletal muscle precursor cell; ULN, upper limit of the normal range.

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Subjects and Methods

The institutional review boards of Charles Drew University and Boston University approved the protocol. All participants provided written informed consent.

Participants

Subjects evaluated in this study were recruited between May 2003 and June 2005.

Inclusion criteria. Inclusion criteria for subjects were as follows: HIV-positive men, 18–60 yr of age, with objective evidence of HIV infection with low to low-to-normal testosterone levels (<400 ng/dl); documented weight loss within the previous 6 months of between 5 and 15% of body weight or an actual body mass index at screening of between 17 and 20 (equivalent to 85–95% of the lower limit of ideal weight); an energy intake in excess of 80% of the recommended dietary allowance; on stable and potent antiretroviral therapy for at least 12 wk or not starting antiretroviral therapy in the next 4 months; CD4 cell count greater than 50/mm^3 and HIV copy number less than 10,000 copies/ml; and able and willing to provide informed consent and comply with the protocol.

Exclusion criteria. Exclusion criteria were as follows: concurrent severe lipodystrophy; history of prostatic or mammary cancer; significant diarrhea; use of any androgen, GH, or other anabolic or orexigenic agents within the past 6 months; use of systemic corticosteroids, except for topical application; significant cardiac, renal, or hepatic disease; AIDS defining illness (Centers for Disease Control HIV Classification, 1993, Clinical Category C) within the previous 3 months (except HIV wasting syndrome); malignancy, other than Kaposi's sarcoma localized to the skin; involvement in (vigorous) resistance exercise training programs (body building) in the past 3 months; diabetes mellitus; limiting neuromuscular, joint, or bone disease or history of stroke with residual neurological defect that would preclude measurements of muscle strength or physical function; current alcohol or drug dependency; history of hypersensitivity to anabolic steroids or to GH; and any of the following blood test results: liver function test (alanine aminotransferase and aspartate aminotransferase) at least five times the upper limit of the normal range (ULN), alkaline phosphatase greater than five times ULN or more than three times ULN if bilirubin is above normal, cholesterol total (>5× ULN) or triglycerides of at least 700 mg/dl, serum creatinine of at least two times ULN, hemoglobin of no more than 8.0 g/dl or more than 18 g/dl, platelet count of no more than 50×10^9/liter, hematocrit greater than 48%, and prostate-specific antigen of at least 4 ng/ml.

Concomitant medications. Men who have received in the preceding 6 months or are currently using androgenic steroids, recombinant human GH, IGF-I, or other anabolic agents or appetite stimulants were excluded. Also excluded were the following: drugs that affect testosterone secretion or metabolism such as ketoconazole, dilantin, and phenobarbital; use of corticosteroids (except topical); and food supplements that might affect body composition, such as creatine, high-dose amino acid supplements, whey protein supplements, androstenedione, dehydroepiandrosterone, and marinol. Patients who have been on a stable dose of erythropoietin for at least 3 months were allowed to continue.

The prespecified primary was change in leg press strength and physical function.

Randomization and treatment

The eligible subjects were randomly assigned, using a blocking scheme with a block size of 4, to receive by weekly im injections of either a supraphysiologic dose of testosterone enathate (300 mg) (Savient Pharmaceuticals, East Brunswick, NJ) or sesame oil. This dosage of testosterone enathate has an excellent safety record (13). All injections were given in the Clinical Research Center to ensure compliance. Treatment duration was 16 wk.

Testosterone and body composition measurements

Serum total testosterone levels in all samples were assayed, using liquid chromatography, tandem mass spectrometry. Dual-energy x-ray absorptiometry (QDR 4500A; Hologic, Waltham, MA) was used to measure total body and lean body mass at entry and study endpoint (wk 16). The dual-energy x-ray absorptiometry scanner was calibrated weekly using the body composition analysis step phantom.

Muscle sample collection

Percutaneous biopsies of vastus lateralis were obtained at baseline and on treatment d 14, using previously described procedures (9, 14, 15). This sampling time was selected because of the consideration that gene pathways important in mediating anabolic effects would be activated early in the course of muscle accretion.

Microarray methodology

RNA was purified from frozen muscle biopsies, and trace DNA was removed from the RNA samples using the SV Total RNA Isolation kit (Ambion, Austin, TX). RNA concentrations were determined in 2 μl sample by spectrophotometry using a NanoDrop-1000 (NanoDrop Technologies, Wilmington, DE). The initial test set consisted of nine specimens obtained from four placebo-treated and five testosterone-treated subjects (selected randomly), and the complete set consisted of 15 specimens obtained from eight placebo and seven treated subjects. Three to 5 μg total RNA from selected specimens were processed using a U133A 2.0 chip from Affymetrix (Santa Clara, CA).

In an independent aging dataset, skeletal muscle biopsy datasets from seven young subjects and eight older subjects were used to identify differentially expressed genes [Gene Expression Omnibus (GEO) entry GSE362] (16). The age ranges in subjects from which biopsies were obtained were seven younger (range of 21–27 yr) *vs.* eight older (range of 67–75 yr) men. In the gene expression data available, there were 10,379 gene probes present in both the aging dataset and the testosterone treatment dataset that were evaluated for differential expression.

The resulting gene expression profiles were evaluated for trend change in placebo *vs.* testosterone-dependent gene expression and also through the use of model-based hierarchical clustering and leave-one-out cross-validation to identify genes with similar profiles of expression over the baseline testosterone levels in the placebo and treated series. The cluster analysis was performed using a previously described Bayesian approach, CAGED (for Cluster Analysis of Gene Expression Dynamics) (17). Enrichment for gene categories associated with testosterone treatment and/or aging was determined using EASE (Portland, OR) software, as described by Hosack *et al.* (18). The Affymetrix datasets can be accessed at GEO (http://www.ncbi.nlm.nih.gov/geo/). Supplemental information is published as supplemental data on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org.

Cell lines and cultures

Human skeletal muscle precursor cells (SkMCs) (Cambrex Laboratories, Walkersville, MD) were grown in GM medium (DMEM containing 10% fetal bovine serum) and induced to differentiate in DM medium (DMEM containing 2% horse serum). Dihydrotestosterone (DHT) was added to a final concentration of 30 nM for the times indicated.

Real-time quantitative RT-PCR

Human AR, TCF8, ATRX, NCOA3, RBL2, and TCF4 RNA transcripts extracted from total RNA in muscle biopsies were quantified on an ABI 7500 with real-time quantitative RT-PCR using Assays-on-Demand from Applied Biosystems (Foster City, CA) and normalized to endogenous 18S, as described previously (19, 20). Relative RNA levels for each gene were measured as RQ values ($2^{-\Delta\Delta Ct}$), a quantitative value representing the amount of each gene expressed (21).

Western blot analysis

Whole cells were lysed in cell lysis buffer (Cell Signaling Technology, Danvers, MA) containing the protease inhibitor phenylmethylsulfonylfluoride. Whole-cell lysates (10 μg) were separated using by SDS-PAGE on a 4% stacking gel/10% minigel, transferred to nitrocellulose membranes, blocked with 5% nonfat milk, and incubated with primary antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA) at 4 C overnight (in some cases, 2 h at room temperature), washed, and incubated

with secondary horseradish peroxidase-conjugated antibodies (Cell Signaling Technology). The protein bands were visualized using enhanced chemiluminescence detection (ECL-plus; GE Healthcare, Little Chalfont, UK).

Statistical analysis

Clinical variables were evaluated as linear or log-transformed data (because of skewed distribution of some variables), and group means were compared using a two-sample Student's *t* test. Changes in body weight and lean body mass from baseline to end of treatment were compared between the two groups using a two-sample *t* test. Paired differences in phosphorylated p38 and Akt were compared in the two groups using a paired *t* test.

The relative expression level for each validated gene, $2^{-\Delta\Delta Ct}$, measured using RT-PCR in placebo- and testosterone-treated subjects, were correlated with microarray values for the corresponding genes using Pearson's correlation coefficients. Once differentially expressed genes were identified by CAGED, the biologically enriched categories were identified, as described previously (19), by implementing a stand-alone version of the EASE statistical software (18).

Results

Baseline characteristics of the subjects

The two groups did not differ significantly in their baseline age, CD4 T-lymphocyte count, weight loss, and HIV copy number (Table 1). However, subjects in the serum testosterone group had significantly lower baseline testosterone levels ($P = 0.04$).

Change in body weight and composition

Testosterone treatment was associated with greater gains in body weight than placebo at 8 wk compared with 3 wk (+2.05 *vs.* −1.07 kg; $P = 0.003$), increases in lean body mass (2.93 *vs.* 0.35 kg; $P = 0.003$), and decreases in percentage of total body fat (−2.4 *vs.* −0.4%; $P = 0.013$) at 16 wk. This trend was evident in the sample subset that had been randomized for microarray analysis and in the entire specimen set (Fig. 1 and data not shown). The observed weight gain corroborates a large body of evidence supporting the role of testosterone in weight gain among HIV-positive subjects and also validated the efficacy of treatment in this study. Although we did not measure fluid retention in this study, we have done this in several previous studies (7, 8, 22) and found no significant change in the ratio of total body water to lean body mass, indicating that apparent gains in lean body mass were not attributable to water retention in excess of that associated with protein accretion.

Identification of testosterone-responsive genes

Biopsy specimens were randomly selected within each group (test set, four placebo and five testosterone; full set, eight placebo and seven testosterone) for microarray analysis to identify differentially expressed genes associated with treatment. In the initial test set, enriched biological categories included muscle-associated gene sets, immune response, and muscle growth (supplemental Table 1, published as supplemental data on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org).

Based on that initial data, additional specimens were randomly selected and evaluated. In the full set, baseline testosterone values differed significantly in the two groups at study entry (Table 1). Because we were interested in identifying testosterone-dependent changes, each group of arrays was therefore ordered from low to high (left to right) and normalized to the lowest common value within the overlapping range for the placebo- and testosterone-treated groups (*i.e.* baseline A; range for placebo, 265–394 ng/dl; treated, 250–332 ng/dl) or by normalizing to the lowest value within each group (baseline B; range for placebo, 265–394 ng/dl; treated, 144–332 ng/dl). We then analyzed for clusters of gene expression representing coordinate expression patterns that differed between the two groups using the software CAGED (17). Trends in gene expression patterns did not differ substantially based on the choice of baseline.

Of the more than 20,000 gene probes on the chip, 10,969 genes were detected. Among these, 2277 gene probes were differentially expressed in association with testosterone treatment. Five hundred nineteen of the 2277 gene-probe profiles were then selected after inspection of the dendrogram and were evaluated for biological category enrichment (supplemental Table 2, published as supplemental data on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). Enriched biological categories included the IGF-I-mediated [*i.e.* the AKT/mammalian target of rapamycin (mTOR) pathway] and the androgen receptor (AR)-mediated (*e.g.* TCF4/β-catenin) signaling pathways. Many gene sets associated with transcriptional control were also differentially regulated, including chromatin remodeling genes. A selected subset of differentially up-regulated genes in response to testosterone treatment are shown in dendrograms using either the common range baseline (Fig. 2A) or the lowest value for each group as a baseline (Fig. 2B). Notably, the overall trends in gene expression were similar, although more genes were identified using the larger sample size (baseline B).

Statistical enrichment of genes associated with muscle gene expression were detected, using multiple gene lists compiled in our laboratory [Montano Lab Gene Set (MLGS)] or with existing web datasets (Fig. 3A). Notably present were

TABLE 1. Baseline characteristics of the subjects

Characteristic	Placebo group (n = 22)				Testosterone group (n = 22)				*P* value
	Mean	SD	Min	Max	Mean	SD	Min	Max	
Baseline									
Age (yr)	43.3	6.5	30.8	58.7	43.0	6.9	30.1	55.8	0.43
Weight loss (at entry)	12.9	3.1	8.0	18.0	15.0	3.9	8.0	20.0	0.91
Weight at entry	162.3	23.4	120.0	208.0	168.1	19.0	146.0	227.5	0.82
CD4 count (cells/ml)	460.1	228.6	157.0	940.0	355.3	218.6	61.0	751.0	0.06
Baseline testosterone(ng/dl)	323.2	62.8	194.0	397.0	285.4	75.2	119.0	403.0	0.04[a]
HIV log copies/ml	3.57	1.13	2.60	5.88	3.31	0.91	2.60	5.51	0.18

[a] Statistical significance.

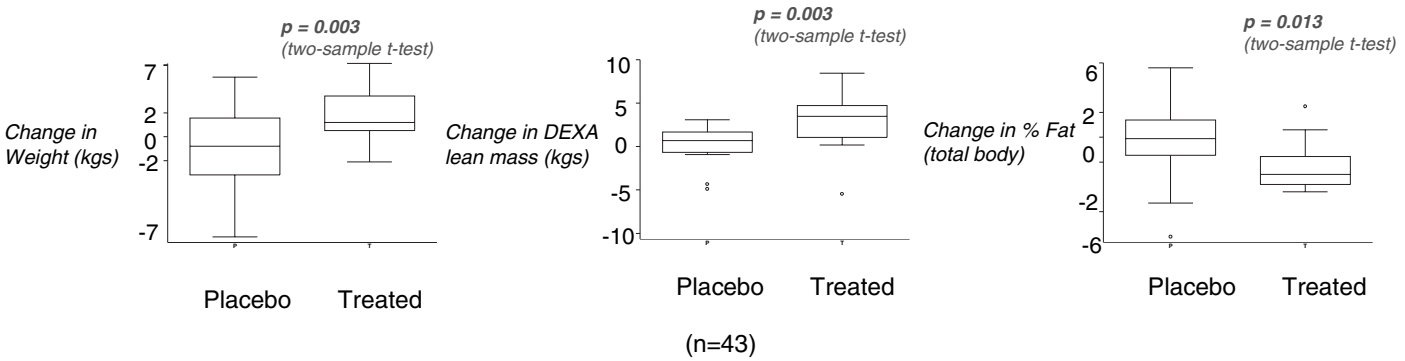


FIG. 1. Changes from baseline in body weight and body composition in placebo- and testosterone-treated men. Subjects in the testosterone treatment group had a significant difference in treatment-associated weight gain than placebo at 8 wk compared with 3 wk (+2.05 *vs.* −1.07 kg; $P = 0.003$), increases in lean body mass (2.93 *vs.* 0.35 kg; $P = 0.003$), and decreases in percentage of total body fat (−2.4 *vs.* −0.4%; $P = 0.013$) at 16 wk. Note that one subject discontinued study after baseline values were obtained; therefore, the sample size became n = 43 for later analysis. Similar results were obtained for the subset of subjects with microarray data (data not shown). *P* values represent significance based on two-sample *t* tests with unequal variance. *Box plots* indicate change from baseline to wk 16. The *horizontal line* within each *box plot* represents the median (50th percentile), and the *lower* and *upper box* boundaries represent the interquartile range (*i.e.* 25th percentile and 75th percentile), respectively. *Lines* extending outside of the *box* represent adjacent data. All *data points* outside of this range are represented by *circles* and are considered outliers. DEXA, Dual-energy x-ray absorptiometry.

genes associated with muscle development and transcriptional regulation (*NFAT5*, *MEF2A*, and *TCF8*), insulin signaling pathway, adipogenic differentiation (*DIPA* and *AMP kinase*), apoptosis, cell cycle events, mitochondrial biogenesis and immune response [*e.g.* chemoattractants, *CCL5* (*RANTES*), *CXCL9* (*MIG*), *CXCL-10* (*IP-10*); activation markers, *CD83*, *CD53*, and *CD44*; and signaling, *IL6R*, *IL6ST*, and *IL-18*] (Fig. 3, B–D). Collectively, these data suggest that myogenic response to androgen treatment represents a complex activation of multiple transcriptional pathways associated with muscle remodeling and immune regulation.

Two fundamental signaling pathways that are activated during muscle differentiation and growth are the p38 MAPK and the phosphatidylinositol-3 kinase/Akt pathway and downstream target mTOR, collectively known as the Akt/mTOR pathway (23–25). We therefore measured protein expression of the active forms of p38 MAPK and Akt in paired samples. Phosphorylated p38 MAPK was detectable in all specimens (data not shown), whereas phosphorylated Akt was up-regulated in the testosterone-treated subjects ($P = 0.004$) (Fig. 4B).

Validation of androgen-associated gene expression by quantitative, real-time PCR

Microarray data are semiquantitative. To validate expression, selected genes in Fig. 2 (*AR*, *TCF8*, *ATRX*, *NCOA3*, *RBL2*, and *TCF4*) were evaluated for quantitative RNA expression using quantitative RT-PCR. The Pearson's correlation coefficients between RNA expression levels measured by using RT-PCR and those obtained from microarray analysis for the same gene-probe locations were high (Table 2).

Confirmation in SkMCs of changes in myogenic gene expression induced by testosterone in HIV-positive muscle biopsies

Because many genes up-regulated by testosterone were implicated in muscle remodeling and myogenesis, we evaluated "early" skeletal muscle expression of MyoD, MEF2A, and myogenin in response to DHT treatment in myogenic precursor cells derived from human SkMCs (Cambrex Laboratories) and cultured in differentiation media *in vitro*. These cells, when treated with DHT, displayed a time-dependent increase in protein expression of the myogenic factors MyoD and myogenin relative to expected α-tubulin increase during differentiation (Fig. 4A).

Common gene sets regulated during androgen treatment and aging

Because aging is associated with loss of skeletal muscle mass and decline in testosterone levels, we speculated that at least some of the changes in gene expression in the skeletal muscle in association with androgen treatment may also resemble those that differentiate older and young men (26, 27). To evaluate this, we identified a published dataset for skeletal muscle gene expression that had used the same microarray chips that were used in this study (GEO entry GSE362) (16). Differential gene expression using BADGE (for Bayesian Analysis of Differential Gene Expression), followed by EASE enrichment analysis, was conducted for both datasets (for a listing of enriched gene categories for each dataset, see supplemental Table 3, published as supplemental data on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org).

Fifteen enriched biological categories were identified as common to both datasets (Fig. 5A). Fifty-four genes were differentially expressed and common to both datasets (supplemental Table 4, published as supplemental data on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). In addition to categories associated with myogenesis, the category "HIV Associated" was enriched in both datasets. Inspection of the gene composition within this category in both datasets identified six overlapping genes associated with immune response (CD14, CCL5/RANTES, and PSMB8) and muscle growth (SDC2, SLC14A, and FN1) (Fig. 5B). These data suggest a common theme of immune

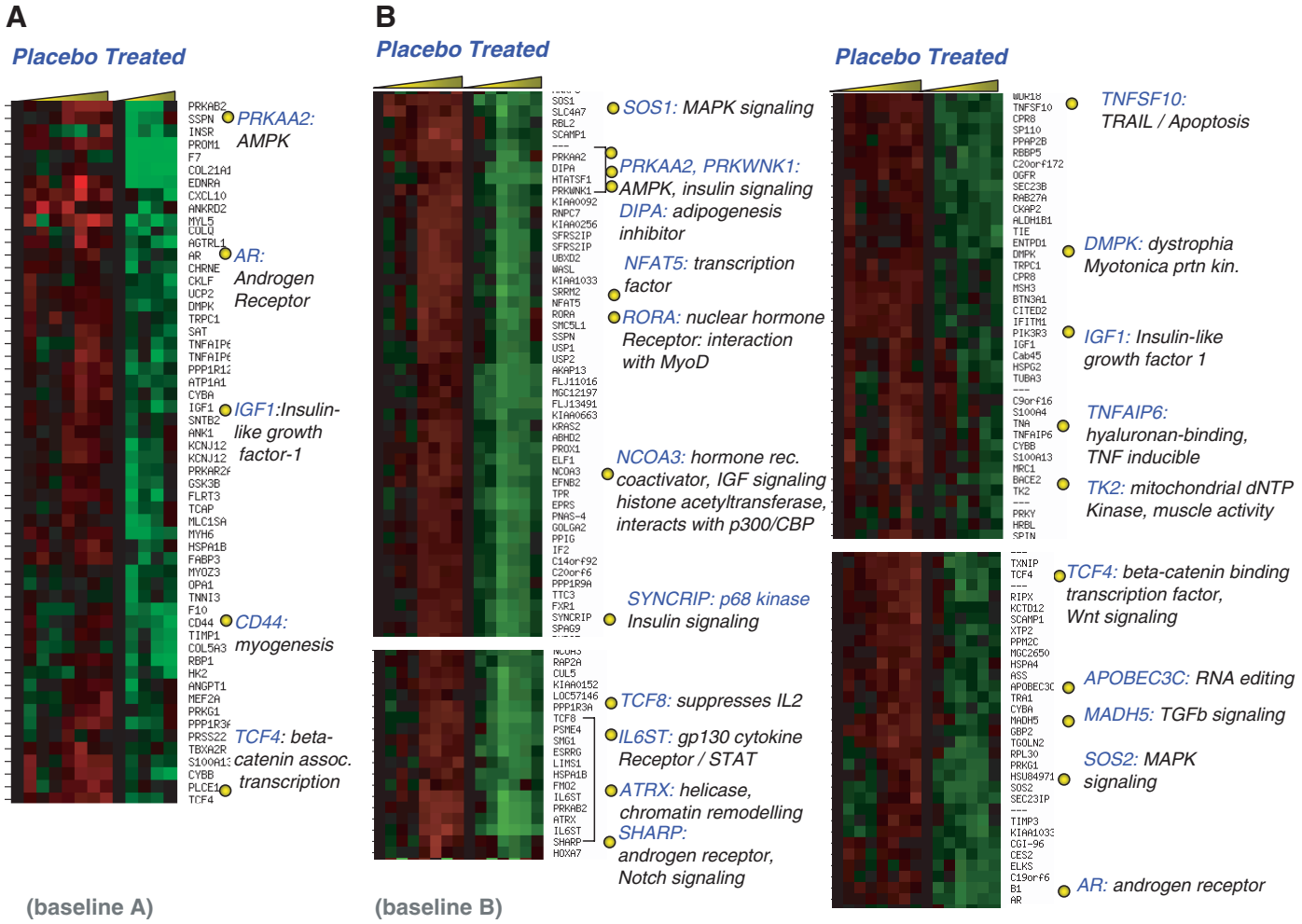


FIG. 2. Selected gene expression profile for placebo- and testosterone-treated subjects 2 wk after treatment. Subjects were arranged based on randomized group (placebo, testosterone), arranged left to right, and normalized to the array specimen with the lowest testosterone values (*i.e.* lowest shared value, baseline A; lowest within group value, baseline B). *Red* represents down-regulated expression, and *green* represents up-regulated gene expression. Selected genes are highlighted to the *right* with common names, and associated functions are indicated.

regulation and myogenic feedback in conditions associated with loss of muscle mass.

Discussion

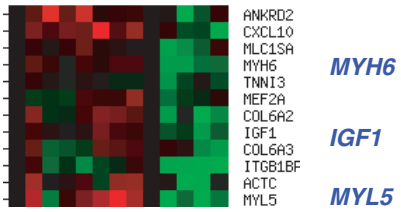
Although weight gain and lean mass accretion during androgen treatment in HIV-negative and HIV-positive subjects is well established (8, 28–31), the profile of gene networks that underlie androgen action has not been well defined. This study represents the first systematic effort to identify coordinately regulated patterns of gene expression in human skeletal muscle (vastus lateralis) obtained from a group of HIV-positive men experiencing weight loss who were randomized, in a parallel double-blind study, to receive either testosterone or placebo. In this analysis, many genes associated with insulin signaling (*AMPK* and *p68 kinase*), AR signaling (*AR*, *RORA*, *NCOA3*, and *SHARP*) and transcription factors associated with tissue remodeling (*TCF4*, *NFAT5*, and *SHARP*) were up-regulated in association with testosterone treatment. In addition, MAPK activation (*SOS1* and *SOS2*), inhibition of adipogenesis (*DIPA*), and activation of chromatin remodeling genes (*NCOA3* and *ATRX*) were coordinately and differentially regulated in response to testosterone treatment (Fig. 2). These data imply that, in addition to genes involved in feedforward androgen signaling (*e.g.* up-regulation of AR itself) and myogenic differentiation, testosterone affects gene sets involved in the modulation of chromatin remodeling, transcriptional control, metabolic regulation, muscle protein synthesis (*e.g.* MAPK and pAkt), and immune regulation. Notably, there were many similarities in the expression of im gene activated by testosterone with those that differentiate older sarcopenic men from young men.

Interestingly, specific genes up-regulated in response to treatment included *IGF-I*, *AR*, and *TCF4* (β-catenin binding transcription factor). IGF-I has been linked previously with proliferation of myoblasts (32) and skeletal muscle hypertrophy (33, 34). Testosterone administration up-regulates AR expression in human skeletal muscle and multipotent cell lines (35, 36). Similarly, a role for TCF4 in muscle remodeling has also been proposed (37). Testosterone and DHT by promoting the association of AR with β-catenin and TCF4 have been shown to activate a number of Wnt target genes in

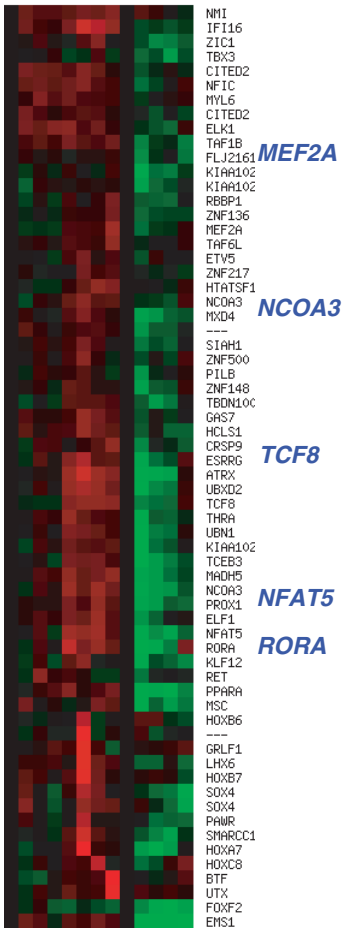
A

System	Gene Category	Hits	Probability
GO Cellular Component	extracellular matrix	49	4.8E-11
GO Biological Process	immune response	64	3.7E-04
GO Molecular Function	structural constituent of muscle	8	8.3E-03
GO Biological Process	chromatin remodeling	6	1.2E-02
GO Biological Process	development	119	2.5E-02
GO Biological Process	chromatin modification	8	4.1E-02
MLGS-Muscle related	Muscle-HS (Homo sapiens)	69	5.0E-02

B **GO category:** *muscle development:*



C **GO category:** *transcription*



D **GO category:** *Immune response*

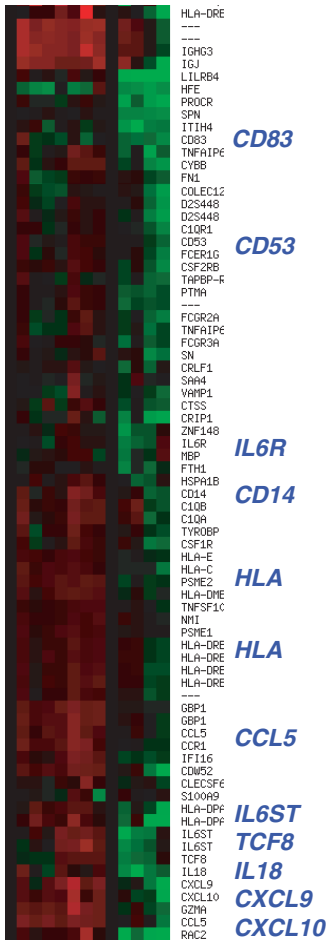


FIG. 3. Enrichment analysis of muscle-associated genes. A, Gene lists were generated based on the literature and on existing Gene Ontology (GO) web datasets and used to evaluate statistical enrichment with an adjusted Fisher's exact test, as described previously (18, 19). Many gene sets associated with muscle, chromatin, and immune response were highly significant. B–D, Subcategories of activated muscle gene sets (B), transcription (C), and immune response (D) that were up-regulated in subjects receiving testosterone supplementation. MLGS, Montano Lab Gene Sets are based on Pub-Med searches.

androgen-responsive cell lines (38). Our observations that testosterone administration up-regulates AR expression are consistent with previous data demonstrating AR up-regulation in muscle biopsies of healthy men treated with testosterone (9, 39, 40) and in C3H 10 T1/2 multipotent cells treated with testosterone or DHT. The results of this study represent the first demonstration that these genes are regulated early during testosterone treatment in the human skeletal muscle from HIV-infected men.

In addition, genes involved in structural components of muscle and the extracellular matrix (ECM) were up-regulated by testosterone treatment; muscle accretion would be expected to be associated with changes in the ECM and structural proteins. Changes in the ECM have been described as being critical mediators of mesenchymal stem cell activity, possibly within the stem cell niche (41). The gene expression profiles were studied only in the vastus lateralis; we do not know whether changes in gene expression profile are similar or different in other muscle groups. The extent to which the stem cell microenvironment is potentially influenced by androgens, as well as distinct cellular subsets within muscle tissue, will require finer analysis in future studies.

The activation of immune response genes in association with androgen treatment is provocative. We observed up-regulation of the following: macrophage-associated chemoattractants *CCL5* (*RANTES*), *CXCL9* (*MIG*), and *CXCL-10* (*IP-10*); the macrophage marker CD14; activation markers *CD83*, *CD53*, and *CD44*; and signaling intermediates *IL6R*, *IL6ST*, and *IL-18* (Fig. 3, B–D). In the aging comparison, both datasets identified six overlapping genes associated with immune response (CD14, CCL5/RANTES, and PSMB8/Macropain) and muscle growth (SDC2/Syndecan, SLC14A/amino acid transporter, and FN1/Fibronectin) (Fig. 5B). Although there was no apparent effect on CD4 count or viral load, these data suggest a common theme of immune regulation and myogenic feedback in conditions associated with loss of muscle mass and will require direct evaluation of immune markers on lymphoid and myeloid subsets in future

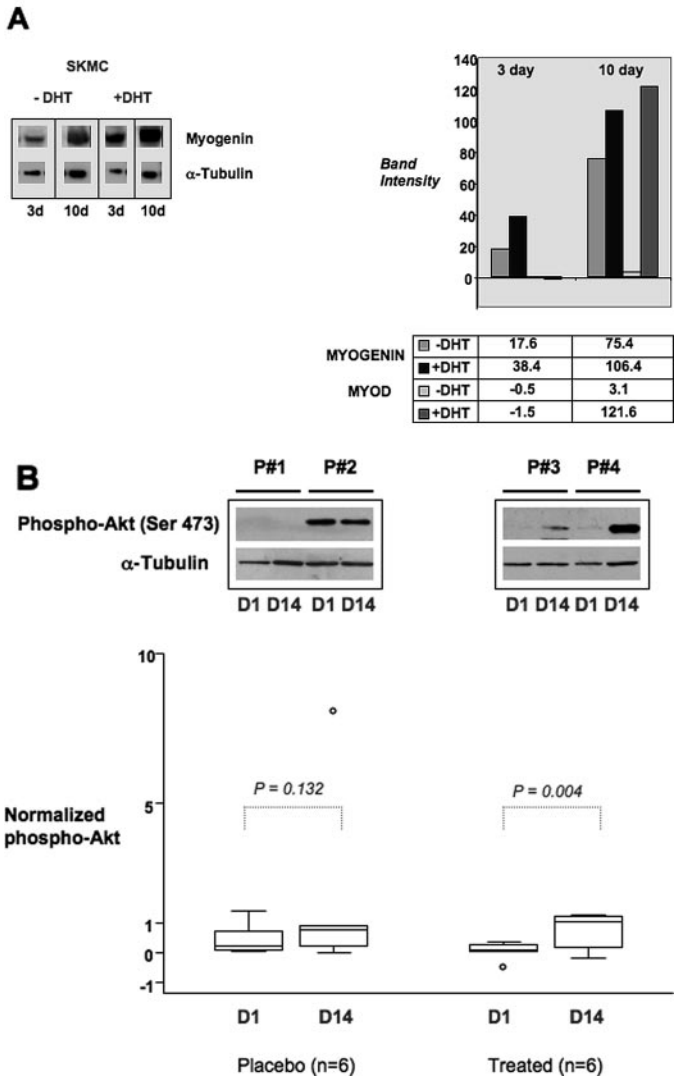


FIG. 4. Expression of myogenic proteins in human SkMCs treated with DHT and in muscle biopsies. A, Protein expression of myogenin and MyoD were measured in Western blots from SkMCs treated with DHT or placebo for the indicated times (3 and 10 d), normalized background intensity, and displayed in bar graph with numeric table below and α-tubulin reference. There were no obvious differences in cell morphology or density under phase microscopy (data not shown). B, Western blot determination of phospho-Akt in skeletal muscle biopsies in 12 subjects (six placebo, six treated) randomly selected from the study population based on sample availability. In most cases, samples did not overlap with array samples. Paired d 1 (baseline) and d 14 box plots are shown, normalized to α-tubulin levels. The placebo specimens did not differ significantly ($P = 0.132$), whereas treated subjects were significantly up-regulated at d 14 ($P = 0.004$). Representative blots are shown above box plots for two placebo subjects (P#1, P#2) and two testosterone-treated subjects (P#3, P#4), wherein placebo specimens did not display time-dependent changes in contrast to up-regulated specimens from the testosterone group.

studies. These data are consistent with a growing body of evidence for muscle repair through cytokine-mediated signaling and crosstalk with muscle cells to promote myogenesis (42–45).

Results in this study suggest that short-term treatment with testosterone positively influences lean muscle mass and myogenic gene expression in skeletal muscle. It remains unclear what effects altered dose schemes or longer-term use of androgens might have on muscle. However, adverse events associated with longer-term use of such drugs limit their use in future studies and require the development of novel agonistic compounds. There is a strong motivation to develop selective androgen receptor modulator agonists (46, 47) that promote muscle mass accretion but do not promote androgen-associated cancers (such as prostate cancer). Because accumulating evidence also supports a role for macrophage immune modulators in myogenesis, the identification of specific initiators of myogenesis mediated by immune factors could provide new targets for the design of what may be termed selective immunomyogenic modulators. A better understanding of the overlap between selective androgen receptor modulator/selective immunomyogenic modulator gene networks and myogenic differentiation represents an important new area worthy of additional investigation. The potential for new treatment modalities for muscle wasting in clinical scenarios, such as HIV infection, muscular dystrophy, and sarcopenia, collectively represent compelling incentives to understand the precise signaling intermediates that guide muscle remodeling and stem cell commitment.

TABLE 2. Selected genes were evaluated for quantitative RNA expression using quantitative RT-PCR

Probe ID	Gene ID	Placebo (n = 8)	Treated (n = 8)
211110_s_at	*AR*	0.57	0.94
212764_at	*TCF8*	0.63	0.70
208859_s_at	*ATRX*	0.35	0.84
209060_x_at	*NCOA3*	0.46	0.84
212332_at	*RBL2*	0.16	0.94
213891_s_at	*TCF4*	0.56	0.95

We also detected expression of phosphorylated p38 MAPK in all HIV-infected subjects and increased phosphorylation of Akt in the testosterone-treated subjects. Based on results in this study, it is possible that HIV infection itself may contribute to the overall level of activated p38 MAPK in muscle as a stress response, an effect that has been described previously in other cell types (48, 49). Notably, we also observed specific up-regulation of phospho-Akt in the testosterone-treated subjects. Activation of Akt by phosphorylation has been demonstrated to be sufficient to induce muscle hypertrophy, potentially through up-regulation of protein synthesis (25) and has been implicated in the inhibition of downstream targets for p38 MAPK-associated atrophy signaling pathways (24, 50, 51). A more detailed analysis of target genes for p38 and pAkt in larger sample sets and in comparison with HIV-negative subjects will be required to more specifically address the relative roles of these regulators.

In contrast to some previous studies that evaluated changes in selected genes after a more extended treatment duration, we obtained muscle biopsies 2 wk after starting testosterone therapy with the presumption that the important regulatory pathways that contribute to muscle fiber hypertrophy would be activated early in the course of treatment. In fact, it is possible that, once a new steady state with respect to muscle mass has been achieved, the changes in regulatory pathways may revert back to baseline by the end

A

DATABASE	Categories present in both	HIV positive men Androgen / Placebo	HIV negative men Young / Old
System	**Gene Category**	**Probability**	**Probability**
GO Molecular Function	*carbohydrate binding*	5.87E-06	5.87E-02
GO Molecular Function	*cation binding*	2.70E-03	2.61E-03
GO Molecular Function	*copper ion binding*	1.37E-02	1.57E-02
GO Molecular Function	***extracellular matrix structural constituent***	9.94E-13	7.87E-03
GO Molecular Function	*glycosaminoglycan binding*	3.14E-08	6.82E-04
GO Molecular Function	*heparin binding*	1.35E-06	2.57E-04
MLGS	*HIV Associated*	6.69E-04	3.44E-04
GO Molecular Function	***insulin-like growth factor binding***	4.33E-03	2.60E-02
GO Molecular Function	*ion binding*	9.57E-03	3.49E-03
GO Molecular Function	*isomerase activity*	3.06E-02	5.27E-02
GO Molecular Function	*metal ion binding*	9.57E-03	3.49E-03
GO Molecular Function	*pattern binding*	1.05E-07	4.06E-04
GO Molecular Function	*polysaccharide binding*	7.14E-08	3.01E-04
GO Molecular Function	*racemase and epimerase activity*	1.16E-02	4.02E-02
GO Molecular Function	***structural constituent of muscle***	1.32E-02	3.20E-02

B

CCL5: The cytokine RANTES encoded by this gene functions as a chemoattractant for blood monocytes.

CD14: CD14 is a surface protein preferentially expressed on monocytes/macrophages.

FN1: Fibronectin is involved in cell adhesion, wound healing, host defense, and metastasis.

PSMB8: Macropain is induced by gamma interferon and is within the immuno-proteasome.

SDC2: The syndecans mediate cell binding, cell signaling, and cytoskeletal organization and the syndecan-2 protein functions as an integral membrane protein and participates in cell proliferation.

SLC1A4: solute carrier family, glutamate/neutral amino acid transporter

FIG. 5. Comparison of enriched gene sets in skeletal muscle of testosterone-treated HIV-positive men and a separate dataset obtained from young *vs.* elderly healthy men. A, Gene probes present in this dataset (10,379) and an existing sarcopenia dataset (GSE362) including younger (21–27 yr) and older (67–75 yr) were evaluated for differential expression and gene category enrichment. Shown are the 15 gene categories present in both datasets with their associated probabilities (all $P < 0.05$). Note the category HIV Associated. B, Biological function of the six genes common to both datasets within the category: HIV Associated. A total of 54 genes were differentially expressed in both datasets (supplemental Table 4). MLGS, Montano Lab Gene Sets are based on Pub-Med searches.

of the 16-wk treatment period, which was not evaluated by microarray analysis in this investigation.

Human studies such as these that are based on analyses of muscle biopsies have some inherent limitations. Because of the burden imposed on the participants, only limited time points can be evaluated. Thus, the changes in gene expression observed in muscle biopsies obtained 2 wk after treatment should not be extrapolated to other time points. The exact timing of testosterone activation of target genes will require additional investigation in longitudinal studies. Although the Affymetrix microarrays contained more than 20,000 markers, they did not include all human genes. Therefore, it is possible that additional genes regulated by testosterone might have been missed because they were not represented on these microarrays. As previous experience has shown, the microarray analysis can be very useful in generating novel hypotheses and providing new leads that might not be apparent from analysis of specific candidate gene expression studies. However, microarray analysis can only unveil associations but cannot establish cause and effect relationships. Although we observed a clear dichotomy in expression between the two groups, evidence for coordinately expressed differences in expression patterns for selected genes will require validation. The subjects were receiving a wide range of antiretroviral drugs and the small sample did not allow meaningful evaluation of the effect of antiretroviral drugs. Although there was no obvious effect on viral load in the placebo- compared with testosterone-treated groups, the potential interactive role of androgens and antiretroviral drugs on gene expression remains unclear and will require careful evaluation in future studies.

The expression profile observed in this study may have larger relevance for the role of androgens in muscle remodeling, based on the similarity in regulated gene sets that we observed in the aging-associated sarcopenia dataset (Fig. 5). The congruent gene activity in both datasets may point toward a link between immune cells and myogenic signaling in the pathogenesis of aging-associated sarcopenia. The observed effects of testosterone on multiple pathways unveiled by this study may help to explain why the use of androgen deprivation therapy in treatment of prostate cancer and other conditions has been associated with various adverse conditions, including diabetes (52), cardiovascular disease (52), risk of bone fracture (53), and osteoporosis and obesity (54).

The possibility that gene networks activated by testosterone may also be involved in other conditions such as aging that are associated with loss of skeletal muscle mass warrants additional investigation and should help in therapies designed to measure correlated improvements in strength and physical performance.

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Address all correspondence and requests for reprints to: Monty Montano, Ph.D., 650 Albany Street, EBRC 640, Boston University School of Medicine, Boston, Massachusetts 02118. E-mail: mmontano@bu.edu.

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