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# Androgen receptor agonism promotes an osteogenic gene program in preadipocytes

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## Abstract

Androgens regulate body composition by interacting with the androgen receptor (AR) to control gene expression in a tissue-specific manner. To identify novel regulatory roles for AR in preadipocytes, we created a 3T3-L1 cell line stably expressing human AR. We found AR expression is required for androgen-mediated inhibition of 3T3-L1 adipogenesis. This inhibition is characterized by decreased lipid accumulation, reduced expression of adipogenic genes, and induction of genes associated with osteoblast differentiation. Collectively, our results suggest androgens promote an osteogenic gene program at the expense of adipocyte differentiation.

### Keywords

Adipocyte; Osteoblast; Androgen receptor; Gene regulation

## 1. Introduction

The mechanisms by which androgens modulate fat distribution and bone density are clinically important, especially in an aging population where declining testosterone levels are prevalent [1]. Although a risk factor for type 2 diabetes mellitus, obesity is demonstrated to protect against bone loss [2–5]. Recent studies, however, have demonstrated a negative correlation between visceral fat accumulation and bone density, suggesting fat distribution may predict bone density and insulin resistance [6–9]. The interaction between visceral fat accumulation and bone mineral density remains poorly understood; yet, the mechanisms are important to improve healthspan in an aging world population.

Adipocytes and osteoblasts share a common mesenchymal stem cell (MSC) origin [10] with increasing evidence of transdifferentiation between cell types [11,12]. Accordingly, any factor controlling the balance between adipogenesis and osteogenesis in MSCs represents a potential regulator of body composition.

An early event in human adipocyte differentiation is the upregulation of *AR* expression, which opposes fat cell differentiation in an androgen-dependent manner [13]. In order to investigate androgen regulation of preadipocyte commitment more comprehensively, we



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created a 3T3-L1 cell line stably expressing full-length AR and analyzed the gene programs regulated by the androgen/AR axis.

## 2. Materials and methods

### 2.1. Cell culture, differentiation, and preparation of stable cell lines

3T3-L1 cells were maintained at 5% $CO_2$/37 °C in DMEM/F12 (Invitrogen) with 10% fetal bovine serum (FBS; Gemini Bio-Products), 100 U/ml penicillin, and 100 μg/ml streptomycin. Postconfluent cells were differentiated with 5 μg/ml insulin, 1 μM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine in DMEM/F12 medium containing 10% FBS (DMI). After 48 h, the medium was changed to DMEM/F12 containing 10% FBS and 5 μg/ml insulin. Subsequently, the culture medium was replaced with DMEM/F12 containing 10% FBS every 48 h.

Flag-tagged human AR (fAR) was stably expressed at physiologically relevant levels in 3T3-L1 preadipocyte cells using lentivirus, as previously described [13]. Stable clones were selected in puromycin after single cell dilution.

### 2.2. Oil Red O staining

After differentiation, media was removed and 10% formalin was added for 5 min. Formalin was removed and a second volume of 10% formalin was added to wells for 1 h. Wells were then washed with 60% isopropanol and allowed to dry. Oil Red O (2 g/L) was applied 10 min, followed by extensive washing with distilled water. All steps were performed at room temperature. Images were acquired using a digital camera.

### 2.3. Antibodies and western blotting

Western blot analysis was performed with whole cell lysates run on 4–12% Bis-Tris NuPage® (Invitrogen) gels and transferred onto Immobilon-P Transfer Membranes (Millipore). After membrane blocking (SuperBlock, Pierce), primary antibodies (anti-AR rabbit polyclonal, Santa Cruz Biotechnology) were incubated overnight at 4 °C, followed by secondary antibodies for 1 h at room temperature. Immunoreactive bands were visualized by chemiluminescence. β-actin (mouse monoclonal, Sigma Chemical Co.) was used as the invariant control.

### 2.4. RNA extraction and qPCR analysis

RNA was extracted from cells using the RNeasy kit (Qiagen) following manufacturer instructions. To measure relative mRNA expression, qPCR was performed using the Taqman RT-PCR one-step master mix in conjunction with an ABI 7500 real-time PCR system (Applied Biosystems). Each sample was tested in duplicate in two independent experiments. β-actin was used as the invariant control. The following primer and probes (Roche Universal Probe Library) were used:

*AR*: 5′-tgtcaactccaggatgctctact-3′; 5′-tggctgtacatccgagacttg-3′. Probe: 5′-6-FAM-ttcaatgagtaccgcatgc-BHQ-3′.

FABP4: 5′-ggatggaaagtcgaccacaa-3′; 5′-tggaagtcacgcctttcata-3′. Roche probe #77.

*CEBPB*: 5′-aagatgcgcaacctggag-3′; 5′-cagggtgctgagctctcg-3′, Roche probe #67.

*Pref1*: 5′-cgggaaattctgcgaaatag-3′; 5′-tgtgcaggagcattcgtact-3′, Roche probe #80.

PPARG: 5′-gaaagacaacggacaaatcacc-3′; 5′-gggggtgatatgtttgaacttg-3′, Roche probe #7.

*CEBPA*: 5′-aaacaacgcaacgtggaga-3′; 5′-gcggtcattgtcactggtc-3′, Roche probe #67.

*Ank*: 5′-tcaccaacatagccatcgac-3′; 5′-actgcatcctccttgactgc-3′; Roche probe #32.

*ENPP1*: 5′-cggacgctatgattccttaga-3′; 5′-agcacaatgaagaagtgagtcg-3′; Roche probe #72.

*Notch*: 5′-ctggaccccatggacatc-3′; 5′-aggatgactgcacacattgc-3′; Roche probe #80.

*SPP1*: 5′-cccggtgaaagtgactgatt-3′; 5′-ttcttcagaggacacagcattc-3′; Roche probe #82.

*Chrd*: 5′-tcactgcccacctccttg-3′; 5′-atcttttaccacgccctgag-3′; Roche probe #66.

*Cyr61*: 5′-ggatctgtgaagtgcgtcct-3′; 5′-ctgcatttcttgccctttt-3′; Roche probe #66.

### 2.5. DNA microarrays

Microarrays were performed by the Baylor College of Medicine Microarray Core Facility using Affymetrix Mouse Gene 1.0 ST arrays (Affymetrix). 3T3-L1 cells stably expressing human AR were differentiated in the presence or absence of 10 nM R1881 for 24 h, followed by total RNA isolation. All RNA samples were analyzed with a Bioanalyser 2100 (Agilent Technologies) before microarray hybridization.

### 2.6. Microarray analysis

Normalized gene expression values were calculated by the Robust Multiarray Averaging method using R.2.10 and Limma 2.19 analysis package, as previously described [13]. The false discovery rate was controlled by Benjamini and Hochberg correction to account for multiple testing error.

### 2.7. Statistical analysis

Student's *t*-test was used for statistical analyses of qPCR data. A *p*-value cutoff of 0.05 was used to determine significance.

## 3. Results

### 3.1. Exogenous expression of AR is required for androgen signaling in 3T3-L1

*AR* mRNA is latently expressed during 3T3-L1 adipogenesis, leading to limited androgen responsiveness in these cells [13–15]. We investigated the relationship between *AR* and 3T3-L1 adipogenesis by measuring relative mRNA levels of *AR* and *aP2* between days 0 and 8 after dexamethasone/IBMX/insulin (DMI) induction, representing preadipocytes and mature adipocytes, respectively. *AR* was weakly expressed in days 1, 2, and 3, reaching highest levels in days 6 through 8. As a reference, *aP2* was upregulated 900-fold at day 6 while *AR* exhibited 6-fold induction (Fig. 1A), consistent with reported expression patterns [14]. To test the effect of androgens on 3T3-L1 adipogenesis, we added DHT or R1881 to 3T3-L1 cells beginning on day 0 of induction. In contrast to a previous report [16] and consistent with our recent findings [13], these ligands did not inhibit 3T3-L1 adipogenesis (Fig. 1A). We also evaluated *AR* expression levels in 3T3-L1 cells and mouse primary fat tissues (Fig. 1C). *AR* was expressed at very low levels in differentiated 3T3-L1 cells (day 8) compared to subcutaneous fat, epididymal fat, retroperitoneal fat and brown fat. Based on these results, we reasoned low levels of AR expression in 3T3-L1 prevented an inhibitory effect of androgens on adipogenesis.

Next, we generated 3T3-L1 stable cell lines constitutively expressing Flag-tagged human AR (fAR). Briefly, 3T3-L1 cells were infected with lentivirus encoding fAR with verification of AR protein levels by Western blotting (Fig. 1D). AR was detected in pooled 3T3-L1 stable cells, single stable 3T3-L1 clone #14, and LNCaP cells. Clone #25 showed a weak band, indicating a low level of AR expression. AR protein in parental 3T3-L1 cells was undetectable, in agreement with the androgen-refractory nature of these cells.

Subsequently, Clones #14 and #25 were induced to differentiate for 8 days in the presence of vehicle (EtOH), 10 nM R1881 or 1 μM hydroxyflutamide (OHFL), an AR antagonist, to test for androgen responsiveness (Fig. 1E). Conventional adipocyte differentiation conditions (EtOH) promoted adipogenesis in both clones. However, differentiation of Clone #14 cells was completely blocked by R1881, but not by OHFL. In contrast, adipogenesis in Clone #25 cells was not affected by either androgen or anti-androgen (OHFL). These results indicated adipogenesis of Clone #14 cells was inhibited by AR activation.

### 3.2. Androgen alters adipogenic genes when AR is expressed in 3T3-L1 cells

Adipogenic stimuli activate C/EBPβ, C/EBPδ and glucocorticoid receptor (GR) to induce genes encoding PPARγ and C/EBPα [17–20]. Marking the commitment phase, early activation of C/EBPβ, C/EBPδ and GR during 3T3-L1 differentiation has been shown to repress expression of the preadipocyte marker *Pref-1* [21–23] Subsequently, PPARγ mRNA and protein expression are robustly induced in a feed-forward loop with C/EBPα to induce or repress adipose-specific genes [24,25].

We measured the expression of several adipogenic marker genes by qPCR to determine whether reduced Oil-Red-O staining in R1881-treated cells (Fig. 1E) resulted from androgen/AR-mediated alterations in the 3T3-L1 differentiation program. Stable expression of fAR in 3T3-L1 (Clone #14: fAR cells) followed by differentiation in the presence of 10 nM R1881 significantly reduced the induction of adipogenic marker genes, including *PPARγ* (Fig. 2A), *C/EBPα* (Fig. 2B), and *aP2* (Fig. 2C).

Upregulation of *C/EBPβ* (Fig. 2D) and *C/EBPδ* (Fig. 2E) by dexamethasone in the early stages of adipocyte differentiation [17,18] was abrogated by R1881 treatment as early as 2 h post DMI treatment. However, the kinetics of *Pref-1* downregulation (Fig. 3F) were unaffected under these conditions, suggesting androgen/AR signaling did not block the differentiation program through maintenance of the preadipocyte state [21–23].

### 3.3. Androgens promote an osteogenic gene signature in 3T3-L1 expressing AR

Our data suggested stable expression of AR in 3T3-L1 cells (fAR cells) inhibited adipocyte differentiation in an androgen-dependent manner. We next characterized the androgen-dependent transcriptome in fAR cells. Three treatment groups were used to determine the effect of androgen on gene expression: vehicle (no induction); DMI+EtOH (ethanol, E); and 10 nM R1881+DMI (R1881). fAR cells were treated for 24 h, followed by RNA isolation, microarray, and follow-up qPCR analysis. We detected 2578 probe sets (Supplemental File 1) differentially expressed (2-fold; $q < 0.05$) in three contrast groups (Fig. 3A): probe sets changed after 24 h in response to DMI (E v. NT), 10 nM R1881+DMI (R v. NT), or the difference between E v. NT and R v. NT (R v. E). We hypothesized the last contrast (R v. E) identified a subset of genes uniquely regulated by androgen during adipogenesis (Fig. 3B).

We next used gene ontology (GO) analysis to identify biological pathways enriched in each treatment group. Comparison of biological pathways altered by DMI+EtOH (E) or DMI +R1881 (R) relative to undifferentiated fAR cells demonstrated enrichment of mitosis-related biological processes (Fig. 3C), reflecting the requirementin 3T3-L1 cells of mitotic clonal expansion prior to terminal differentiation [26]. However, when we compared DMI +EtOH (E) to DMI+R1881 (R), three GO biological process categories associated with bone development were significantly enriched (Fig. 3D): regulation of ossification, osteoblast differentiation, bone development.

To study the bone phenotype at the gene expression level, we selected genes for qPCR analysis which exhibited the most significant fold change between DMI+EtOH (E) and DMI +R1881 (R) treatments: *Ank* (regulation of ossification), *Cyr61* (osteoblast differentiation),

*Chrd* (regulation of ossification, osteoblast differentiation), *Enpp1* (regulation of ossification), *Notch1* (osteoblast differentiation), and *Spp1* (osteoblast differentiation). For qPCR validation, fAR cells were treated for 48 h with vehicle (EtOH), DMI, 10 nM R1881, or 10 nM R1881+DMI. DMI treatment alone repressed expression of *Ank* (Fig. 4A), *Cyr61* (Fig. 4B), *Chrd* (Fig. 4C) and *Spp1* (Fig. 4F). *Enpp1* (Fig. 4D) and *Notch1* (Fig. 4E), genes associated with 'regulation of ossification' and/or 'osteoblast differentiation' were unchanged by DMI treatment alone. In the presence of R1881 alone, or in combination with DMI, three patterns of response were observed when compared to the vehicle control. Genes were either (i) not repressed by androgen treatment *(Ank);* (ii) induced by androgen independently of DMI *(Cyr61, Chrd, Enpp1, Notch1);* or (iii) more significantly repressed by R1881 *(Spp1).* In summary, our gene expression analysis established androgen treatment in fAR cells promoted an osteoblast phenotype at the expense of adipogenesis.

## 4. Discussion

The role of androgens and AR has expanded from reproductive tissues to multiple endocrine targets, including bone and adipose tissue [27]. Clinically, testosterone deficiency in aging men is associated with accumulation of central adiposity, decreased bone mineral density and type 2 diabetes mellitus [28]. Indeed, testosterone therapy has been shown to improve insulin sensitivity, reduce visceral fat mass, plasma triglycerides, cholesterol and fasting blood glucose levels, and increase bone density [29–31]. These facts underscore the importance of androgens in modulating energy balance and body composition.

We initially sought to characterize the roles of AR and androgen during 3T3-L1 preadipocyte differentiation. Consistent with previous data [14], we observed upregulation of *AR* late in the differentiation process, correlating with induction of the mature adipocyte marker, *aP2.* However adipogenesis was not affected by R1881. While AR modulates mature adipocyte function [32], our findings collectively suggested a limited role of AR and androgens in 3T3-L1 differentiation. Supporting this notion, *AR* mRNA levels in 3T3-L1 cells were extremely low compared to primary fat depots, consistent with time-resolved expression of nuclear receptors during 3T3-L1 adipogenesis [14,15], microarray profiling [33], and our recent work [13].

C/EBPβ, C/EBPδ, and GR co-operatively induce *C/EBPα* and *PPARγ* mRNA to initiate adipogenesis [17–20]. Then, C/EBPα and PPARγ coordinately activate the transcription of adipocyte-specific genes [25,26,34]. 3T3-L1 cells stably expressing fAR (clone #14) showed reduced mRNA levels for key adipogenic transcription factors, *PPARγ* and *C/EBPα*, and concomitant reduction of the adipocyte marker *aP2* (Fig. 2). Other adipocyte-determining transcription factors, including *C/EBPδ* and *C/EBPβ*, were less affected.

Previous studies have shown dexamethasone drives preadipocytes toward a novel intermediate cellular state through transient activation of C/EBPβ and C/EBPδ, leading to repression of *Pref-1* [21–23]. In this study, downregulation of *Pref-1* was not affected by androgen treatment, suggesting activation of C/EBPβ and C/EBPδ was intact. Collectively, these results indicate androgens block adipogenesis prior to the induction of *PPARγ* and *C/EBPα*, but downstream of *Pref-1* repression by dexamethasone.

Transcriptomic profiling of short-term (24 h) treatment with the synthetic androgen R1881 treatment on differentiating 3T3-L1 cells stably expressing fAR (Fig. 4) showed unique pathways enriched upon gene ontology analysis. When we compared R1881 and differentiation (EtOH) treatments to undifferentiated cells, expected pathways associated with mitotic clonal expansion [26] were enriched: M phase, mitotic cell cycle, cell cycle process. However, ontology analysis of genes differentially expressed between fAR cells

differentiated in the presence of R1881 or differentiated in the presence of vehicle, showed enrichment of processes associated with bone development and osteogenesis.

Validation of key genes in each of these ontology categories substantiated the positive effect of androgens on bone differentiation. *Ank* [35,36], *Chrd* [37,38], *Cyr61* [39,40], *Enpp1* [35,41,42], *Notch1* [43,44], and *Spp1* [45] represent genes marking osteoblast function and bone remodeling, each showing regulation by R1881 alone or in combination with DMI. Collectively these findings position androgen signaling in preadipocytes as a mediator of osteogenic gene transcription.

Osteoblasts and adipocytes arise from a common mesenchymal stem cell (MSC) origin, as illustrated by the ability of MSCs to differentiate into either lineage [10]. Thus, we propose AR modulation may mediate the negative relationship between visceral fat and bone [7] to improve body composition.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.03.078.

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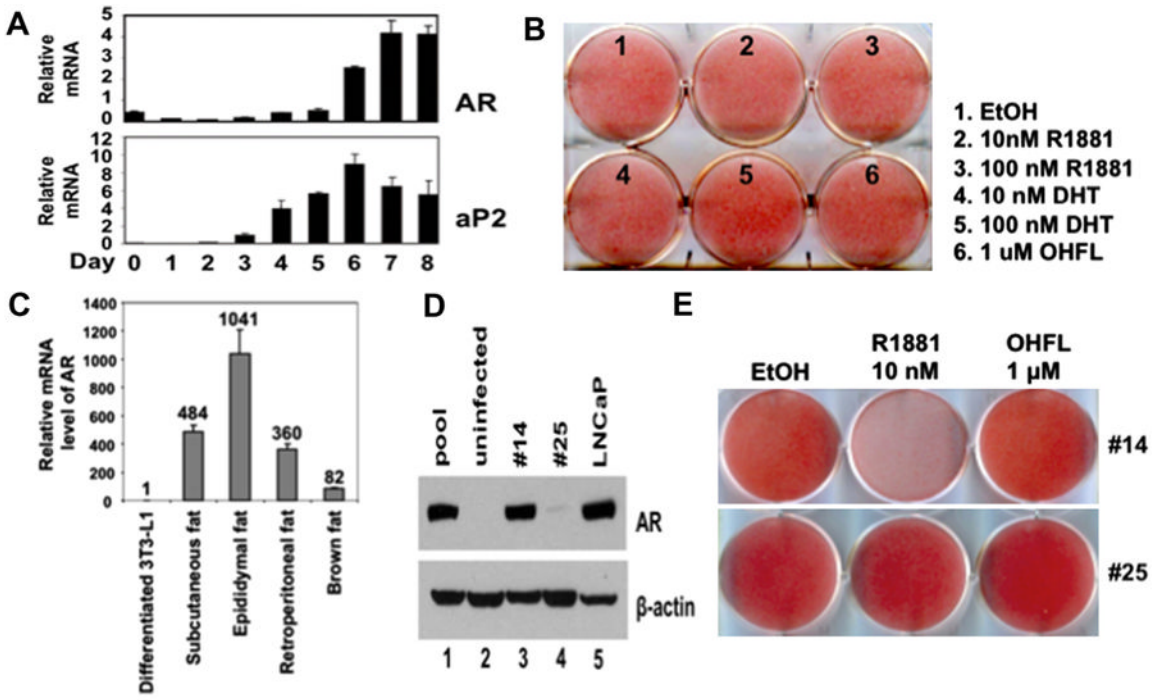


**Fig. 1.**

AR expression and action in mouse adipocytes. (A) Induced *AR* and *aP2* mRNA levels in wild-type 3T3-L1 cells during adipogenic differentiation. (B) Wild type 3T3-L1 cells were treated with adipocyte differentiation cocktail in the presence of androgens for 8 d followed by Oil Red O staining. (C) Relative *AR* mRNA for *in vitro* differentiated 3T3-L1 cells and murine adipose tissues. (D) 3T3-L1-AR stable cell lines were created by infecting 3T3-L1 cells with lentivirus expressing flag-tagged AR. Western blot comparison of AR protein levels in pooled 3T3-L1 infected with fAR lentivirus and uninfected 3T3-L1 with 3T3-L1-fAR stable clones #14 and #25. LNCaP, a prostate cancer cell line with endogenous AR expression, was used for comparison of relative AR expression levels. (E) 3T3-L1 fAR stable clones #14 and #25 were differentiated in the presence of ethanol (EtOH), 10 nM R1881, or O-hydroxyflutamide (O-HFL) for 8 d. Oil-Red-O was used to determine effects of hormones on adipogenesis in both clones.

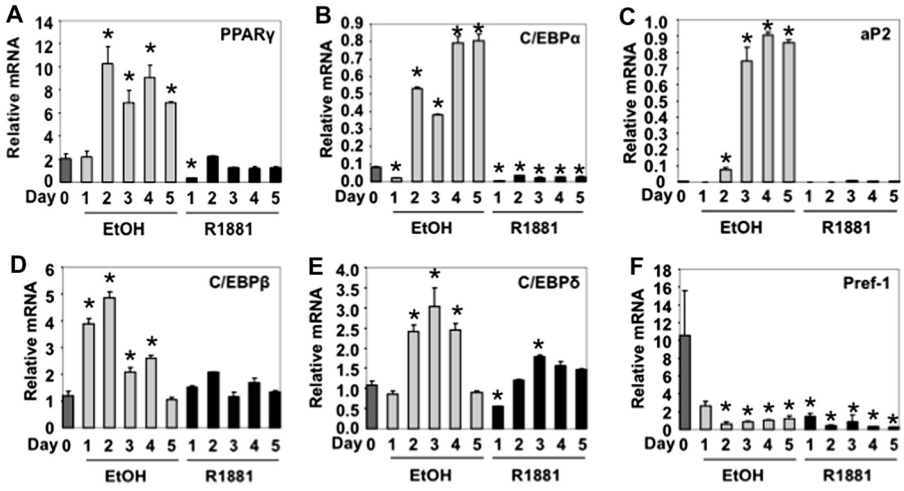


**Fig. 2.**

Effect of androgen treatment on the expression of key genes in adipogenesis using 3T3-L1-fAR stable cell line (#14). 3T3-L1 fAR cells were differentiated for up to 5 days in the presence or absence of 10 nM R1881. qPCR was used to measure mRNA expression for (A) *PPARγ*, (B) *C/EBPα*, (C) *aP2*, (D) *C/EBPβ* (E) *C/EBPδ*, and (F) *Pref-1.* All results are $n = 2 \pm$ S.E.M. Asterisks indicate mRNA statistically different from the vehicle control (*$p < 0.05$).

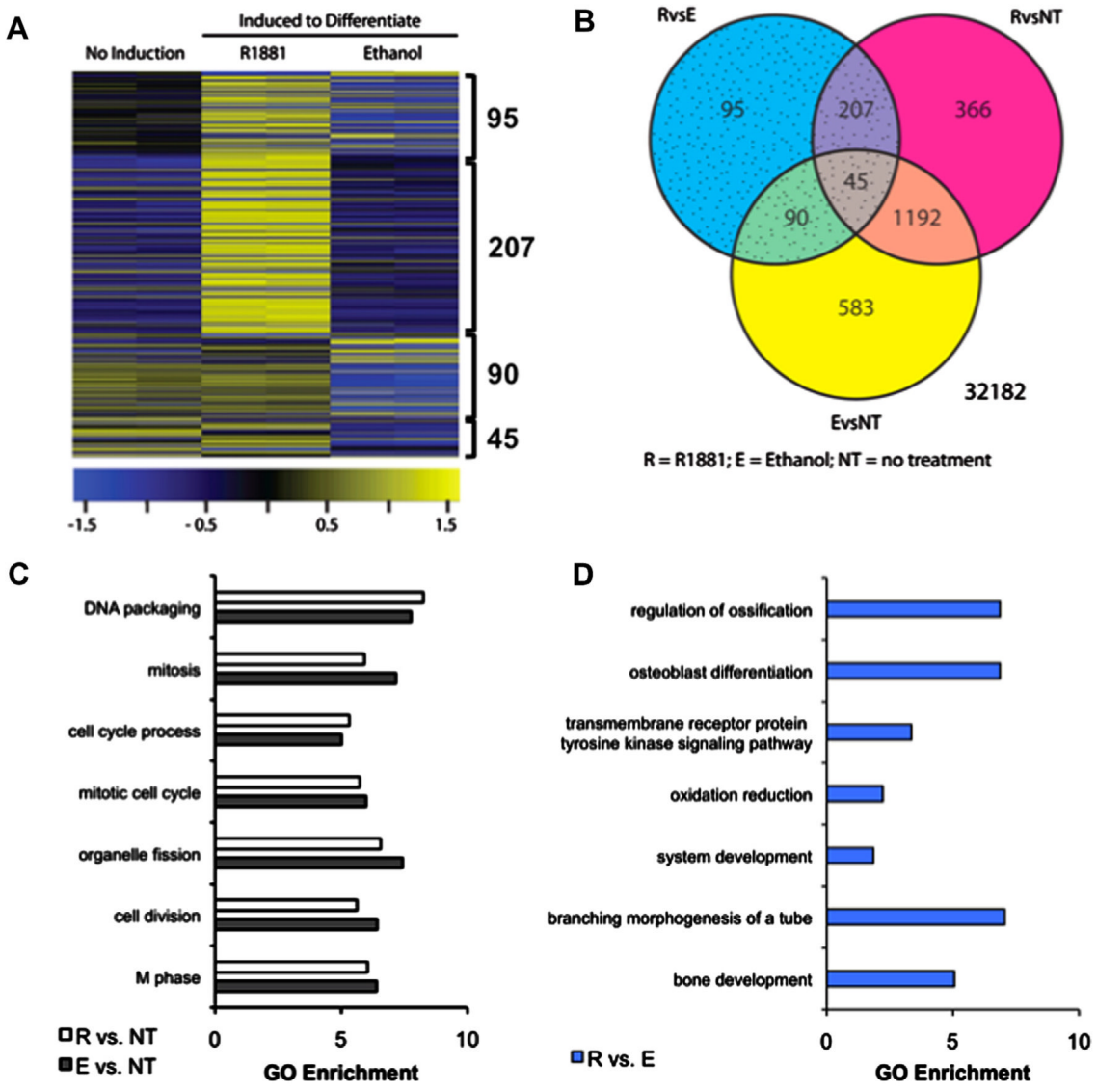


**Fig. 3.**

Androgen regulation of genome-wide gene expression during adipogenesis in 3T3-L1-fAR cells. Confluent 3T3-L1-fAR cells (clone #14) were induced to differentiate for 24 h in the presence of 10 nM R1881 (R), EtOH (E) or were left undifferentiated (no treatment; NT). (A) Shown are array probes significantly different between cells differentiated in the presence or absence of androgen; FC > 2; *q*-value < 0.05. Significantly changed probe sets in the R v. E contrast are highlighted. (B) Genes shared and unique to each treatment group represented by Venn diagram. (C) Gene Ontology (GO) analysis of R1881 (R) and ethanol (E) treatments versus NT controls showed no pathways were differentially enriched. (D) Further GO analysis of genes between R and E showed pathways associated with bone differentiation significantly enriched. Genes with FC > 2 and FDR < 0.05 were used for GO analysis.

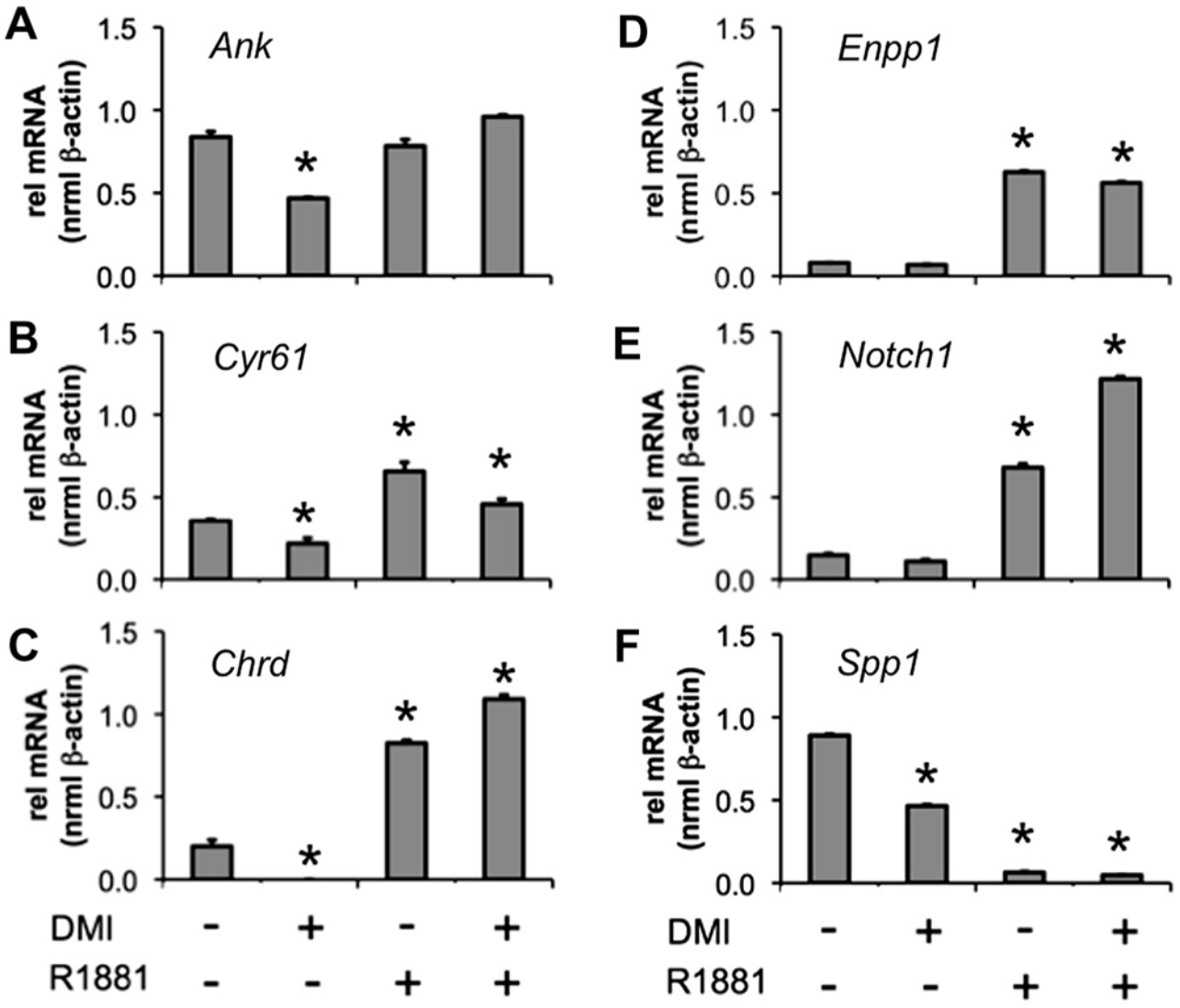


**Fig. 4.**

Androgen promotes expression of genes involved in osteogenesis. 3T3-L1 fAR cells were differentiated for 48 h in the presence or absence of 10 nM R1881. qPCR was used to measure mRNA expression for (A) *Ank*, (B) *Cyr61*, (C) *Chrd*, (D) *Enpp1*, (E) *Notch*, and (F) *Spp1.* All results are $n = 2 \pm$ S.E.M. Asterisks indicate mRNA statistically different from the vehicle control ($*p < 0.05$).