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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.

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 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

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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]