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Androgens induce sebaceous differentiation in sebocyte cells expressing a stable functional androgen receptor

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ABSTRACT

Androgens act through non-genomic and androgen receptor (AR)-dependent genomic mechanisms. AR is expressed in the sebaceous gland and the importance of androgens in the sebaceous function is well established. However, the *in vitro* models used to date have failed to evidence a clear genomic effect (*e.g.*, modification of gene expression profile) of androgens on human sebocyte cells. In order to study the impact of active androgens in sebocytes, we constructed a stable human sebocyte cell line derived from SEBO662 [17] constitutively expressing a fully functional AR. In these SEBO662 AR+ cells, dihydrotestosterone (DHT) induced AR nuclear translocation and the strong modulation of a set of transcripts (RASD1, GREB1 . . .) known to be androgen-sensitive in other androgenic cells and tissues. Moreover, we observed that DHT precociously down-regulated markers for immature follicular cells (KRT15, TNC) and for hair lineage (KRT75, FST) and up-regulated the expression of genes potentially related to sebocyte differentiation (MUC1/EMA, AQP3, FADS2). These effects were fully confirmed at the protein level. In addition, DHT-stimulated SEBO662 AR+, cultured in a low-calcium defined keratinocyte medium without serum or any complement, neosynthesize lipids, including sebum lipids, and store increased amounts of triglycerides in lipid droplets. DHT also induces morphological changes, increases cell size, and treatments over 7 days lead to a time-dependent increase in the population of apoptotic DNA-fragmented cells.

Taken together, these results show for the first time that active androgens alone can engage immature sebocytes in a clear lipogenic differentiation process (Graphical abstract). These effects depend on the expression of a functional AR in these cells. This model should be of interest for revisiting the mechanisms of the sebaceous function *in vitro* and for the design of relevant pharmacological models for drug or compound testing.

1. Introduction

In humans, sebaceous glands (SG) develop during the 13–15th weeks of gestation, from “bulges” on the developing hair follicles. Well developed at birth they then atrophy until puberty, when increased androgen output promotes a marked enlargement of the glands. Both basal and differentiating sebocytes in SG express the androgen receptor (AR) with a nuclear location and it is recognized that androgens are critical in the development and regulation of the sebaceous function [1]. An additional proof of the importance of androgens in SG function is that androgen-insensitive subjects who lack functional AR do not produce sebum and do not develop acne [2]. It has been shown that skin, especially through SG, is a steroidogenic tissue and that sebocytes are the key regulators of androgen homeostasis in human skin [3–4], suggesting a possible autocrine regulation of the sebaceous function in addition to systemic regulation [5].

Androgens act through the single nuclear receptor AR. When activated by androgens in the cytoplasm, the translocation of AR to the nucleus takes place, initiating androgen effects *via* genomic signaling [6–7]. These effects can be cell type-dependent and may also depend on a considered physio-pathological status [8]. In addition to direct AR-dependent effects, non-classical effects of

Abbreviations: AR, androgen receptor; SG, sebaceous gland; RASD1, RAS, dexamethasone-induced 1; GREB1, growth regulation by estrogen in breast cancer 1; CYP24A1, cytochrome P450, family 24, subfamily A, polypeptide 1; RT-qPCR, reverse-transcription-real-time-PCR; NHEK, normal human epidermal keratinocytes.

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testosterone have also been demonstrated in several tissues, in particular in the reproductive, cardiovascular, immune, and musculoskeletal systems [9].

After isolation from *e.g.*, androgen-sensitive prostate tumors and *in vitro* culture, only very few established cell lines keep a stable expression of AR and a spontaneous significant response to androgens [7]. It is thus possible that in many cases the absence of a clear response to androgens comes from a defect in functional AR expression in the considered models.

Increased cell volume, accumulation of lipid droplets in the cytoplasm, and nuclear degeneration are phenomena indicating terminal differentiation of human sebocytes followed by holocrine secretion and cell death [10–11].

In all previous works, testosterone alone failed to induce the full program of sebaceous differentiation and lipogenesis [12–14] and other coregulators for sebaceous lipogenesis such as peroxisome proliferator-activated receptors (PPARs) and their ligands have been identified as important for sebaceous function [15]. A clear transcriptional effect of androgens in sebocyte cell models *in vitro* has never been evidenced. Moreover, it has been shown that DHT did not induce apoptosis of the immortalized sebocyte cell line SZ95 [11] but stimulated its proliferation [12,16]. Other studies have reported androgen-mediated proliferative effects, but curiously with differences regarding the body site from which sebaceous glands were isolated [13]. Taken together, these data indicate a potential pro-proliferative differentiation-limiting effect of androgens on sebocytes, at least *in vitro* in the most studied immortalized cell line SZ95. Although a non-genomic mechanism cannot be excluded, these proliferative effects seem to be directly linked to AR function since AR interfering molecules such as siRNA slow them [16].

We recently immortalized a new human sebocyte cell line, SEBO662, able to grow in keratinocyte defined media, allowing phenotypic and functional comparison with keratinocytes [17]. SEBO662 cultured in defined kSFM or Epilife® low calcium keratinocyte media do not exhibit significant lipogenic differentiation, excepted when they are stimulated by complex lipogenic mixes or cultured as 3D-epithelia, at the air-medium interface [17]. As many human cell lines sub-cultured *in vitro*, SEBO662 express AR to a background level and do not respond to androgens. In order to highlight the potential impact of androgens in sebocyte cells, we constructed a stable SEBO662 AR+ cell line which constitutively expresses a fully functional AR. In this model the active androgen dihydrotestosterone (DHT), tested alone in basal (non-complemented) keratinocyte SFM medium, induces strong specific responses, including the expression of androgen-sensitive genes but also the induction of a clear sebocyte lipogenic differentiation program. The main effects of DHT on differentiation markers, lipogenesis, cell size/morphology, proliferation, and death are reported.

2. Materials and methods

2.1. Normal skin tissue and cells

The tissues used for AR mRNA profiling were obtained from aesthetic surgeries, within the context of a contract with the hospital of Poitiers (France). Skin samples and primary keratinocytes (NHEK) were obtained from breast surgeries; hair follicles and SG were microdissected from face lifts. NHEK and sebocyte cells were amplified and maintained in low calcium keratinocyte SFM (kSFM, Life Technologies, Saint Aubin, France) supplemented with 1 ng/ml epidermal growth factor (Life Technologies), 2 ng/ml keratinocyte growth factor (Millipore, Saint Quentin en Yvelines, France) and 25 μg/ml gentamycin sulfate (Sigma–Aldrich, Saint Quentin Fallavier, France). For AR mRNA profiling (Fig. 1A), the skin, hair, SG, and NHEK samples consisted in pools from 3 different donors.

2.2. SEBO662, construction of SEBO662 AR+, cell culture, and treatments

The E6/E7-immortalized sebocyte cell line SEBO662 was already described [17] and is referred as the control, "parent" or AR(−) cell line. For SEBO662 AR+ construction, low (4) passage number SEBO622 were seeded at 300,000 cells/75 cm^2 flask, then transduced with full length AR gene-expressing lentiviral vector. This experimental part was performed by Vectalys (Toulouse, France). Cells were then amplified in supplemented kSFM medium, characterized for AR expression and referred as the AR(+) cell line.

In all further testing conditions, the kSFM complements were fully removed, so the media only consisted in a low calcium kSFM base (complements used only in amplification and seeding/pre-treatment steps). AR(+) cells were used at passage 7 in the early characterization studies (Fig. 1) and at passages ranging from 15 to 25 in all the other experiments. DHT (Sigma–Aldrich) at the final concentration of 10 nM was used as the androgen stimulation. Unless otherwise stated, the cells were treated for 24 h for gene expression (mRNA) studies, 3 days and/or 7 days for protein and lipid analysis and up to 14 days for metabolism or apoptosis studies. Media were changed every 3 or 4 days.

2.3. mRNA analysis

For mRNA studies, cells were cultured at confluency, in triplicate, and treated or not (controls) with DHT for 24 h. RNA extraction, quality control, quantification, and RT-qPCR were performed as previously described [18]. Official gene names, abbreviations and primers for qPCR are listed in Table S1.

Whole cell transcriptome analysis was performed as recommended by Affymetrix (High Wycombe, Great Britain), using the hU219 chip and the GeneAtlas platform. All the reagents and devices were from Affymetrix. Quality controls were performed at each step of the process using an Agilent Bioanalyzer 2100 system (Massy, France). After CEL file generation and normalization, data were transferred into an EXCEL file. An overall representation of the different comparisons is given through dedicated scatter plots (Fig. 1C).

2.4. in situ *labeling of cell monolayers*

Cells were seeded at confluency (50,000 cells/well) in 96-well plates and treated or not with DHT for the indicated time. Different types of labeling were performed, using an automated InCell Analyzer 1000® bioimaging system (GE Healthcare, Orsay, France). Specific biomarker labels were coupled to nucleus counterstaining by blue fluorescent Hoechst 33842 dye (Sigma–Aldrich). All the primary antibodies, fluorescent probes and kits are described in Table S2.

For immunofluorescence labeling of protein markers, cell monolayers were fixed in 4% paraformaldehyde for 20 min, then permeabilized for 10 min with 0.1% Triton X-100 (Sigma–Aldrich) in phosphate-buffered saline solution, blocked for 1 h in 1% bovine serum albumin /0.05% Tween 20 (Sigma–Aldrich). The cells were then labeled using the selected primary antibody (Table S2), then revealed using a dedicated Alexa-coupled secondary antibody (Life Technologies), together with Hoechst dye.

Lipid (mainly triglyceride droplets) detection in fixed cell layers was performed by incubation with Bodipy dye (Sigma–Aldrich).