The orphan nuclear receptor NR4A1 regulates transcription of key steroidogenic enzymes in ovarian theca cells

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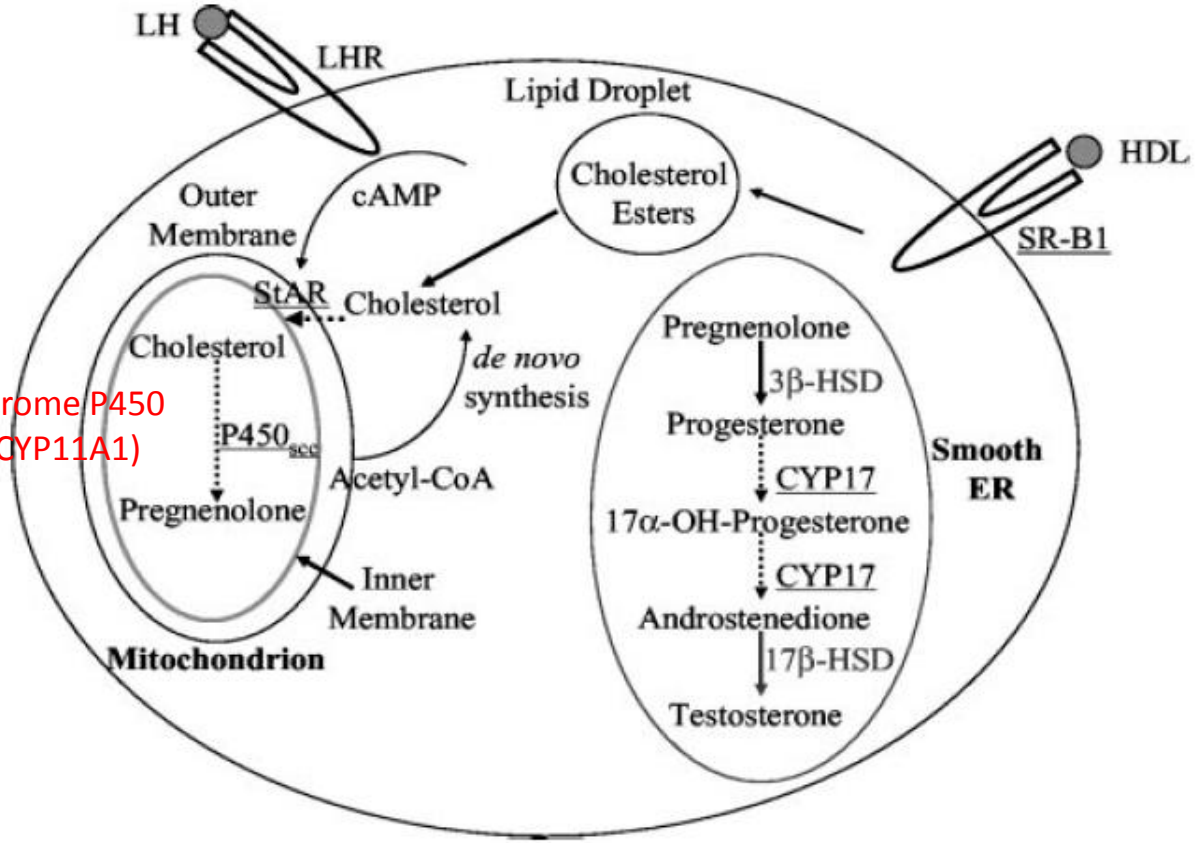
Introduction

❖ The ovarian follicle is composed of two unique types of steroidogenic cells:

- Granulosa Cells
  - Within the follicle surround the oocyte; their numbers increase directly in response to heightened levels of circulating gonadotropins or decrease in response to testosterone.
  - They also produce peptides involved in ovarian hormone synthesis regulation. Follicle-stimulating hormone (FSH) induces granulosa cells to express leutenizing hormone (LH) receptors on their surfaces; when circulating LH binds to these receptors, proliferation stops.
- Theca Cells
  - The endocrine cells associated with ovarian follicles that play an essential role in fertility by producing the androgen substrate required for ovarian estrogen biosynthesis. Theca cells differentiate from the interfollicular stroma in response to proteins secreted from growing follicles.

❖ Androgens are synthesized by primary theca cells in response to LH and then diffuse across the basement membrane to serve as immediate substrates for estradiol synthesis by granulosa cells in response to FSH.



Cytochrome P450 11A1 (CYP11A1)

FIG. 1. Testosterone biosynthesis in the rat testis. Cholesterol is produced *de novo* in the testis or acquired from plasma lipoproteins. StAR protein transports cholesterol from the outer to the inner mitochondrial membrane, where it is converted to pregnenolone by $P450_{SCC}$. Pregnenolone is converted to testosterone by a series of enzymatic reactions in the smooth endoplasmic reticulum. Genes previously shown to have diminished expression in the fetal testis after DBP treatment are *underlined*. Events or reactions affected by DBP treatment are shown with *broken arrows*.

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❖ The androgen-secreting theca cells express cytochrome P450 cholesterol side-chain cleavage enzyme (P450scc, CYP11A1), 17-hydroxylase; 17,20 lyase (CYP17) and 3-hydroxysteroid dehydrogenase (HSD3B2), all of which are key enzymes required for androgen biosynthesis.

❖ Androgens can be regulated by the expression of StAR protein which facilitates cholesterol entry into the mitochondria.

Introduction

❖Nuclear transcription factors participate in the transcriptional regulation of steroidogenic enzyme genes in endocrine tissues: SF- 1, GATA4, C/EBP, SREBP, COUP-TFII, SP1, CREB/CREM, members of the AP-1 family (c-FOS and c-JUN), DAX-1, LRH-1, DLX, and NR4A1.

❖NR4A1 (NGFI-B, Nur77 and TR3) is a member of the NR4A family of orphan nuclear receptors, which also includes NR4A2 (Nurr1) and NR4A3 (Nor1).

- ❖Members of this family were shown to bind as homodimers, heterodimers or monomers to specific binding elements containing the core motif (AAAGGTCA) and they mainly act as transcriptional factors to regulate gene expression both positively and negatively.

❖NR4A1 is widely expressed in many different tissues and plays an important role in a variety of biological processes, including regulation of cell apoptosis, neural differentiation, inflammatory response, atherogenesis, glucose and lipid metabolism, and steroidogenesis.

❖NR4A1 as an essential regulator of the expression of a number of steroidogenic enzyme genes in the hypothalamo-pituitary–adrenal/gonadal axises.

❖To activate expression of corticotropin-releasing hormone (CRH) in the hypothalamus and pro-opiomelanocortin in the pituitary.

❖In the adrenal gland, a role for NR4A1 in the regulation of CYP21, CYP11B2 and HSD3B2 genes transcription has also been demonstrated suggesting it may regulate cortisol, aldosterone and androgen production.

❖NR4A1 activates the promoter of several genes involved in testosterone biosynthesis in Leydig cells, including the human HSD3B2, rat CYP17, and mouse StAR promoter.

Objective

Using mouse follicle culture model to study the role of NR4A1 in the regulation of primary steroidogenic enzymes gene expression and steroidogenesis in ovarian theca cells.

Materials and Methods

1. Plasmids, bacteria, cell lines, and reagents
2. Immunohistochemistry
3. Construction of recombinant adenovirus
4. Follicular isolation and culture
5. Infection of follicle theca cells with recombinant adenoviruses
6. Protein extraction and Western blot analysis
7. Quantitative real-time RT-PCR analysis
8. Hormone assays
9. Statistical analysis

Immunohistochemical localization of NR4A1 in healthy human ovaries

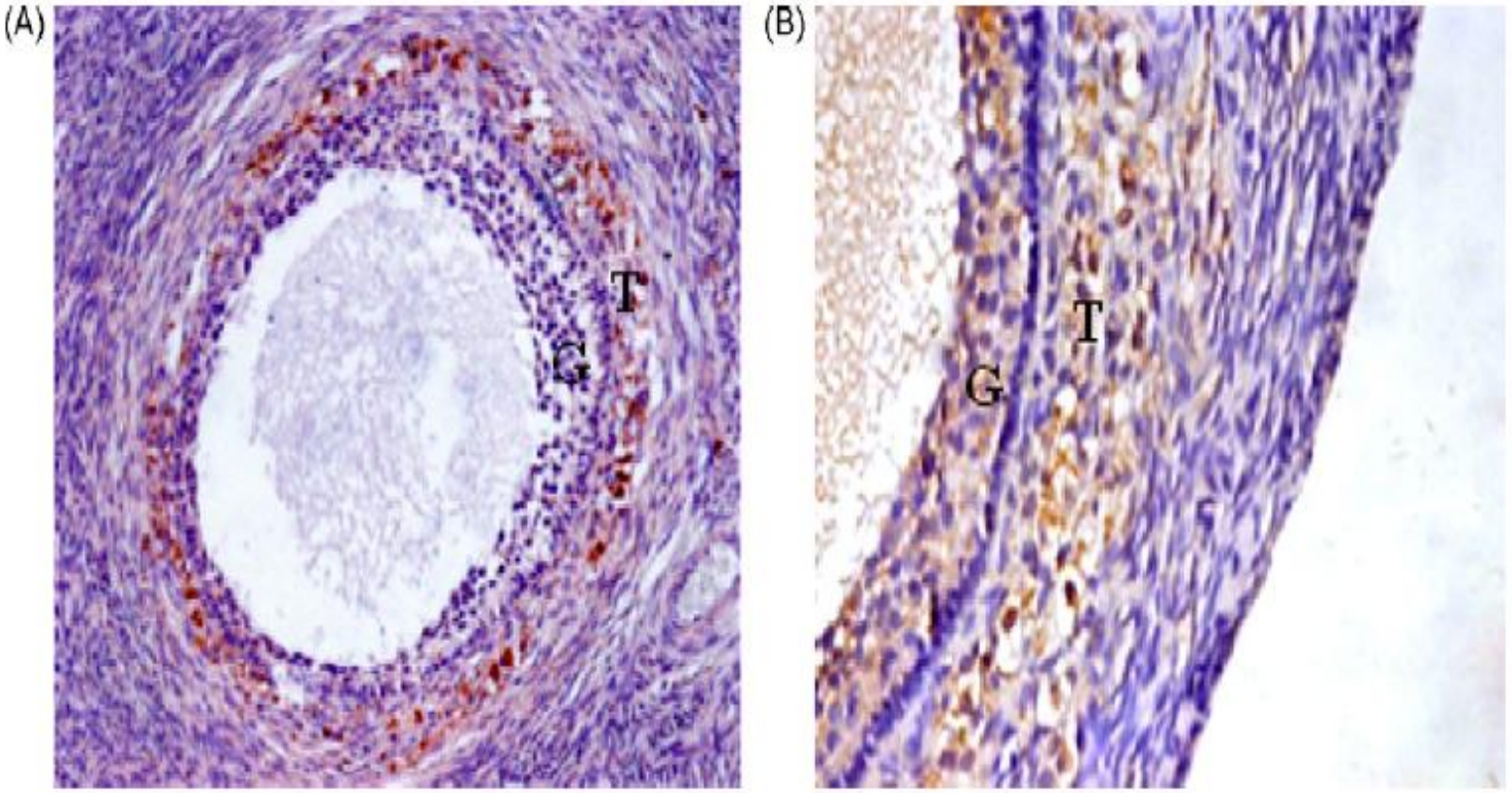


Fig. 1. Immunohistochemical analysis of NR4A1 in human ovaries was performed as described in Section 2. Within the ovary, NR4A1 was extensively expressed in theca cells and granulosa cells (A: original magnification of 20×; B: original magnification of 40×). Positive immunoreactive signals were visualized as brown stain. T = theca cells, G= granulosa cells.

Table 1
Primers used for real-time RT-PCR.

Gene	Primer[a]	Sequence	Size(bp)
NR4A1	Forward Reverse	ATCCGGGCACACTTGGACTC CCCACTTTCGGATAACGTCCAG	157 bp
CYP11A1	Forward Reverse	CAGACGCATCAAGCAGCAA CTGGAGGCAGGTTGAGCAT	209 bp
CYP17	Forward Reverse	TCTGGGCACTGCATCACG GCTCCGAAGGGCAAATAACT	124 bp
HSD3B2	Forward Reverse	GCTTCCAAACGCTGACACCA GGCCCTGTGATCCATCCAATAG	145 bp
StAR	Forward Reverse	CCACCTGCATGGTGCTTCA TTGGCGAACTCTATCTGGGTCTG	142 bp
β-Actin	Forward Reverse	CCGTAAAGACCTCTATGCC CTCAGTAACAGTCCGCCTA	278 bp

[a] Sequences are from 5′ to 3′ ends.

Effect of NR4A1 overexpression on steroidogenic enzyme gene expression and steroidogenesis in theca cells

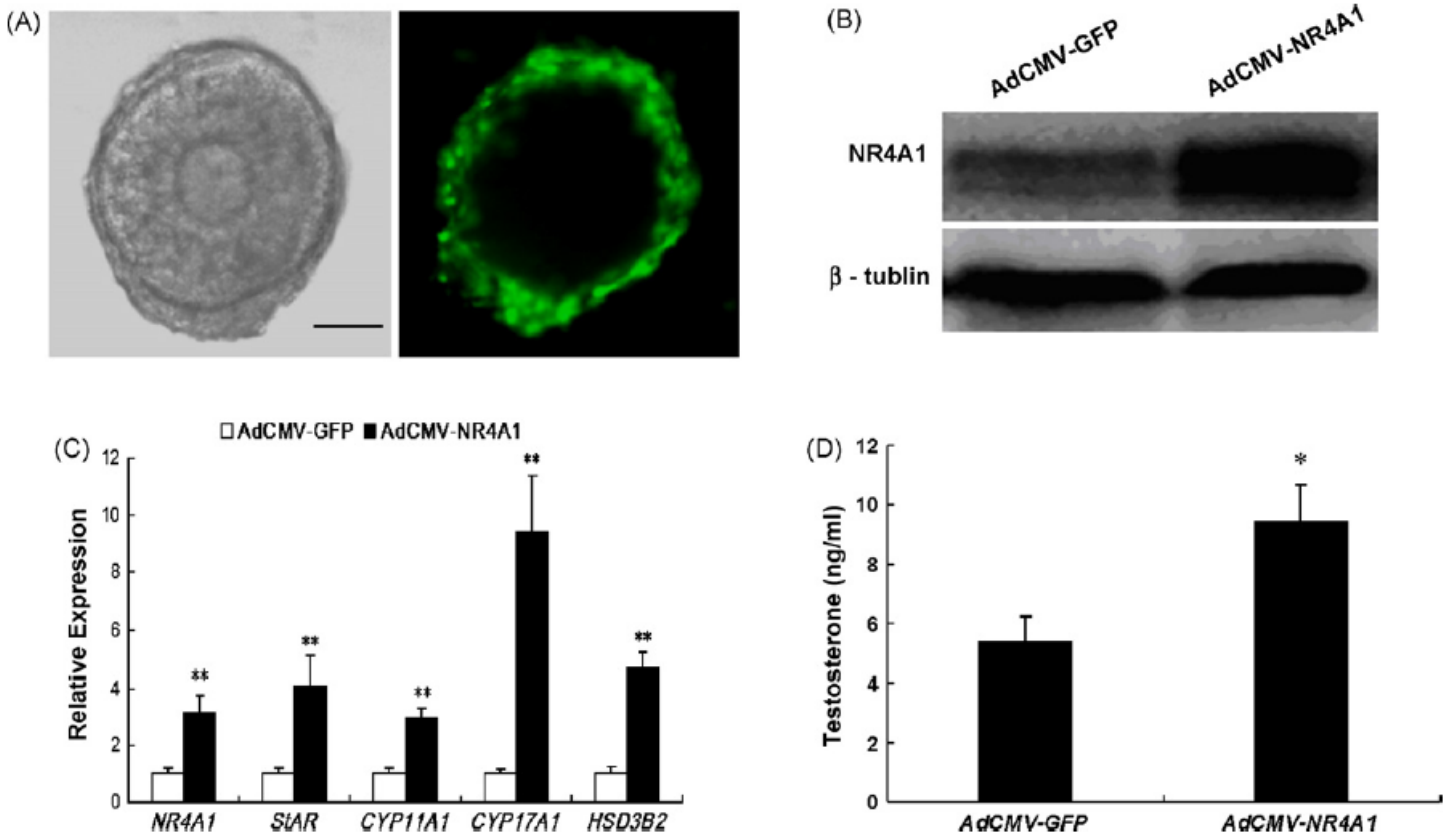
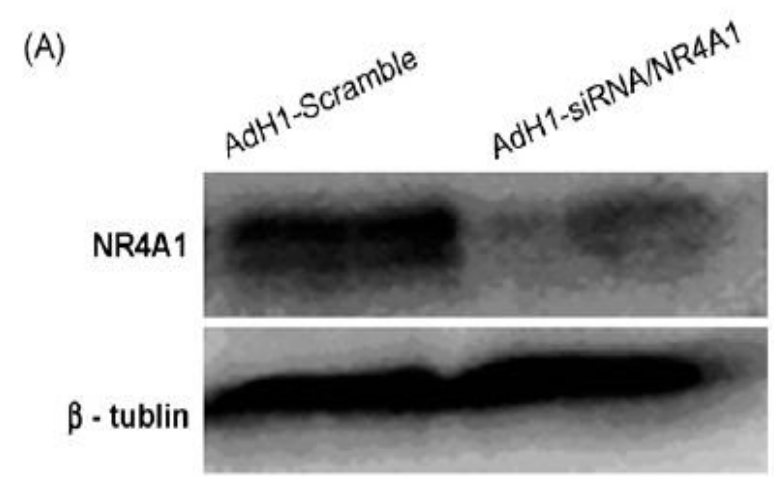
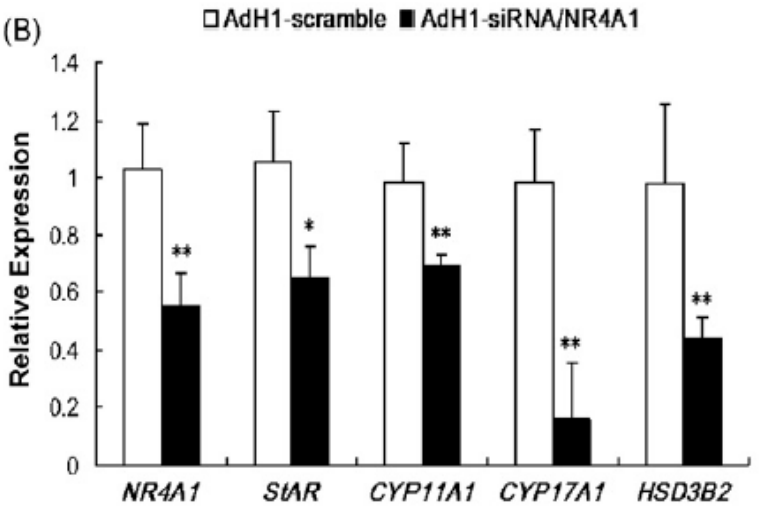


Fig. 2. Effect of NR4A1 overexpression on steroidogenic enzyme gene expression and steroidogenesis in theca cells. (A) Infection of follicular theca cells with recombinant adenoviruses. Follicles were grown as described in Section 2. On the first day of culture, follicles were infected with recombinant adenovirus AdCMV-NR4A1 or AdCMV-GFP and cultured for another 48 h. More than 90% of theca cells were infected as judged by the expression of GFP under a fluorescence microscope. Left: light microscope; right: fluorescence microscope. Bar = 100 μm. (B) Western blot analysis of NR4A1 protein expression in recombinant adenovirus infected follicles. Follicles were infected with AdCMV-NR4A1 or AdCMV-GFP for 48 h. The expression of NR4A1 protein was analyzed by immunoblotting. AdCMV-NR4A1 infected follicles expressed increased levels of NR4A1 relative to AdCMV-GFP infected follicles. β-Tublin were used as internal controls. Forty follicles from the same treatment group were pooled and served as one sample. (C) Effect of NR4A1 overexpression on StAR, CYP11A1, CYP17 and HSD3B2 expression. Follicles were infected with AdCMV-NR4A1 or AdCMV-GFP for 24 h. The expression of StAR, CYP11A1, CYP17 and HSD3B2 were analyzed by real-time RT-PCR. AdCMV-NR4A1 infected follicles exhibited a significant increase in StAR, CYP11A1, CYP17 and HSD3B2 expression as compared to those receiving AdCMV-GFP. β-Actin were used as internal controls. (D) Effect of NR4A1 overexpression on theca cell testosterone production. Follicles were infected with AdCMV-NR4A1 or AdCMV-GFP for 48 h. The amount of testosterone accumulated in the media was evaluated by RIA. AdCMV-NR4A1 infected follicles synthesize increased amount of testosterone compared with AdCMV-GFP infected controls. Results are presented as mean ± SEM from at least 3 independent experiments. Sample sizes were 20 follicles per treatment, per experiment. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. AdCMV-GFP infected controls.

NR4A1 act as transcription activator to modulate steroidogenic enzyme transcription as well as testosterone production in ovarian theca cells.

Effect of NR4A1 silencing on steroidogenic enzyme gene expression and steroidogenesis in theca cells





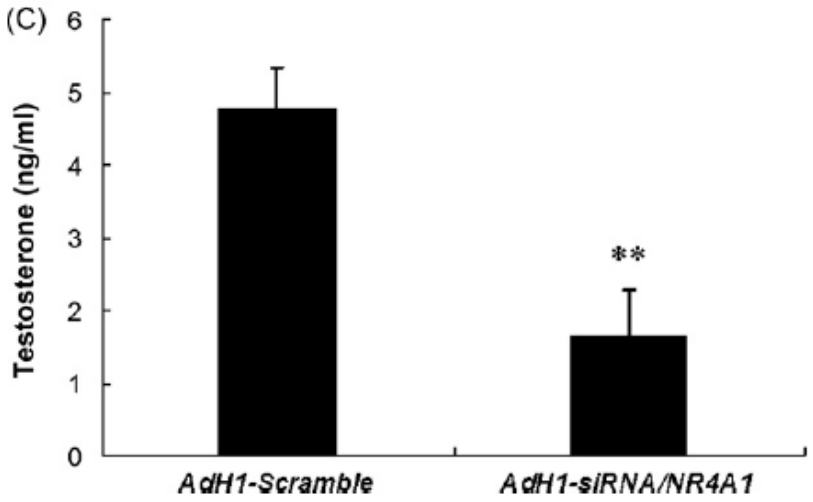


Fig. 3. (A) Western blot analysis of NR4A1 protein expression in recombinant adenovirus infected follicles. Follicles were infected with AdH1- NR4A1 or AdH1-scramble for 48 h. The expression of NR4A1 protein was analyzed by immunoblotting. Endogenous NR4A1 protein expression was significantly reduced in AdH1-NR4A1 infected follicles as compared with AdH1-scramble infected controls. Tublinwereused as internal controls. Forty follicles from thesametreatment group were pooled and served as one sample. (B) Effect of NR4A1 silencing on StAR, CYP11A1, CYP17 and HSD3B2 expression. Follicles were infected with AdH1-NR4A1 or AdH1-scramble for 24 h. The expression of StAR, CYP11A1, CYP17 and HSD3B2 were subjected to real-time RT-PCR. AdH1-NR4A1 infected follicles exhibited a significant decrease in StAR, CYP11A1, CYP17 and HSD3B2 expression as compared to those receiving AdH1-scramble.-Actin were used as internal controls. (C) Effect of NR4A1 silencing on theca cell testosterone production. Follicles were infected with AdH1-NR4A1 or AdH1-scramble for 48 h. The amount of testosterone accumulated in the media was evaluated by RIA. AdH1-NR4A1 infected follicles exhibited decreased testosterone secretion relative to AdH1 scramble infected controls.

The functional study strongly demonstrated that orphan nuclear receptor NR4A1 plays a positive role in modulating steroidogenic enzyme expression, hereby leading to increased testosterone synthesis in ovarian theca cells.

Time-dependent effects of FSK treatment on NR4A1, StAR, CYP11A1, CYP17 and HSD3B2 expression

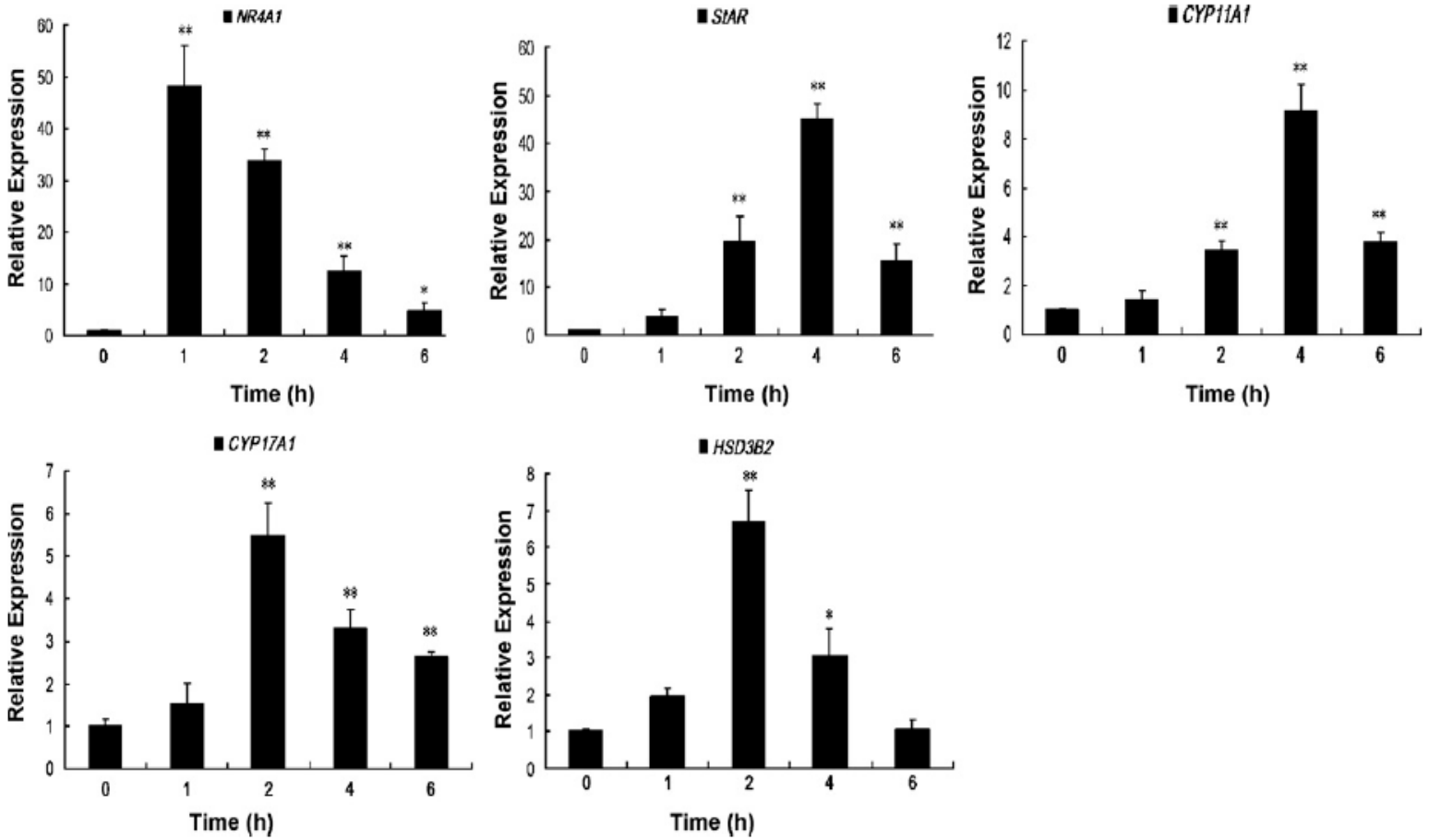


Fig. 4. Follicles were treated with FSK (10 nM), an activator of cAMP. Relative expression of NR4A1, StAR, CYP11A1, CYP17 and HSD3B2 were determined by quantitative RT-PCR. NR4A1 was rapidly induced following 1 h of incubation with FSK. Expression of the NR4A1 gene clearly precedes that of StAR, CYP11A1, CYP17 and HSD3B2. B-Actin were used as internal controls. Results are presented as mean ± SEM from at least 3 independent experiments. Sample sizes were 10 follicles per treatment, per experiment. *$p < 0.05$ and **$p < 0.01$ vs. respective time controls.

NR4A1 may represent a de novo synthesized transcription factor in response to cAMP signaling and is involved in steroidogenic enzyme gene transcription in the ovary.

Summary

❖Orphan nuclear receptor NR4A1 plays a significant role in upregulation of StAR, CYP11A1, CYP17 and HSD3B2 which leads to the increase in androgen production in ovarian theca cells.

❖To establish a direct link between NR4A1 and steroidogenic enzyme gene transcription in theca cells.

❖Modulation of these steroidogenic enzymes by NR4A1 could influence the capacity of the ovarian theca cells to produce androgen.

❖In vitro follicular culture methods coupled to an adenoviral gene-manipulation procedure has been established and proven to be a useful approach to assess the role of specific genes in ovarian biology.

Future Studies

❖The underlying mechanism by which NR4A1 mediated StAR, CYP11A1, CYP17 and HSD3B2 transcription is currently unknown, data from other literature indicated that NR4A1 may activate their target gene transcription through interaction with coactivators and corepressors that link receptors to the transcriptional machinery.

❖The relative expression levels of such presumed cofactors in the ovary and their effect on the regulation of steroidogenic enzyme gene transcription, however, is an area which requires further investigation.

❖The effect of hyperandrogenism on the expression of NR4A1 and its concomitant impact on other clinical manifestations of PCOS.