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Androgen upregulates NR4A1 via the TFAP2A and ETS signaling networks

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ABSTRACT

Hyperandrogenism is one of the clinical and biochemical characteristics of polycystic ovary syndrome (PCOS). Our previous studies confirmed that nuclear receptor subfamily 4 group A member 1 (NR4A1), as a differentially expressed gene in the ovaries of PCOS patients, was upregulated by increased androgen. However, the potential mechanism of NR4A1 upregulation remains unknown. To elucidate the molecular mechanisms involved in NR4A1 regulation, we cloned and characterized the promoter regions of the NR4A1 gene using a series of truncated promoter plasmids in luciferase reporter assays. We identified two unique core promoters of NR4A1 located within the +1055/+1251 and +3183/+3233 regions relative to the transcription start site. TFAP2A downregulated NR4A1 expression, while five ETS transcription factors, ETS1, ELK1, ERG, FLI1 and SPI1, could upregulate NR4A1 promoter activity in HeLa cells. Of these transcription factors, ETS1 and ELK1 were the most effective ones. Moreover, all six transcription factors were confirmed to interact directly with the NR4A1 promoter. In conclusion, this study presents the first description that TFAP2A and ETS family signaling networks are involved in the androgen-mediated transcriptional regulation of NR4A1, which contributes to the understanding of the molecular mechanisms involved in the TFAP2A-NR4A1 and ETS-NR4A1 signaling networks in PCOS.

1. Introduction

Polycystic ovary syndrome (PCOS), a multifactorial and polygenic endocrine disorder that is common among women of reproductive age, is characterized by anovulation, hyperandrogenism and polycystic ovarian morphology (Krishnan and Muthusami, 2017; Qi et al., 2017; Wood et al., 2003). The adverse effects of PCOS on the female endocrine system and metabolism harm reproductive health, leading to infertility (Ehrmann et al., 1995; Legro et al., 1998). Previous studies have shown that NR4A1 is an upregulated protein in ovarian granulosa cells in PCOS patients (Pan et al., 2018).

NR4A1 was the first identified member of the NR4A subfamily of orphan nuclear receptors, which are classified as early-response genes and can be induced by a wide range of physiological signals (Herschman, 1991; Maruyama et al., 1998). NR4A1 has been shown to play an essential role in endocrine-related diseases (Ohno et al., 2009). Previous studies have shown that NR4A1 regulates androgen receptor (AR) transcription by binding to the NGFI-B response element (NBRE) in the AR promoter (Dai et al., 2012). Additionally, AR has been demonstrated to suppress the transcriptional activity of NR4A1 on steroidogenic enzyme gene promoters (Song et al., 2012). In human hair follicles, both dihydrotestosterone (DHT) and testosterone (T) increase the expression of NR4A1 (Xie et al., 2016). A daedal network exists among NR4A1, steroidogenesis and hyperandrogenism related to PCOS pathophysiology. All these studies indicate that NR4A1 plays a vital role in androgen-related signaling pathways. However, the potential mechanism of NR4A1 upregulation by androgen in PCOS remains unknown.

In the present study, we investigated whether androgen can increase NR4A1 expression in KGN cells and the underlying molecular mechanisms of this effect. We found that androgen upregulated the transcription of NR4A1, and identified the binding proteins controlling the transcript variant 3 expression of NR4A1, which will enhance the understanding of NR4A1 regulation as a possible mechanism underlying the pathogenesis of PCOS.

Abbreviations: NR4A1, nuclear receptor subfamily 4 group A member 1; PCOS, polycystic ovary syndrome; TFAP2A, transcription factor AP-2 alpha; ETS1, ETS proto-oncogene 1, transcription factor; ERG, ETS-related gene; ELK1, ETS domain-containing protein Elk-1; FLI1, FLI-1 proto-oncogene, ETS transcription factor; SPI1, SPI-1 proto-oncogene

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2. Materials and methods

2.1. Cell culture

HeLa and KGN cells were purchased from the American Type Culture Collection (ATCC). The cell cultures were maintained in high-glucose DMEM (for HeLa cells) or DMEM/F12 (for KGN cells) containing 10% fetal bovine serum (FBS, Invitrogen, USA) and 1% antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin; HyClone, USA) at 37 °C under a 5% CO2 atmosphere. Testosterone at 10^{-7} M concentration was used to create the hyperandrogenic model while ethanol as a control. The culture medium was changed every other day.

2.2. Transient cell transfection

Transient cell transfection was performed as previously described (Xu et al., 2016). Briefly, promoter activity was analyzed using pGL3-Basic vector as a negative control, and the plasmid pRL-TK (Promega, USA) was cotransfected as an internal control. All plasmids were propagated in *Escherichia coli* DH5α and isolated using a QIAprep Spin Miniprep Kit (Qiagen, Germany). HeLa cells were plated on 96-well plates 24 h before transfection, and triplicate wells were used for each group. A total of 100 ng of pGL3 vector containing the different truncated DNA fragments and 4 ng of pRL-TK vector was cotransfected into the cells in each well using Lipofectamine™ 3000 Transfection Reagent (Thermo Fisher, USA), according to the manufacturer's instructions.

2.3. Plasmid construction

Three different regions (-2048/+216, +185/+1454, +2235/+3332) containing the proximal promoter of human NR4A1 transcript 3 (NCBI Reference Sequence: NM_001202233.1) were PCR-amplified with PrimeSTAR® HS (Clontech, USA) using the primer sets F1-1/R1, F2-1/R2, and F3-1/R3, which contained three pairs of restriction endonuclease sites: KpnI/MluI, KpnI/NheI, and KpnI/HindIII. The amplified fragments were cloned into the polycloning site preceding the luciferase coding sequence in the pGL3-Basic plasmid (Promega, USA), generating the vectors pGL-KM-P1 (2265 bp), pGL-KN-P1 (1270 bp) and pGL-KH-P1 (1098 bp). A series of truncated fragments of the regulatory region of the NR4A1 gene were created by PCR amplification using corresponding primers and the three fragments above as templates; these truncated fragments were inserted into pGL3-Basic luciferase reporter vectors using the same pairs of restriction endonucleases described previously. The plasmids containing the truncated fragments were designated pGL-KM-P2 through pGL-KM-P5, pGL-KN-P2 through pGL-KN-P5 and pGL-KH-P2 through pGL-KH-P4. NR4A1 gene promoter site mutants were created by PCR amplification using two pairs of corresponding primers, pGL-KN-P3 and pGL-KN-P3, as the templates. All constructs were confirmed by DNA sequencing.

2.4. Luciferase assay

Luciferase assay was performed as previously described (Xu et al., 2016). Briefly, at 48 h after transfection, cell lysates were prepared for measurement of luciferase activity using a Dual-Luciferase® Reporter Assay System (Promega, USA). The pGL3-Basic vector was used as a control, while the pRL-TK plasmid (Promega, USA), which expressed Renilla luciferase, was used as an internal reference to standardize the luciferase activity. Silencing or overexpression plasmids for different factors were cotransfected into HeLa cells with 100 ng of promoter reporter plasmid and 2.5 ng of pRL-TK plasmid. The transfection experiments were performed in triplicate. At least three independent experiments were performed.

2.5. Chromatin immunoprecipitation (ChIP)

ChIP assays were performed using an EZ-ChIP kit (Millipore, USA). Briefly, approximately 10^7 cells were fixed with 1% formaldehyde and sonicated on ice using ten 15 s bursts with 8 s intervals between bursts. Optimization experiments were performed to obtain an optimal length of chromatin fragments consisting of 200–1000 bp of genomic DNA. The target chromatin fragments were enriched with 2 μg of each primary antibody and immunoprecipitated with protein G agarose beads. Nonimmunized rabbit IgG was used as a negative control. After extensive washing of the beads, DNA was released following protease K digestion. The DNA was purified and analyzed using PCR with primers encompassing the putative binding sites of the NR4A1 promoter.

2.6. RNA extraction and real-time quantitative PCR

Total RNA from HeLa or KGN cells previously subjected to different treatments was extracted using the TRIzol reagent (Invitrogen, USA), and 1 μg of total RNA was reverse-transcribed in a 20 ul reaction using a High Capacity RNA-to-cDNA Kit (Applied Biosystems, USA) following the manufacturer's instructions. SYBR® FAST qPCR Kits (Takara, USA) were used in combination with a real-time PCR detection system. The relative fold change in gene expression was calculated by the $2^{\Delta\Delta CT}$ method. GAPDH was used as an internal reference for normalization of the real-time PCR results. The PCR products were analyzed on 2% agarose gels. Primers were showed in Supplementary Table 1.

2.7. Construction of siRNA and lentiviral overexpression vectors

siRNAs for TFAP2A, ETS1, ELK1, ERG, FLI1, and SPI1 (KeyGEN, China) and lentiviral overexpression vectors for TFAP2A, ETS1, ELK1, ERG, FLI1, and SPI1 were purchased (GeneChem, China) for transfection experiments in HeLa and KGN cells.

2.8. Statistical analysis

The average and standard deviation (SD) were calculated for each experimental group. All statistical analyses were performed with SPSS 19.0 software. One-way analysis of variance (ANOVA) was used to evaluate quantitative data in more than two groups. Data for which ANOVA indicated a significant difference were further analyzed using Student's *t*-test for one-to-one comparisons. Statistical significance was set $P < 0.05$.

3. Results

3.1. The expression of NR4A1 is increased in KGN cells under hyperandrogenic conditions

To determine the function of androgen on NR4A1 expression, KGN cells were treated with high androgen levels for 48 h and then harvested for real-time PCR and western blot analysis. Based on the previous experiments in our laboratory, 10^{-7} M of testosterone chosen from a wide range of testosterone frome 10^{-8} to 10^{-5} M represents an optimal dose, and subsequently used in all of our experiments. Also, the expression of NR4A1 was significantly increased at both mRNA and protein levels after androgen treatment (Fig. 1A,B).

3.2. Identification of NR4A1 transcripts 3 and 4 and discovery of increased NR4A1 transcript 3 expression in KGN cells under hyperandrogenic conditions

Four transcript variants were identified in the PubMed database and four corresponding primers were designed for the four transcript variants of NR4A1 (Supplement Table 1). We confirmed that only two of the four NR4A1 transcript variants were expressed in KGN cells (TV-3

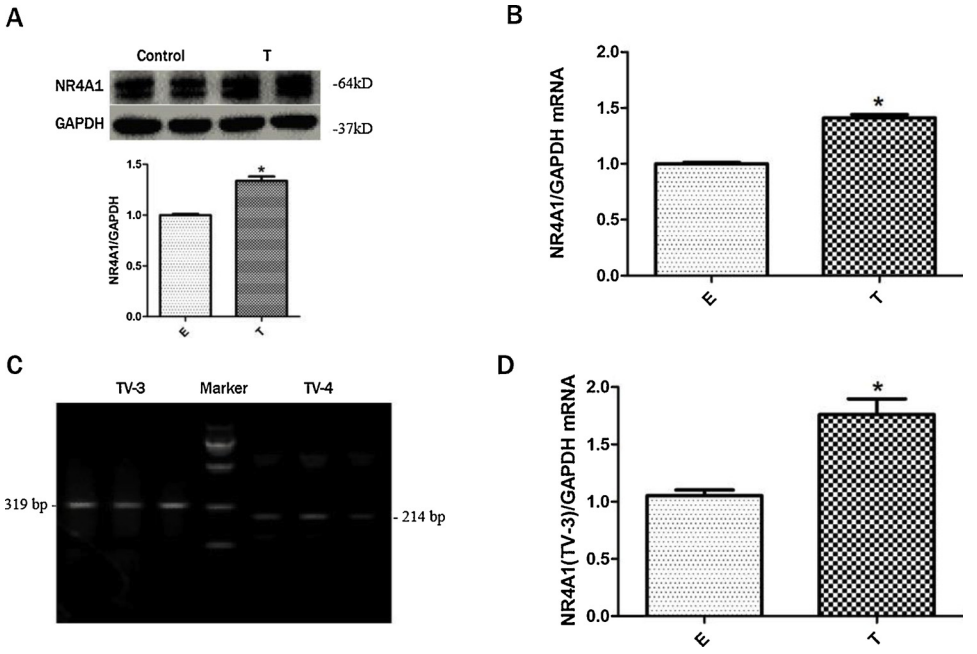

Fig. 1. Expression of NR4A1 and NR4A1 transcript variant (TV) 3 in KGN cells after androgen (T) stimulation (A, B) Expression of NR4A1 in KGN cells with testosterone (T as 10^{-7} M or 100 nM of testosterone concentration) or ethanol (E as Control) treatment were measured by real-time PCR and western blot (In picture A, the lanes 1 and 2 on left are parallel control samples, the lanes 3 and 4 on right are parallel experimental group samples treated with testosterone) analysis (*, $P < 0.05$). (C) NR4A1 TV-3 and TV-4 were detected by PCR in KGN. Three pairs of independent samples were performed in one agarose gel. The five bands from top to the bottom in the middle lane of the picturse is Marker (Successively represent the molecular sizes of 1000 bp, 750 bp, 500bp, 250 bp and 100 bp) (D) The relative mRNA levels of NR4A1 TV-3 in KGN cells after androgen treatment were assayed by real-time PCR (*, $P < 0.05$). The results are presented as the mean ± SD.

and TV-4) (Fig. 1C) while all four transcript variants were detected in Hela cells. RT-PCR was used to detect mRNA expression of TV-3 in KGN under hyperandrogenic conditions. The results revealed significant increases in NR4A1 TV-3 level (Fig. 1D).

3.3. The identification of NR4A1 transcript variant 3 (TV-3) core promoter region

The Promoter 2.0 Prediction Server was used along with the NCBI database to identify the transcription start site (52022732) of NR4A1 TV-3. Then we selected the 2000bp sequence upstream of TSS and constructed five different truncated fragments which were amplified by PCR and subsequently cloned into the pGL3-Basic luciferase reporter vector (producing pGL-KM-P1, pGL-KM-P2, pGL-KM-P3, pGL-KM-P4, and pGL-KM-P5) distinguished by the restriction endonucleases KpnI and MluI. However, none of the five fragments displayed significant luciferase activity in HeLa cells (Fig. 2A).To further determine the core promoter of NR4A1, we checked the Ensemble database and reselected two fragments with high promoter activity at the downstream of the TSS (Supplement Fig. 1A) and further constructed five (distinguished by restriction endonucleases KpnI + NheI) and four (distinguished by restriction endonucleases KpnI + HindIII) truncated sequences respectively. Interestingly, the truncated promoters from the two different regions displayed completely different activity patterns. Deletion to +1251bp from the TSS (pGL-KN-P4) caused a significant increase in promoter activity, while the remaining truncated sequences of pGL-KN displayed no luciferase activity, indicating the presence of at least one negative regulatory element in the +1055/+1251 region (pGL-KN-P3) (Fig. 2B). In contrast, deletion to +3233bp from the TSS (pGL-KH-P4) resulted in a significant decrease in promoter activity, while the other truncated sequences of pGL-KH displayed significant luciferase activity, indicating the presence of positive regulatory elements in the +3183/+3233 region (pGL-KH-P3) (Fig. 2C).

3.4. Androgen upregulates transcriptional activity of NR4A1 core promoter with TFAP2A binding site

To verify the effect of androgen on NR4A1 transcriptional activity, pGL-K**N**-P3 was cotransfected into HeLa cells with or without androgen treatment (10^{-7}M) (with ethanol as control). The results showed that androgen significantly enhanced the transcriptional activity of NR4A1 (Fig. 3A). Then we used JASPAR to predict transcription factor binding sites in pGL-KN-P3 and a TFAP2A binding site in pGL-KN-P3 was found (Fig. 3B). Site-directed mutagenesis was performed to determine whether this putative site is functional and responsible for the regulation of core promoter activity. As expected, mutation of the TFAP2A binding site increased the NR4A1 transcriptional activity by approximately three-fold compared with the parental pGL-KN-P3 (Fig. 3B). Furthermore, we detected the expression of TFAP2A in KGN cells after 48 h after hyperandrogen treatment. Moreover, the expression of TFAP2A was significantly decreased under hyperandrogenic conditions (Supplement Fig. 2) which further confirmed that high hyperandrogen could inhibit TFAP2A expression and abolish its inhibition of NR4A1 expression.

3.5. Inhibitory effect of TFAP2A on NR4A1

To further study the effect of TFAP2A on the transcriptional activity and expression of NR4A1, we cotransfected pGL-K**N**-P3 and si-TFAP2A into HeLa cells. The luciferase activity showed that downregulation of TFAP2A expression significantly increased the NR4A1 transcriptional activity (pGL-K**N**-P3) (Fig. 3C). Similarly, the protein level of NR4A1 also increased in KGN treated with siRNA-mediated knockdown of TFAP2A (Fig. 4D). On the other hand, to confirm the inhibitory effect of TFAP2A on NR4A1, we co-transfected pGL-KN-P3 and a lentiviral vector with the TFAP2A overexpression and the result showed that high expression levels of TFAP2A significantly reduced NR4A1 transcriptional activity (Fig. 3E). Consistent with the change in transcriptional activity of NR4A1 promoter, TFAP2A overexpression inhibited NR4A1 in KGN cells as well (Fig. 3F). Further, we discovered direct interactions between TFAP2A and NR4A1 by ChIP assays (Fig. 3F).

3.6. Androgen upregulates transcriptional activity of NR4A1 core promoter with ETS binding site

After we confirmed the inhibition of NR4A1 caused by TFAP2A, we further studied the effect of ETS in NR4A1 regulation. pGL-KH-P3 (with a ETS family binding site) was cotransfected into HeLa cells with or without androgen treatment (10^{-7} M). The results showed that androgen significantly enhanced the transcriptional activity of this fragment (Fig. 4A). To determine whether ETS is due to the upregulation of NR4A1 transcriptional activity, a plasmid containing a mutation in ETS

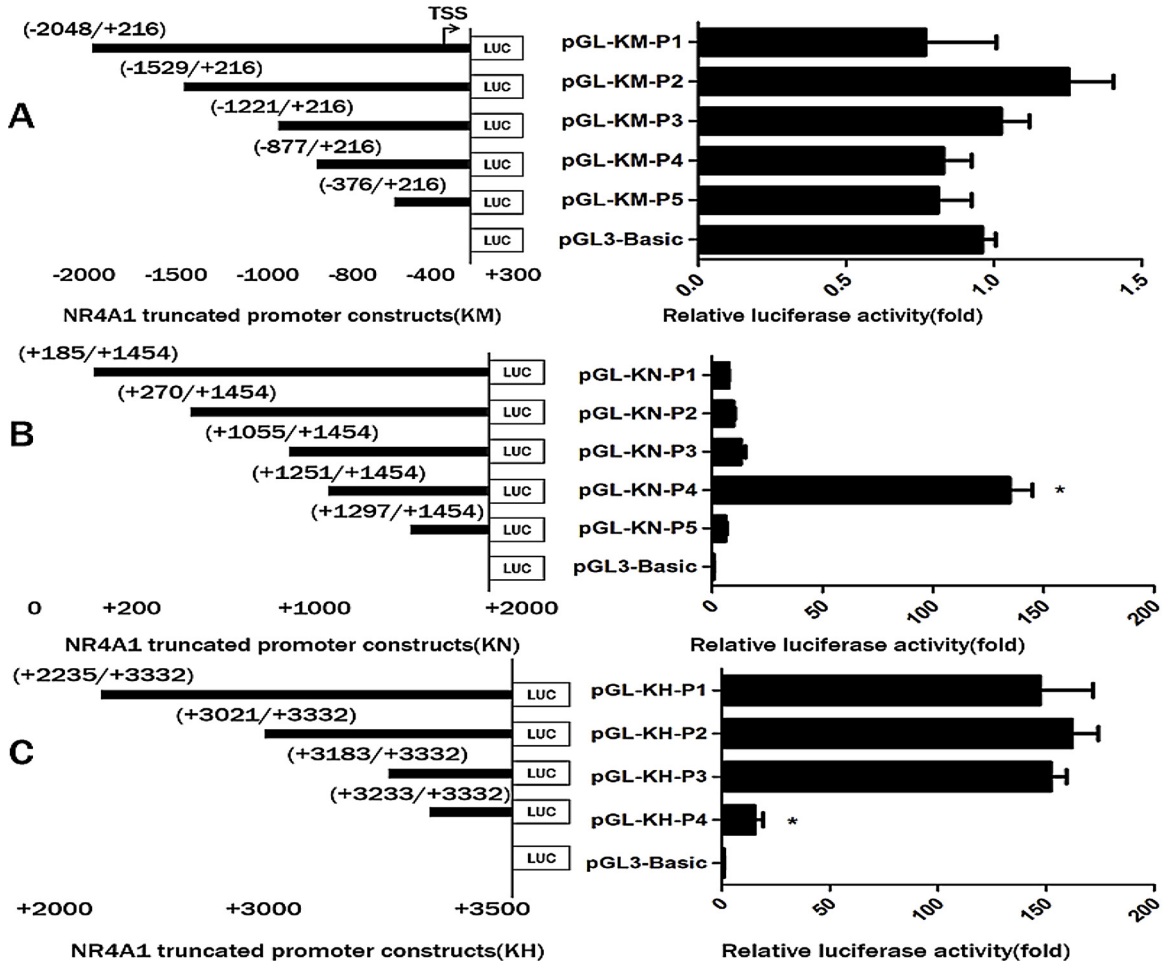

Fig. 2. The locations and relative luciferase activity of NR4A1 TV-3 promoter constructs in three different promoter areas. (A) Different lengths (KN-P1 to KN-P5) in the second promoter area (−2048/+216) with their relative luciferase activity (*, $P < 0.05$). (B) Different lengths (KN-P1 to KN-P5) in the second prediction area (+185/+1454) along with their relative luciferase activity (*, $P < 0.05$). (C) Different lengths with different lengths (KH-P1 to KH-P4) in the third prediction area (+2235/+3332) along with their relative luciferase activity (*, $P < 0.05$). All three experiments were performed in Hela cells. The results are presented as the mean ± SD of triplicate transfections and are expressed in arbitrary units based on normalization of the firefly luciferase activity to the Renilla luciferase activity. The bars represent the mean ± SD of three independent transfection experiments.

family binding site was constructed. As we predicted, the mutation of the ETS family binding site led to a 60% reduction in the promoter activity of NR4A1 (pGL-KH-P3) (Fig. 4B). Furthermore, we detected the expression of five members of ETS family (ETS1, ELK1, ERG, FLI1 and SPI1) in KGN cells with androgen treatment (10^{-7} M). It showed that the expression of all five factors was significantly increased under hyperandrogen which indicated androgen promote NR4A1 expression through multiple factors of ETS family (Supplement Fig. 2).

3.7. Stimulating effect of ETS1, ELK1, ERG, FLI1 and SPI1 on NR4A1

To further research the effect of ETS family members on the transcriptional activity and expression of NR4A1, we cotransfected pGL-KH-P3 and si-ETS1, si-ERG, si-ELK1, si-FLI1, or si-SPI1 into HeLa respectively. Downregulation of ETS1, ELK1, ERG, FLI1 and SPI1 expression significantly decreased the promoter activity of NR4A1 (Fig. 4C). The protein level of NR4A1 was also detected in KGN with siRNA-mediated knockdown of ETS1, ELK1, ERG, FLI1 and SPI1 (Fig. 4D and Supplement Fig. 2). It shows that the knockdown of all five factors of ETS family could lead to inhibition on NR4A1 expression. Also, within the five factors, the effects of ETS1 and ELK1 are most apparent. Therefore, we cotransfected pGL-KH-P3 and lentiviral vector with the overexpression of ETS1 or ELK1 in Hela respectively. ETS1 or ELK1 overexpression both can significantly increase NR4A1 transcriptional activity (Fig. 4E). Correspondingly, increased protein expression of NR4A1 was discovered in KGN cells treated with overexpression of ETS1 or ELK1 (Fig. 3F). At last, we confirmed the direct interactions of all five factors with NR4A1 (Fig. 3F).

4. Discussion

In this study, we confirm the role of androgen in promoting NR4A1 expression in human granulosa cell. Two of four transcript variants of human NR4A1 gene were identified in KGN, and it is noteworthy that The PCR primers used in Fig. 1B detected both TV3 and TV4, and the observed 1.4 fold increase could be due to increase in at least one variant. Since subsequent experiment (Fig.1D) indicated that TV3 increased 1.8 fold following androgen induction, it is speculated that androgen may have little or no effect on the expression of TV4. Then we identified two distinct sequence fragments (+1055/+1251 and +3183/+3233) with obvious transcriptional activity downstream of TSS and found that the androgen-regulated effects of these two fragments were controlled by two types of transcription factors: TFAP2A and ETS family. Next, we confirmed TFAP2A inhibits NR4A1 transcriptional expression by binding to +1055/+1251 region. We also confirmed that ETS1, ELK1, ERG, FLI1 and SPI1 play a role in stimulating the transcriptional expression of NR4A1 by binding to +3183/+3233 region and further confirmed that both signal pathways are

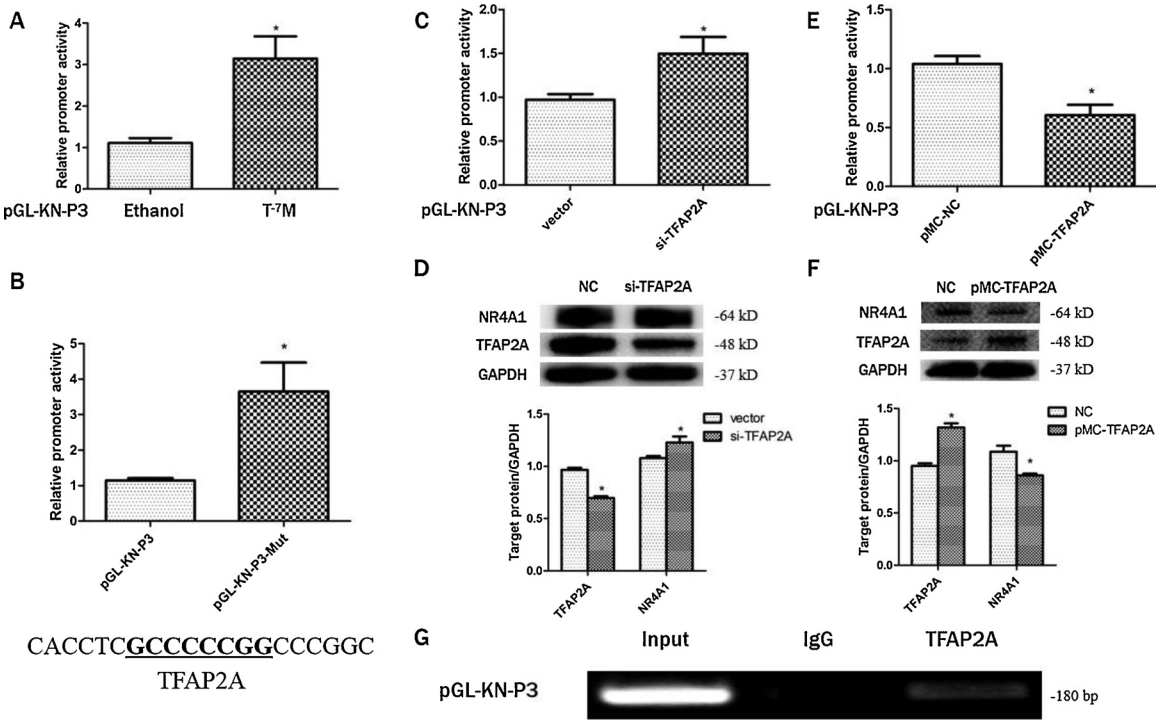

Fig. 3. Inhibitory effect of TFAP2A on NR4A1 expression (A) The effect of hyperandrogen on luciferase activity of pGL-KN-P3, ethanol as control (*, $P < 0.05$). (B) The effect of TFAP2A binding site mutation in pGL-KN-P3 on luciferase activity (*, $P < 0.05$). (C, D) The promoter activity and expression of NR4A1 increased with si-TFAP2A treatment (*, $P < 0.05$). (E, F) The promoter activity and expression of NR4A1 inhibited by TFAP2A overexpression (*, $P < 0.05$). (G) ChIP assay to examine the association of TFAP2A with NR4A1. The experiments of A、B、C and E were performed in Hela cells. The experiments of D、F and G were performed in KGN cells. Data are presented as the mean ± SD of three independent experiments.

regulated by androgen which suggests that the TFAP2A-NR4A1 and ETS family factors-NR4A1 networks may have something to do with the pathological development of PCOS characterized with hyperandrogenism.

The existing researches of NR4A1 mainly focus on its effects on cell proliferation and apoptosis, the regulation of tumor growth and

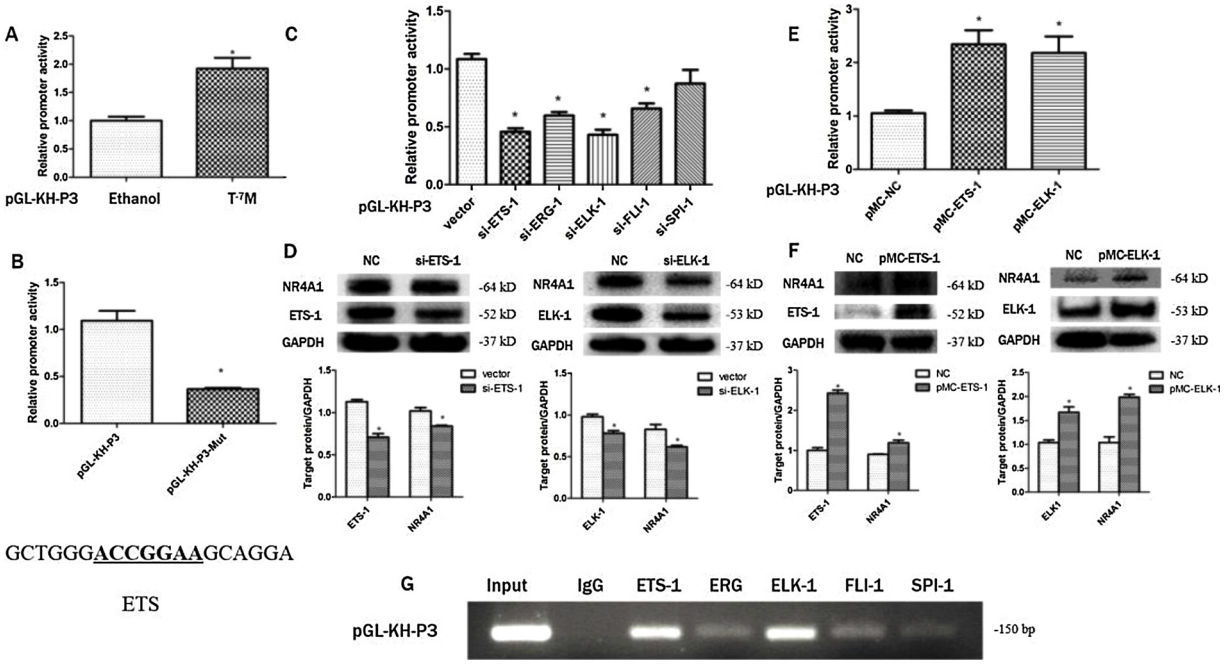

Fig. 4. Expression Promotion of ETS family members on NR4A1 expression (A) The effect of hyperandrogen on luciferase activity of pGL-KH-P3, ethanol as control (*, $P < 0.05$). (B) The effect of ETS family members binding site mutation in pGL-KH-P3 on luciferase activity (*, $P < 0.05$). (C, D) The promoter activity and expression of NR4A1 decreased with si-ETS-1, si-ERG, si-ELK-1, si-FLI-1, or si-SPI-1 treatment independently (*, $P < 0.05$). (E, F) The promoter activity and expression of NR4A1 increased by ETS1 and ELK1 overexpression respectively (*, $P < 0.05$). (G) ChIP assay to examine the association of ETS1, ELK1, ERG, FLI1, and SPI1 with NR4A1. The experiments of A–C and E were performed in Hela cells. The experiments of D、F and G were performed in KGN cells. The results are presented as the mean ± SD.

migration, and the effect on steroid hormone synthesis (Hazel et al., 1988; Kagaya et al., 2005, Maxwell and Muscat, 2006). However, few studies reported the regulation of NR4A1 itself, and most are based on mouse and rat (Choi et al., 2004; Soker and Godecke, 2013; Tenga et al., 2016). Besides these studies, the regulation of human NR4A1 gene is still unclear. Unlike these two species, four different transcript variants are encoded by NR4A1 gene in human while only single transcription is fully proved in mouse or rat in GenBank. In our present study, we identified two of the four transcript variants of human NR4A1 gene in human granulosa cell: transcript variant 3 and 4. We have further confirmed that transcript variant 3 is the main type regulated by androgen. These results may help to build a better understanding the regulation of NR4A1 in human bodies and a new level of study of human diseases.

Generally, the gene promoter region is located upstream of its transcription start site (TSS), which regulated by transcription factors through binding to their cis-acting elements. However, in a few cases, some of the promoter regions with binding sites are located at downstream of TSS. For example, recent study has shown that ZFX plays a regulatory role by binding to a sequence located downstream +240bp of its target gene TSS (Rhie et al., 2018). Interestingly, in our study, we confirmed that no transcriptional activity has shown at the upstream region of TSS in NR4A1 transcript variant 3. However, correspondingly, we identified two non-sequential DNA sequences with promoter transcription activity: + 1055/+1251bp and + 3183/+3233bp at downstream of TSS, and further identified the corresponding transcription regulators: TFAP2A and ETS family factors (ETS1, ELK1, ERG, FLI1, SPI1) which expression were detected in Hela cells in previous studies. Our finding of this rare phenomenon may play an important role in further study to clarify the way of gene regulation in the future.

TFAP2A belongs to the AP-2 family of transcription factors, which consist of five members: TFAP2A-E. The five proteins have significant sequence similarities and a specific structural organization that is involved in DNA binding and dimerization (Dimitrova et al., 2017; Wang et al., 1997). The effect of TFAP2A can be divided into two aspects: promoting or inhibiting the expression (Liu et al., 2007; Milunsky et al., 2008; Pihlajamaa et al., 2014; Seberg et al., 2017). In our study, TFAP2A showed obvious inhibitory effects on NR4A1 regulation, including the regulation of promoter activity of NR4A1 or the inhibition of NR4A1 protein expression. We also confirmed that androgen inhibited the transcriptional expression of NR4A1 by down-regulating the expression of TFAP2A. Therefore, we believe that TFAP2A may plays a vital role in the pathological changes caused by androgen elevation in polycystic ovary syndrome.

The ETS family, as the transcription factors, consists of 28 members, and there is a highly conserved ETS domain in these transcription factors, recognizing the core (A/C)GGA(A/T) motif of the promoters and enhancers of downstream target genes (Seth and Watson, 2005). Previous studies have reported the ETS proteins are involved in the migration, invasion, proliferation or apoptosis of cancer cells, and also participate in the development and function of angiogenesis and immune cells (Choi et al., 2011; Hollenhorst et al., 2011; Plotnik and Hollenhorst, 2017). Also recent studies have shown that ERG plays an important role in the invasion and migration of prostate cancer with high levels of androgen expression (Cao et al., 2018). The phenomenon of gene fusion in tumors characterized by hyperandrogenism is also mentioned in similar studies (Wang et al., 2017). However, no previous studies have shown any correlation between ETS and PCOS. In the present study, we confirmed the direct interaction of several ETS family genes with NR4A1, a differentially expressed gene in PCOS. This finding suggests that ETS family may be involved in PCOS pathological changes. Notably, androgen treatment increased the expression of ETS family members, including ETS-1, ELK-1, ERG, FLI-1 and SPI-1, suggesting androgen regulates ETS transcription factors in human granulosa cell which partly explains the hyperandrogenic characteristics of PCOS. For the first time, we propose a new point of view that TFAP2A and the ETS family members are involved in pathological changes of PCOS characterized by hyperandrogen.

In conclusion, transcript variant3 is the main transcript of human NR4A1 gene in granulosa cell. We demonstrated androgen through TFAP2A and ETS family members binding to two different promoter regions located at downstream of TSS to regulate transcription of NR4A1. The pathophysiological significance of TFAP2A- and ETS family-mediated NR4A1 activation largely explains the regulation of NR4A1 by androgen. The upstream signal that controls the expression of TFAP2A and ETS family members needs to be identified in future studies. Given the extensive involvement of NR4A1 in PCOS, the identification of TFAP2A and ETS family members as potent NR4A1 regulators, which are regulated by androgen, is an important discovery in the pathophysiology of PCOS. Further investigation into the TFAP2A-NR4A1 and ETS-NR4A1 signaling networks may provide useful information for the development of new therapeutic modalities for PCOS.

Acknowledgments

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biocel.2019.05.015.

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