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Research paper

Identification of *SEPTIN12* as a novel target of the androgen and estrogen receptors in human testicular cells

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ABSTRACT

SEPTIN12 (SEPT12) is a testis-enriched gene that is downregulated in the testis of infertile men with severe spermatogenic defects. While *SEPT12* is involved in spermatogenic failure and sperm motility disorder, *SEPT12* transcriptional regulation is still unknown. Here we report the promoter region of *SEPT12* as a 245 bp segment upstream of the transcription start site. One androgen receptor (AR) and two estrogen receptor α (ERα) binding sites in this region were initially identified by bioinformatics prediction and confirmed by chromatin immunoprecipitation assay. Truncated ERα or AR binding sites decreased the promoter activity, which indicated that the ERα and AR are essential for the *SEPT12* promoter. On the other hand, the promoter activity was enhanced by the treatment with 17β-estradiol (E2) and 5α-dihydrotestosterone (5α-DHT). Thus, one androgen and two estrogen hormone responsive elements located in the promoter of *SEPT12* gene can regulate *SEPT12* expression.

Two single nucleotide polymorphisms (SNPs), rs759992 T > C and rs3827527 C > T, were observed in the *SEPT12* gene promoter region and were able to decrease the promoter activity. In conclusion, the current work identified the promoter of the human *SEPT12* gene and provided key evidence about its transcriptional regulation via E2 and 5α-DHT. Since *SEPT12* has an important role in spermatogenesis, *SEPT12* expression analysis can be developed as a potential tool for the assessment of environmental or food pollution by hormones or for the evaluation of the risk of endocrine-disrupting chemicals (EDCs) in general.

1. Introduction

Spermatogenesis is a complex and dynamic process that male germ cells undergo to expand and develop into mature spermatozoa in the seminiferous tubules of the testes. During spermatogenesis, spermatogonial stem cells undergo mitotic division into spermatocytes [1]. Afterward, diploid spermatocytes go through meiosis and differentiate into haploid round spermatids and then develop into elongated spermatids through a unique differentiation, spermiogenesis, and mature into spermatozoa [2,3]. However, it is believed that the regulation of the expression of the majority of the genes involved in spermatogenesis still not fully understood.

SEPTIN12 gene (*SEPT12*) is a member of the *SEPTIN* gene family that has a highly conserved, polymerized, cytoskeletal GTP-binding domain in mammalian species [4]. It is highly expressed in testicular tissue [5], especially in post-meiotic germ cells [6]. During the seventh stage of mouse spermatogenesis, *SEPT12* proteins start to

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express and form a filamentous structure around the outer part of the acrosome. *SEPT12* proteins are located at the annulus of mature spermatozoa and interact with *SEPT1/4/6/7* to construct the *SEPT* ring structure [5]. The complex *SEPT1/4/6/7/12* ring structure forms the major component of mature spermatozoa annulus that is involved in the cortical organization and terminal differentiation of spermatozoa [7]. However, the transcriptional regulation of *SEPT12* gene expression in the process of spermatogenesis is still unclear.

SEPT12$^{+/-}$ chimeric mice were found to have severe spermatogenic defects with immotile, bent tail, round-headed, and acrosomal defects of sperm [5]. In addition, two different *SEPT12* mutations have been identified in infertile men who have a defective sperm annulus with a bent tail [8,9]. Furthermore, *SEPT12* filaments, located at the sperm annulus, were connected to sperm motility [8]. These results indicated that *SEPT12* plays a critical role in spermatogenesis and sperm structure [10].

Spermatogenesis in mammals is essentially regulated by the action of a complex interaction of steroid sex hormones, including androgens, estrogens, and progestogens [11,12]. As steroid hormones enter the cell, they bind to the steroid hormone nuclear receptor, causing the receptor to form a dimer. The ligand-inducible nuclear receptor dimer, as a transcription factor, translocates to the nucleus and regulates the transcriptional expression of the target genes by binding to the specific hormone response element sites in the promoter region of the target genes [13]. For example, testosterone and 5α-dihydrotestosterone (5α-DHT) in the testis bind to the androgen receptor (AR) to regulate gene expression in spermatogenesis [14–16]. Estrogen is secreted by Leydig cells interact with the estrogen receptor (ER) to regulate specific gene transcription [17,18].

In the current study, we aimed to identify the promoter region of *SEPTIN12* and to investigate the transcription regulation of the *SEPT12* gene in the process of spermatogenesis. We proposed that the transcription of the *SEPT12* gene might be regulated through the interaction of estrogens, androgens, and their nuclear receptors.

2. Material and methods

2.1. Clinical information

This study was approved by the Institutional Review Board of Kuo General Hospital and National Cheng Kung University Hospital in Taiwan. Total 160 infertile men with abnormal semen parameters and 200 fertile men with normal semen parameters were enrolled in the study from January 2005 to July 2007. The infertile men presented at least one of the following semen parameters: sperm concentration $<20 \times 10^6$/mL, motile sperm <50% [19], normal morphology <14% based on Kruger strict criteria [20]. All patients underwent a hormone profile, physical examination, detailed history, and ruled outpatients with Y-chromosome microdeletions [21]. The control subjects had fathered at least one child within 2 years without assisted reproductive technologies.

2.2. Genotyping and prediction of transcription factor binding sites for SEPT12 promoter region

Genomic DNA was extracted from peripheral blood samples using a Puregene DNA isolation kit (Gentra, Minneapolis, MN, USA). Polymerase chain reaction (PCR) reactions were used to amplify the *SEPT12* promoter region. The primer sequences were as follows: human SEPT12-P, forward 5′CCCGGTAATCCAGCTACTTGG3′ and reverse 5′GGCAGAGAGAGAGAGAAGCAG3′. The PCR reaction consisted of 35 cycles of gene amplification as follows: 95 °C for 30 s, 64 °C for 30–90 s, and 72 °C for 1 min. The PCR products were resolved by electrophoresis on a 1% agarose gel and the appropriate band were excised and purified for the subsequent steps of cloning or sequence determination by ABI 377 automated DNA sequencer (Applied Biosystems, Foster City, California, USA). Putative transcription factor binding sites in the promoter region of *SEPT12* were identified using the Promoter 3.0 Prediction Server (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3).

2.3. Plasmid preparation

Reporter plasmids were constructed by inserting various lengths PCR products of 5′-upstream regions for human *SEPT12* between the *Bgl*II and *Kpn*I sites of pGL3-Basic Vector lacking the promoter (Promega Corp., Madison, WI). An approximately 1.1-kb fragment of the *SEPT12* promoter spanning nucleotides −1038 to +75 (−1038/+75) was sub-cloned into the pGL3-Basic Vector. The deletion constructs (−1038/+75, −721/+75, −481/+75, −245/+75) were generated by PCR. The sequences of PCR primers were: SEPT12-SD4 F: 5′GGGGTACCACTAAACTCCAG3′; SEPT12-SD3 F: 5′GGGGTACCAGCAAGGGCAA3′; SEPT12-SD2 F: 5′CCCCTACCACCTCCTCAC3′; SEPT12-SD1 F: 5′GGGGTACTGGGTTTGCTCA3′ and used the same reverse primer, SEPT12-R: 5′GAAGATCTTGTGACACCTGGT3′. The fidelity of all constructs created was confirmed by nucleotide sequence analysis. The pRL-TK vector (Promega Corp.) containing the Renilla luciferase gene under the thymidine kinase promoter was used as a control. Human androgen and estrogen expression plasmids pEGFP-C1-AR (Addgene, plasmid #28235) and pEGFP-C1-ERα (Addgene, plasmid #28230) were obtained from Addgene (http://www.addgene.org), for the cell overexpression experiments.

2.4. Cell culture

The NT2/D1 cell line (a pluripotent human testicular embryonal carcinoma cell line, ATCC number CRL-1973) and the HeLa cell line (human cervical epithelial adenocarcinoma cell line, ATCC number CRM-CCL-2) were obtained from the Bioresource Collection and Research Center (BCRC) of the Food Industry Research and Development Institute (Taiwan). The cells were routinely maintained in Dulbecco's Modified Eagle's Medium (Gibco Laboratories Inc., Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Life Technologies, Grand Island, NY, USA, 10437-028), 100 U/mL penicillin, and 100 μg/mL streptomycin (Corning Inc Life Sciences, Acton, MA) in a humidified atmosphere at 37 °C and 5% CO_2.

2.5. Transient transfection and luciferase assay

For the promoter activity assay, 7×10^3 cells were seeded into 0.1 mL culture medium supplemented with 10% (*v*/*v*) FBS in each well of a 96-well culture plate and cultured for 16–18 h in a humidified atmosphere at 37 °C and 5% CO_2. Following the 16 h incubation, each well was transfected with 0.3 μg each of Sep12 promoter-pGL3-Basic reporter plasmid and 0.03 μg pRL-TK vector per well using Lipofectamine 3000 Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's manual. About 24 h transfection, firefly and Renilla luciferase activities were measured by using the Dual-Luciferase Reporter assay system, according to the manufacturer's instructions (Promega Corporation, Madison, WI, USA). Renilla luciferase data were used to normalize the firefly luciferase data by calculating the ratio of firefly/Renilla values for transfection efficiency. Data were analyzed using the GraphPad Prism 5.0 statistical software (GraphPad Software, San Diego, CA) [22].

2.6. Chromatin immunoprecipitation (ChIP)

Chromatin extracted from NT2/D1 cells was sheared to fragments of 200–500 bp long by ChIP-IT®Express Enzymatic (Active Motif, Carlsbad, CA) and immunoprecipitated with anti-ERα (Cell Signaling, #8644, Beverly, MA, USA) and anti-AR monoclonal antibodies (Cell Signaling, #5153, Beverly, MA, USA). The immunoprecipitated DNA was purified and subjected to PCR amplification of 169 bp fragments of the *SEPT12* promoter and a 276 bp fragment, about 3 kb upstream of the *SEPT12* promoter, as a negative control. The sequences of PCR primers were: CHIP-hSEPT12-F: 5′CTGGGGT CATTGTGGGGATT3′(-129/-110); CHIP-hSEPT12-R: 5′GGAGCTGCCTT GTCCTGG3′(+22/+39); CHIP-hSEPT12-up3000-F: 5′GCCATAAGACC CACCAATGT3′(-3229/-3210); and CHIP-hSEPT12-up3000-R: 5′AAG CCTGGATGGAATCACAC3′(-3012/-2993). The PCR amplification cycle consisted of an initial denaturation step at 95 °C for 10 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 59 °C for 30 s, extension at 72 °C for 30 s, and a final extension step at 72 °C for 5 min. The PCR products were analyzed by 2.5% (w/v) agarose gel electrophoresis and confirmed by use of ABI 377 automated DNA sequencer (Applied Biosystems, Foster City, California, USA).

2.7. Site-directed mutagenesis

The truncated ER1, ER2, and AR binding sites *SEPT12* and point mutations of SNPs (rs759992 T > C, rs3827527 C > T) in the *SEPT12* promoter were carried out using pGL3-Basic-SEPT12-SD1 as template according to manufacturer's instructions of the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA). The sequences of site-directed mutagenesis primers were: ER1: SEPT12-mut-ER1-F: 5′GTATTATTAGGCTGG GAAGCTTGTGGGGATTA3′, SEPT12-mut-ER1-R: 5′CTAATCCCCACA AGCTTCCCAGCCTAATAATAC3′; ER2: SEPT12-mut-ER2-F: 5′GCCTC CAGGTGGGATCCACAGCAAGTGCAGATG3′, SEPT12-mut-ER2-R: 5′CATCTGCACTTGCTGTGGATCCCACCTGGAGGC3′ and AR: SEPT12-mut-AR-F: 5′CAGGCCCTGCCAGGGTCGACCAGCTCCTGGAAG3′, SEP T12-mut-AR-R: 5′CTTCCAGGAGCTGGTCGACCCTGGCAGGGCCTG3′. rs759992: SEPT12-SNP-rs759992-F: 5′CCTGGTTCTACTTGGGGATC ATTGTATTATTAGGCTGGG3′, SEPT12-SNP-rs759992-R: 5′CCCAGCCT AATAATACAATGATCCCCAAGTAGAACCAGG3'; rs3827527: SEPT12-SNP-rs3827527-F: 5′CACCAGGCCCTGCCAGGATAAGGCAGCTCCTG G3′, SEPT12-SNP-rs3827527-R: 5′CCAGGAGCTGCCTTATCCTGGCA GGGCCTGGTG3'. The pGL3-Basic-SEPT12-SD1$^{\Delta ER1}$, pGL3-Basic-SEPT12-SD1$^{\Delta ER2}$, pGL3-Basic-SEPT12-SD1$^{\Delta AR}$ represent the truncated ER1, ER2, and AR binding sites in *SEPT12* promoter of pGL3-Basic-SEPT12-SD1, respectively. The sequence fidelity of all mutation constructs was confirmed by use of ABI 377 automated DNA sequencer (Applied Biosystems, Foster City, California, USA).

2.8. 17β-estradiol (E2) and 5α-dihydrotestosterone (5α-DHT) treatment

NT2D1 cells were seeded into 0.1 mL culture medium containing 5% (v/v) charcoal stripped FBS in each well of a 96-well culture plate(7×10^3/well) in a humidified atmosphere at 37 °C and 5% CO_2 for overnight. The charcoal-stripping of fetal bovine serum (FBS) was intended for supplementation of cell culture medium to remove a variety of endogenous compounds, including steroid, peptide, and thyroid hormones [23,24]. Each well was transfected with 0.3 μg *SEPT12* reporter plasmid and 0.03 μg pRL-TK vector per well using Lipofectamine 3000 reagent for 24 h. Cells were treated with E2 and 5α-DHT before luciferase assay for 24 h. The cells were lysed with lysis buffer, and the supernatants were used for the measurement of firefly and Renilla luciferase activities, and RT-qPCR and protein analysis. Promoter activity was calculated as the ratio of firefly/Renilla values. Data were analyzed using the GraphPad Prism 5.0 software. E2 and 5α-DHT were stored in 100% ethanol at a concentration of 1 μM and 10 μM, respectively. Statistical analysis was carried out using *t*-test. Means were considered significantly different at $p < 0.05$.

2.9. Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

RT-qPCR was performed essentially as described before [25]. The forward/reverse primers for RT-qPCR were designed across one intron (68 bp) to eliminate the problems of genomic DNA contamination in the samples. The primer sets were: human *SEPT12* forward, 5′TGGTGAACGGGAGGTGTGT3′ and reverse, 5′ CGCCATGTTCTCCACTTCAA 3'; β-actin forward, 5′GGCACCCAGCA CAATGAAG3′ and reverse, 5′ CCGATCCACACGGAGTACTTG 3'. The RT-qPCR reactions were performed in a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).

2.10. Western blot analysis

Western blot analysis was performed as detailed before [22] using *SEPT12* purified MaxPab mouse polyclonal antibody (Abnova, Taipei, Taiwan) and mouse monoclonal anti-β-actin antibody (Sigma, A9044). β-actin was used as a control in Western blot analysis.

2.11. Statistical analysis

The values shown were mean ± S.D. from three independent experiments in triplicate. Data were analyzed by student's t-test. The significance levels for all statistical tests were at $P > 0.05$ by the GraphPad Prism software (http://www.graphpad.com/prism/prism.htm).

3. Results

3.1. Promoter activity in the 5′-flanking region of the human SEPT12 gene

In this study, we confirmed the human *SEPT12* promoter by transfecting a series of deletion constructs, 5′-upstream region from positions −1038 to +75 bp (−1038/+75) of human *SEPT12*, cloned in the pGL3-Basic vector, transfected into human testicular embryonal carcinoma NT2/D1 cells (AR^+, $ER\alpha^+$) and cervical epithelial adenocarcinoma HeLa cells (AR^-, $ER\alpha^+$), and analyzed with a dual luciferase reporter system for promoter activity assay. Up to −245 bp had a substantial increase in the promoter activity in NT2/D1 and HeLa cells (Fig. 1 A, B). These findings indicated that the −245/+75 bp upstream region of the human *SEPT12* is essential for the transcription activity in testicular cells in addition to some negative regulatory elements in the −1038/-245 bp upstream region of *SEPT12* gene.

3.2. The human SEPT12 promoter region contains estrogen receptor α (ERα) and androgen receptor (AR) binding sites

Bioinformatics analysis was available several consensus transcription factors binding sites in the human *SEPT12* 5′-flanking sequence by the PROMO 3.0 Prediction Server (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) (Fig. 1C). There are putative binding sites for ERα (−125/-121, −61/-57) and AR (+25/+33) within the human *SEPT12* promoter region. These estrogen or androgen receptor binding sites within the

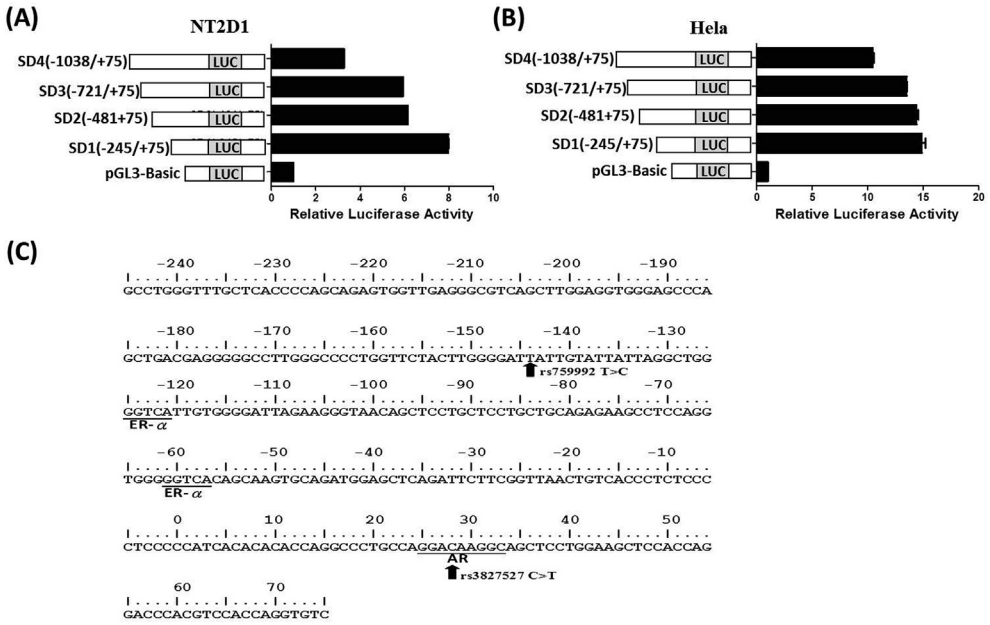

Fig. 1. Promoter identification of the *SEPT12*. Segments spanning various lengths of the *SEPT12* 5′ flanking region (−1038/+75, −721/+75, −481/+75, −245/+75) were sub-cloned into the pGL3-Basic luciferase reporter vector. Nucleotide numbering starts with +1 corresponding to the transcription start site (TSS) which was determined according to NM_001154458. (**A**) NT2/D1 and (**B**) HeLa Cells were transfected with either the empty vector or a *SEPT12* promoter reporter vector in conjunction with the control Renilla luciferase pRL-TK vector for normalization. Extracts were taken 24 h after transfection and the luciferase activity was determined. Luciferase activities were normalized to the Renilla luciferase activities and shown as fold of pGL3-Basic. Mean ± S.D. of triplicates from three independent experiments are shown. (**C**) The sequence of the *SEPT12* promoter region, SNPs and prospective transcriptional factor binding sites, as predicted by the PROMO 3.0 Prediction Server (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3). The binding sites of known transcription factors are underlined. Empty arrows indicate the SNPs. AR: androgen receptor, ER-α: estrogen receptor α.

human *SEPT12* promoter might play an important role in transcriptional regulation.

3.3. Estrogen receptor α (ERα) and androgen receptor (AR) bind to the human SEPT12 promoter

We hypothesized that ERα and AR bind to the SEPT12 promoter to regulate the gene transcription under endogenous estrogen and androgen. To evaluate this hypothesis, we used chromatin immunoprecipitation analysis (ChIP) to determine that ERα and AR directly bind to the ERα and AR sites within the SEPT12 promoter. As shown in Fig. 2A and C, the predicted 169 bp PCR product in *SEPT12* promoter was amplified from samples derived from ERα and AR immunoprecipitation, but not found in rabbit control IgG and up3000 control PCR amplicon (−3229 ~ −2993). ChIP with rabbit control IgG did not enrich *SEPT12* promoter regions, demonstrating the specificity for ERα and AR transcription factors on *SEPT12* promoter regions. No PCR amplicon in the up3000 reaction was demonstrated that ChIP was specific for *SEPT12 nearby region* (Fig. 2 A, C). The sequence of the ChIP-PCR product was identical to the human *SEPT12* promoter region (Fig. 2B, D). These data demonstrated that ERα and AR interacted with the human *SEPT12* promoter.

3.4. SEPT12 promoter activity is regulated by estrogen and androgen

Since estrogen and androgen regulate downstream gene expression through their binding to ERα and AR, we further verified whether ERα and AR directly regulate *SEPT12* transcription. We generated four luciferase reporter constructs in pGL3-Basic, including pGL3-Basic-SEPT12-SD1 (non-mutant, wild), which contained the −245/+75 segment of the *SEPT12* promoter, and pGL3-Basic-SEPT12-SD1$^{\Delta ER1}$, pGL3-Basic-SEPT12-SD1$^{\Delta ER2}$, pGL3-Basic-SEPT12-SD1$^{\Delta AR}$, in which the truncated ERα or AR binding sites of *SEPT12* promoter are located, respectively. As shown in Fig. 3, these truncated SD1 had substantially decreased promoter activity than integral SD1 in NT2/D1 and HeLa cells. When ERα or AR binding sites of *SEPT12* promoter were truncated, *SEPT12* lost its transcription activity even in the presence of endogenous estrogen and androgen hormones (Fig. 3). Thus, estrogen and androgen hormones were essential for the proper transcription activity of *SEPT12* promoter. These data indicated that the full basal *SEPT12* promoter activity in NT2/D1 cells requires the conserved ERα and AR binding sites.

Steroid hormones (estrogen, androgen) as ligand-dependent transcription factors bind to the receptor to form the hormone-receptor complexes that bind to the promoter region of target genes and stimulate gene transcription. We hypothesized that estrogen, androgen and their related receptors (ERα, AR) form hormone-receptor complexes and bind to *SEPT12* promoter to regulate *SEPT12* gene expression. To verify this hypothesis, 10 nM of 5α-dihydrotestosterone (5α-DHT) or 1 nM of 17-β-oestradiol (E2) were used to treat the pGL3-Basic-SEPT12-SD1-transfected cell. The 5α-DHT or E2 had substantially increased the promoter activity of *SEPT12* (Fig. 4A). The expression of *SEPT12* significantly increased under androgen (5α-DHT) or estrogen (E2) treatment by quantitative real-time reverse transcription-polymerase chain reaction analysis (Fig. 4B) and immunoblotting (Fig. 4C and D). These results demonstrated that transcriptional activity and gene expression of *SEPT12* were regulated by 5α-DHT or E2.

Overexpression of AR or ERα by co-transfection with pEGFP-C1-ERα and pGL3-Basic-SEPT12-SD1 increased the promoter activity in NT2/D1 cells (AR$^+$, ERα$^+$) (Fig. 5A and B) and HeLa cells (AR$^+$, ERα$^-$) (Fig. 5C and D). Furthermore, the transcription activity of the *SEPT12* promoter was significantly increased when AR or ERα was overexpressed in the presence of 5α-DHT or E2. However, the promoter activity of *SEPT12* was significantly decreased when AR or

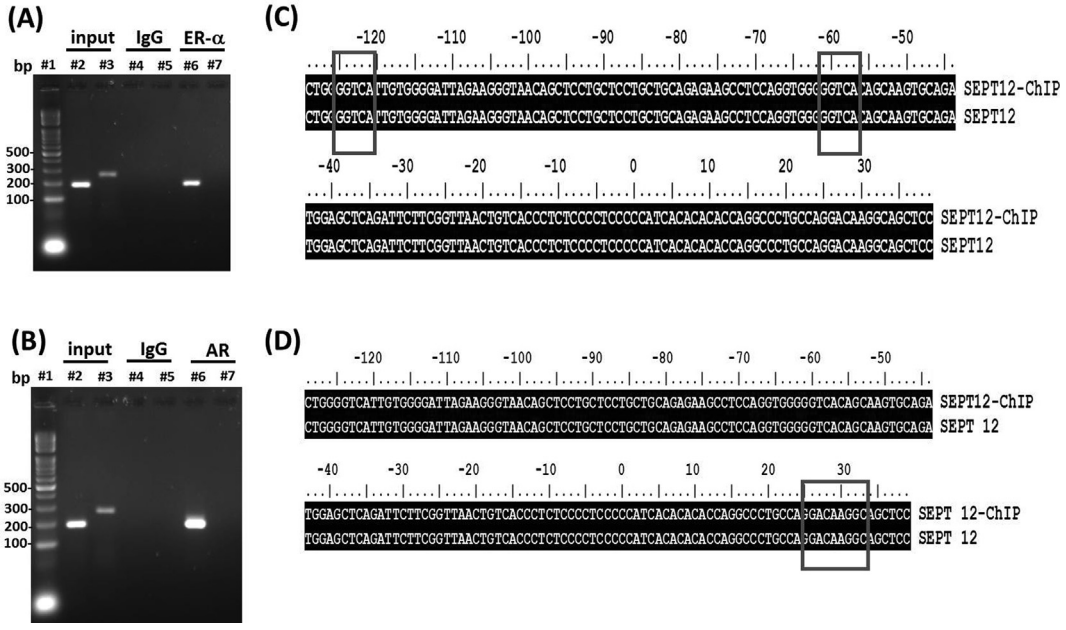

Fig. 2. Estrogen receptor alpha (ERα) and androgen receptor (AR) bind to the *SEPT12* promoter. Formaldehyde-crosslinked chromatin prepared from NT2/D1 cells was immunoprecipitated (IP) with anti- ERα (**A**, lanes 6, 7) or androgen receptor (AR) (**B**, lanes 6, 7) antibodies for the *SEPT12* promoter. The rabbit IgG was used as a negative control of antibody specificity (lane 4, 5). A fragment of comparable size amplified from a site about 3000 bp upstream of *SEPT12* transcription start site was used as a negative control for *SEPT12* specificity of immunoprecipitated chromatin (lane 5, 7) or input DNA (lane 3). Sequence alignment of the *SEPT12* promoter and the ChIP PCR product with anti-ER-α (**C**) or AR (**D**) antibodies for the *SEPT12* promoter. The ERα or AR binding sites are boxed. Approximately 0.4% of the input DNA (without immunoprecipitation) (lane 2, 3) and 5% of the precipitated DNA (lane 4–7) were used as templates for each PCR analysis using primers targeting promoter (lane 2, 4, 6) and up3000 bp region of *SEPT12* (lane 3, 5, 7).

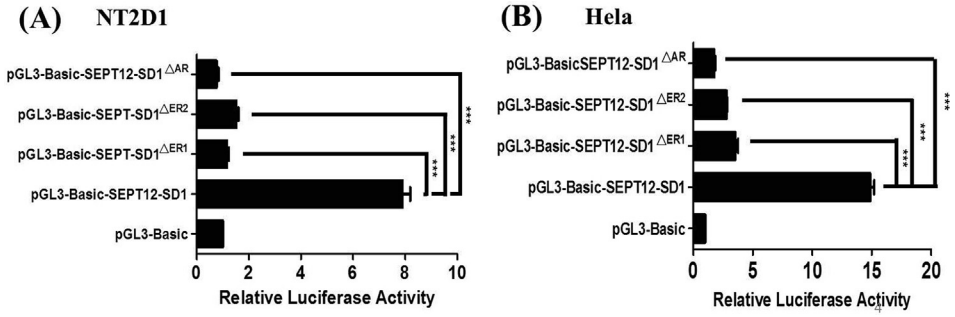

Fig. 3. The binding sites of ERα and AR **are essential for *SEPT12* promoter activity.** (**A**) NT2/D1 and (**B**) HeLa cells were transiently transfected with luciferase reporter plasmids: empty pGL3-Basic, pGL3-Basic-SEPT12-SD1, and pGL3-Basic-SEPT12-SD1$^{\Delta ER1,}$ pGL3-Basic-SEPT12-SD1$^{\Delta ER2,}$ pGL3-Basic-SEPT12-SD1$^{\Delta AR}$ contained truncated ER1, ER2, and AR binding site of *SEPT12* promoter in pGL3-Basic. The *SEPT12* reporter activities were normalized by *Renilla* luciferase activity derived from the co-transfected control vector (pRL-TK). Data shown are mean ± S.D. of duplicated samples and are representative of at least three independent experiments. $^{***}p < 0.001$, versus pGL3-Basic-SEPT12-SD1.

ERα binding sites of *SEPT12* promoter were truncated (Fig. 5A and B). Taken together, the above data indicated that AR and ERα were essential for the promoter activity of *SEPT12* in the presence of 5α-DHT or E2. There was a substantial increase in *SEPT12* promoter activity when AR and ERα were overexpressed simultaneously (Fig. 5E).

3.5. Single nucleotide polymorphisms (SNPs) of promoter effect transcription activity of SEPT12

The promoter of *SEPT12* is located 245 bps upstream to the transcription start site (TSS). One single nucleotide polymorphism (rs759992 T > C) in the *SEPT12* gene promoter was identified in our study subjects of Taiwanese, but not more prevalent in the infertile men ($p = 0.5206$) (Table 1 of Supplementary data). Another SNP, rs3827527 C > T (source: NCBI databank), was found in the AR binding site of the *SEPT12* promoter region, but not found in our study subjects. We constructed these two SNPs (rs759992 T > C, rs3827527 C > T) into the pGL3-Basic-SEPT12-SD1 by site-directed mutagenesis, respectively. These two SNPs (rs759992 T > C, rs3827527 C > T) were found to decrease the transcription activity of *SEPT12* (Fig. 6).

4. Discussion

SEPT12 is highly expressed in the post-meiotic male germ cells in the testis. *SEPT12* is expressed mainly at the edge of the nucleus in humans and mice sperm [26]. It was observed that *SEPT12* is significantly downregulated in the testicular tissues of severe spermatogenic defect patients, such as maturation arrest (MA) and Sertoli cell-only syndrome (SCOS) [5,27]. In addition, Septin12$^{+/-}$ chimeric mice were sterile with severe spermatogenic defects [28]. Thus, the *SEPT12* gene plays a crucial role in the spermatogenesis.

However, the transcriptional regulation of *SEPT12* in the spermatogenesis is not well understood. In this study, we explored the *promoter* and the transcriptional regulation of the *SEPT12* gene by

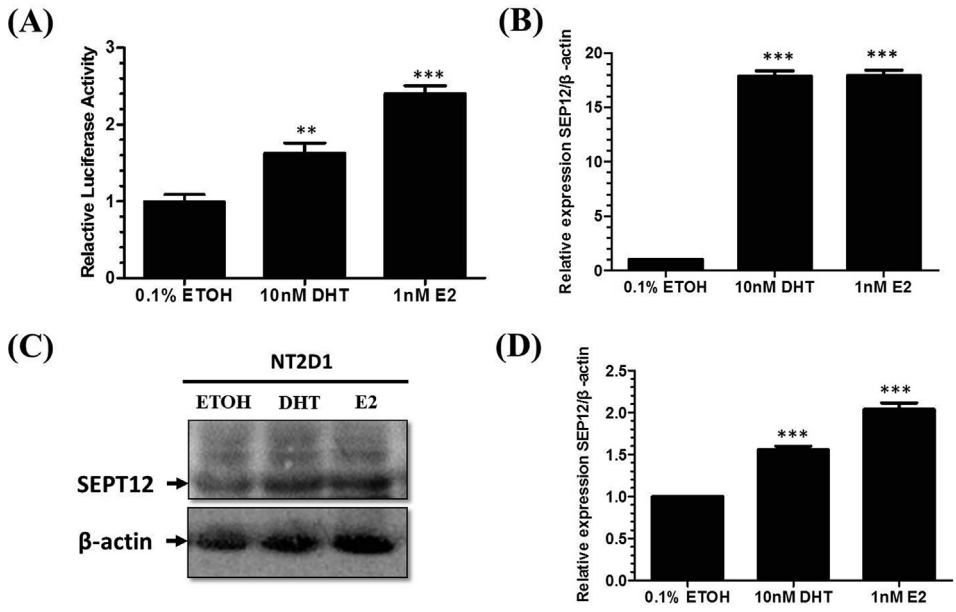

Fig. 4. Enhancement of *SEPT12* transcription expression by 5α-Dihydrotestosterone (5α-DHT) and17-β-Oestradiol (E2). (**A**) NT2/D1 cells were transiently co-transfected with luciferase reporter plasmids (pGL3-Basic-SEPT12-SD1) and pRL-TK reporter plasmids. Twenty four hours after transfection, cells were stimulated with either 10 nM 5α-DHT or 1 nM E2 for 24 h. Luciferase activities were normalized to pRL-TK reporter activities and shown as fold induction compared with vehicle control. (B) NT2/D1 cells were cultured in H-DMEM medium supplemented with 5% charcoal stripping FBS for one day and then treated with 10 nM 5α-DHT 1 nM E2 for 24 h. After incubation, total RNA or protein was isolated from cells and subjected to quantitative real-time reverse transcription-polymerase chain reaction analysis (**B**) and Western blotting analysis (**C**), respectively. β-Actin was used as loading control for Western blots. (**D**) Densitometric analysis of the androgen receptor (AR), estrogen receptor (ERα), and *SEPT12* protein levels relative to β-actin. The gels were quantified using MultiGel-21 (Multi-function Gel Image System) and ImageJ. All data represent the mean ± S.D. of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, versus 0.1% ethanol (EtOH).

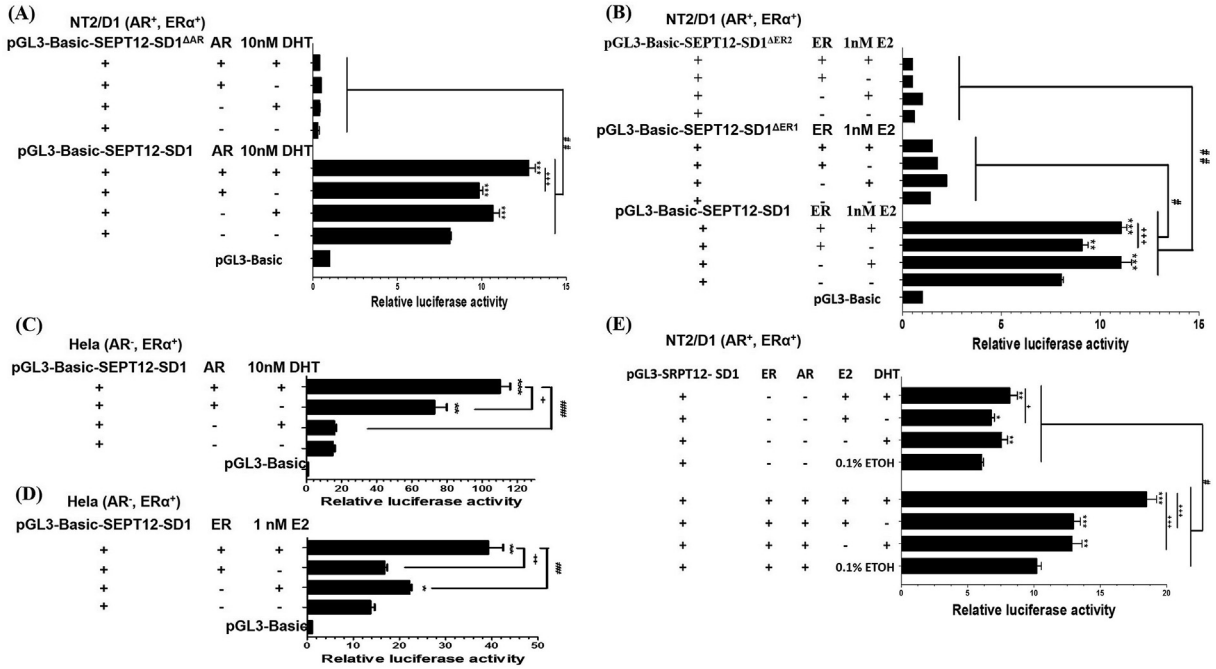

Fig. 5. The AR and ERα are essential for *SEPT12* transcriptional activity. The NT2/D1 (**A, B**) and HeLa (**C, D**) cells were co-transfected with pGL3-Basic-SEPT12-SD1 or truncated pGL3-Basic-SEPT12-SD1$^{\Delta ER1,}$ pGL3-Basic-SEPT12-SD1$^{\Delta ER2,}$ pGL3-Basic-SEPT12-SD1$^{\Delta AR}$, and androgen/estrogen receptor expression vectors pEGFP-C1-AR(**A, C**) or pEGFP-C1-ERα(**B, D**), incubated for 24 h and then treated with 10 nM 5α-DHT (**A, C**) or 1 nM E2 (**B, D**) for 24 h and then luciferase transcription activity was measured. *SEPT12* promoter activity was detected by luciferase assay. AR: pEGFP-C1-AR expression vectors, ER: pEGFP-C1-ERα expression vectors. (**A**) $^{***}p < 0.001$, versus mock (no overexpression and no add hormone) by Student's t-test; $+++p < 0.001$, difference from 10 nM DHT treatment; $^{\#\#}p < 0.01$, pGL3-Basic-SEPT12-SD1 versus pGL3-Basic-SEPT12-SD1$^{\Delta AR}$ by Student's t-test. (**B**) $^{**}p < 0.01$, $^{***}p < 0.001$, versus mock (no overexpression and no add hormone) by Student's t-test; $+++p < 0.001$, difference from 1 nM E2 treatment; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, pGL3-Basic-SEPT12-SD1 versus pGL3-Basic-SEPT12-SD1$^{\Delta ER1}$, pGL3-Basic-SEPT12-SD1$^{\Delta ER1}$ by Student's t-test. (**C**) $^{**}p < 0.01$, $^{***}p < 0.001$, versus mock (no overexpression and no add hormone); $+p < 0.05$, difference from 10 nM DHT treatment; $^{\#\#\#}p < 0.001$, difference from AR overexpression by Student's t-test. (**D**) $^{*}p < 0.05$, $^{**}p < 0.01$, versus mock (no overexpression and no add hormone); $++p < 0.01$, difference from 1 nM E2 treatment; $^{\#\#\#}p < 0.001$, difference from ER overexpression by Student's t-test. (**E**) The NT2/D1 cells were co-transfected with pGL3-Basic-SEPT12-SD1 and pEGFP-C1-AR and pEGFP-C1-ERα expression vectors were incubated for 24 h and then treated with 10 nM 5α-DHT or 1 nM E2 for 24 h and then luciferase promoter activity was measured. $^{*}p < 0.05$,$^{***}p < 0.001$, versus mock (no overexpression and no add hormone); $+p < 0.05$, $+++p < 0.001$, difference from 10 nM DHT treatment; $^{\#\#\#}p < 0.001$, difference from ER and AR overexpression.

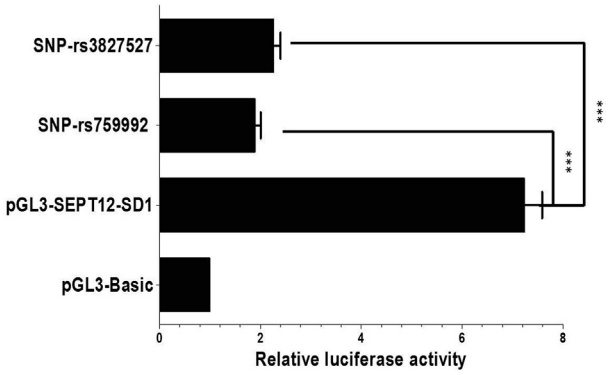

Fig. 6. Single-nucleotide polymorphisms of the *SEPT12* promoter affect transcriptional activity. The SNPs rs759992 T > C and rs3827527 C > T were introduced into the pGL3-SD1-SEPT12 plasmids by site-directed mutagenesis, respectively. NT2/D1 cells were co-transfected with pGL3-Basic-SEPT12-SD1 or pGL3-Basic-SEPT12-SD1-rs759992 T > C (SNP-rs759992), and pGL3-Basic-SEPT12-SD1-rs3827527 C > T (SNP-rs3827527) and the luciferase transcription activity was measured. SEPT12 promoter activity was detected by luciferase assay. Data shown are mean ± S.D. of duplicated samples and are representative of at least three independent experiments. ***$p < 0.001$, versus pGL3-Basic-SEPT12-SD1.

estrogens and androgens. We found that the promoter of the *SEPT12* gene (−245/+75) was located at 245 bp upstream to 75 bps downstream of the transcription start site (TSS). These findings indicated that the −245/+75 bp upstream region of the human *SEPT12* is essential for transcription activity in testicular cells and there are some negative regulatory elements or factors in the upstream region (~1 Kb) of *SEPT12* gene. Two estrogen receptor α (ERα) and one androgen receptor (AR) transcription factor binding sites located in the promoter of the *SEPT12* gene. The interaction of ERα and AR with the *SEPT12* gene was confirmed by the chromatin immunoprecipitation analysis (Fig. 2). The result of chromatin immunoprecipitation analysis (Fig. 2) strongly correlates with the locations of the ERα and AR binding sites on the *SEPT12* promoter determined by luciferase reporter activity. The importance of estrogen and androgen hormones for *SEPT12* promoter activity was confirmed by the ability of exogenous hormones to significantly increase *SEPT12* promoter activity (Fig. 4). In addition, when ERα or AR binding sites of *SEPT12* promoter were truncated, *SEPT12* lost its transcription activity even in the presence of endogenous estrogen and androgen hormones (Fig. 3).

Furthermore, the transcription activity of the *SEPT12* promoter was significantly increased when AR or ERα was overexpressed in the presence of 5α-DHT or E2 and decreased when AR or ERα binding sites of *SEPT12* promoter were truncated (Fig. 5). There was a substantial increase in *SEPT12* promoter activity when AR and ERα were overexpressed under both DHT and E2 simultaneous treatment (Fig. 5E). Thus, *SEPT12* gene expression is regulated by one androgen and two estrogen hormone responsive elements located in the promoter region. These data demonstrated that the transcription factors of two estrogen receptor α (ERα) and one androgen receptor (AR) synergistically interact to regulate the gene expression of *SEPT12*.

Two SNPs were observed in the promoter region, yet there was no significant difference in the incidence rate of SNPs between 160 infertile men with abnormal semen parameters and 200 fertile men with normal semen parameters in the Taiwanese subjects (Table 1 of Supplementary data). The application of site-directed mutagenesis coupled with promoter activity analysis showed that the SNPs rs759992 in the binding site of androgen receptor significantly reduced the promoter activity (Fig. 6). These results indicated that androgen is an important factor for the transcriptional activity of *SEPT12*. Based on this, we hypothesized that these SNPs affect the *SEPT12* promoter activity. However, these SNPs were not ubiquitous in Taiwanese men and they may not have a major role as a cause of non-obstructive azoospermia.

Androgens and estrogens regulate gene expression by binding to their corresponding receptors in the promoter area of their target genes [29–36]. Upon the binding of AR to androgen response elements, multiple co-regulators are recruited and the transcription of androgen-regulated genes involved in cell growth and survival is activated [37–39]. Many genes, which are involved in spermatogenesis are directly or indirectly regulated by androgens and estrogens and so, play a major role in spermatogenesis and males fertility [40–42]. For example, AR knockout (ARKO) male mice were infertile and completely insensitive to androgens. Many genes involved in spermatogenesis are regulated by androgens and the AR via binding to the androgen response element (ARE) of the gene promoter [43–47]. Thus, we suggested that *SEPT12* is one of the genes involved in the AR signals controlling spermatogenesis. While SNP rs3827527 C > T, as sourced from the NCBI databank, was observed in the AR binding site of the *SEPT12* promoter region, it was not identified in our study subjects. Although the promoter activity was significantly decreased when rs3827527 C > T was introduced into the *SEPT12* promoter (Fig. 6), we suggested that SNPs in *SEPT12* promoter are not a major factor for the infertility in our study subjects (Table 1 of Supplementary data).

Estrogen is produced by the testis and acts via ERα and ERβ to activate or repress the transcription of specific genes in testicular development and function in spermatogenesis [48]. The administration of E2 to adult male rats caused the upregulation of 33 genes and the downregulation of 67 genes in the presence of estrogen response elements (EREs) [35]. In addition, E2 was decreased significantly in 2n cells (somatic and germ cells) and in 4n cells (pachytene spermatocytes) and increased in elongated and elongating spermatids [35]. In this study, the two truncated ERα binding sites of the *SEPT12* promoter decreased *SEPT12* transcription activity and demonstrated that ERα regulates *SEPT12* promoter activity in NT2/D1 cells (AR^+, $ER\alpha^+$) (Fig. 5A and B) and HeLa cells (AR^+, $ER\alpha^-$) (Fig. 5C and D).

In general, the androgen receptor as a homodimer, interacts with transcription factors including nuclear factor B (NF-κB) [49,50], AP-1 [51], Smad3 [52], and sex-determining region Y (SRY) [53]. Some studies reported that AR as a heterodimer interacts with the estrogen receptor (ERα) [54], glucocorticoid receptor (GR) [55], testicular orphan receptor 4 (TR4) [56], and alters AR transcriptional activity. In this study, AR and ERα binding sites were identified at the promoter of *SEPT12*. In addition, there was a significant increase in the promoter activity of *SEPT12* when AR and ERα were simultaneously overexpressed (Fig. 5E). However, more evidence is needed to identify ARs interaction, as a heterodimer, with ERα or others. Together, we provided the first clue about the regulatory mechanisms of *SEPT12*. To the best of our knowledge, this is the first report on the association of the transcriptional regulation of *SEPT12* and the susceptibility to AR and ERα control. Since SEPT12 has an important role in spermatogenesis, *SEPT12* expression analysis can be developed as a potential tool for the assessment of environmental or food pollution by hormones or for the endocrine-disrupting chemicals (EDCs) in general.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Author agreement

All authors have made significant contributions to the study, have read, and approved the manuscript. We verify that the

material in the manuscript has not been previously reported, that the manuscript is not under consideration for publication elsewhere, and that it will not be submitted elsewhere while under review by Biochimie.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biochi.2018.11.018.

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