Original Article

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Yangjing Capsule extract promotes proliferation of GC-1 spg cells *via* up-regulated POU3F1 pathway

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Summary **As is similar to glial cell line-derived neurotrophic factor (GDNF), the Yangjing Capsule (YC) extract could also lead to proliferation of spermatogonial stem cells (SSCs) by stimulating the phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) pathway; however, the regulatory effect of YC extract on the expression of POU3F1 still remains unknown. The objective of this study is to determine whether the transcription factor POU3F1 is up-regulated by YC extract through the PI3K/AKT signaling pathway to regulate SSCs survival and proliferation. Cultured GC-1 spermatogonial (spg) cells were treated with 0.01, 0.1, and 1 mg/mL YC extract for 48 h. Cell viability was analyzed using MTT assay, while POU3F1 expression was quantitatively detected using real time-polymerase chain reaction and Western blot analysis. POU3F1, GDNF family receptor alpha1 (GFRα1) short interfering ribonucleic acid (siRNA), and LY294002 (PI3K inhibitor) were applied as blockers to explore the underlying pathway. After 48 h treatment with YC extract, GC-1 spg cells proliferated and POU3F1 expression was significantly increased in a dose-dependent manner. POU3F1 siRNA partially blocked those effects of YC extract. Both GFRα1 siRNA and LY294002, as upstream blockers, reduced POU3F1 expression induced by YC extract. The conclusion is that YC extract promotes proliferation of GC-1 spg cells *via* up-regulation of POU3F1. The potential mechanism is that YC extract triggers the activation of the PI3K/AKT pathway and then up-regulates POU3F1 expression.**

Keywords: Yangjing Capsule (YC), glial cell line-derived neurotrophic factor (GDNF), spermatogonial stem cells (SSCs), POU3F1

1. Introduction

Spermatogonial stem cells (SSCs) are essential for maintaining male fertility as well as species continuity and provide the foundation for spermatogenesis (*1*). The SSCs are the only stem cells in the body that undergo self-renewal throughout the lifetime and transmit genetic information to subsequent generations (*2,3*). Similar to other tissue-specific stem cell populations, the SSCs also maintain tissue homeostasis by retaining the capacity for self-renewal and differentiation (*4*), and their fate decisions are controlled by intrinsic molecular pathways which are activated by extrinsic signals such as growth factor stimuli. Self-renewal and differentiation of rodent SSCs including mouse (*5*), rat (*6,7*), and hamster (*8*), depend on the response to the growth factor glial cell line-derived neurotrophic factor (GDNF). By binding to the glycosylphosphatidylinositol-anchored cell surface molecule of GDNF family receptor alpha1 (GFRα1), GDNF is able to trigger the phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) pathway and up-regulate expression of POU3F1 and eventually lead to survival and proliferation of SSCs (*9-11*) (Figure 1).

The POU domain transcription factors, also known as the octamer (OCT)-binding family of transcription

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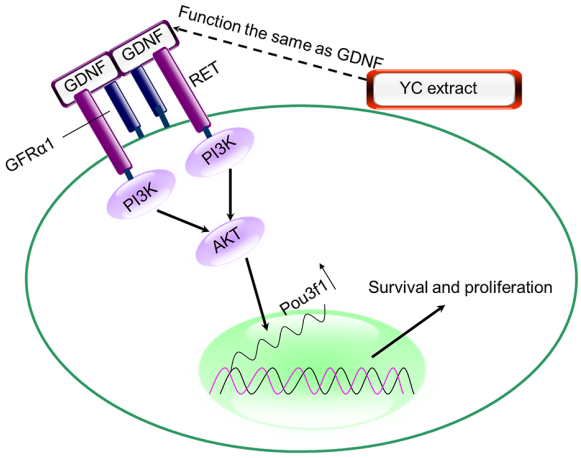

Figure 1. Mechanisms of GDNF and YC extract promoting the proliferation of spermatogonial stem cells (SSCs). GDNF binds with GFRα1 through RET tyrosine kinase to activate PI3K/AKT intracellular signaling pathway and up-regulate the transcription factor POU3F1, and thus leads to SSCs proliferation. The YC extract triggers the activation of PI3K/AKT pathway and then up-regulates POU3F1 expression similar to GDNF.

factors, have played diverse roles in cellular processes and stem cell functions (*12*). POU3F1 (also known as OCT6, TST-1) is a POU-subclass homeobox transcription factor, and it is expressed in testis as well as in brain. In previous studies, POU3F1 has been examined as a regulator of neural cell development (*13-15*). Targeted disruption of POU3F1 expression in mice caused neonatal lethality in which pups displayed abnormal myelination of the axon sheath (*13*). POU3F1 was then first recognized as a candidate involved in SSCs when it was shown to be highly expressed in rat germ cells with enriched SSC activity (*16*). *In vivo*, POU3F1 is expressed by all proliferating spermatogonia (*17*), one previous large-scale microarray analyses revealed that POU3F1 gene expression was regulated by GDNF in cultured SSCs containing $THY1^+$ germ cell populations (*18*). In another study, it was demonstrated that SSCs were induced by the GDNF-PI3K-AKT-POU3F1 pathway in mouse $Thy1^+$ spermatogonial cell cultures (*10*). Moreover, reduction of POU3F1 gene expression by short interfering ribonucleic acid (siRNA) treatment resulted in apoptosis in cultured germ cell populations, and further transplantation analyses revealed impaired SSCs activity *in vitro*. Hence, POU3F1 has been examined as an important intrinsic regulator of GDNF-induced survival and self-renewal of SSCs (*10*).

Yangjing Capsule (YC), a Traditional Chinese Medicine (TCM) formula, primarily contains 11 kinds of herbs (Herba Epimedii Brevicornus, Placenta Hominis, Concha Ostreae, Radix Angelicae Sinensis, Hirudo, Semen Astragali Complanati, Rhizoma Polygonati Sibirici, Radix Rehmanniae preparata, Semen Vaccariae Segetalis, Radix Astragali Mongolici and Semen Litchi). Our team has conducted clinical and experimental studies on YC extract for nearly ten years. It could significantly improve sperm density, vitality, and DNA integrity in infertile males (*19,20*). Moreover, after YC extract therapy, noticeable sperm was found in the semen of patients' with azoospermia who suffered from DAZ gene deletion (*21*). Further investigations indicated that spermatogenesis could be enhanced by YC extract several ways. The YC extract stimulated mouse Leydig tumor cells to secrete testosterone which motivated peritubular myoid cells to produce factors influencing SSCs maintenance (*22,23*). The YC extract could also contribute to proliferation of SSCs by motivating the PI3K/AKT pathway, revealing the same functionality as GDNF (*24*). However, the regulatory effect of YC extract on expression of POU3F1 still remains unknown. Thus, the objective of the present study is to determine whether the transcription factor POU3F1 is up-regulated by YC extract through the PI3K/AKT signaling pathway to regulate SSCs survival and proliferation.

2. Materials and Methods

2.1. *Chemicals*

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, and lyophilized trypsin-ethylenediaminetetraacetic acid were purchased from GIBCO BRL (Grand Island, NY, USA). 3-[4,5-Dimethylthiazolyl-2]-2,5-diphenyltetrazolium bromide (MTT), diethyl pyrocarbonate, dimethyl sulfoxide (DMSO), sodium dodecyl sulfate (SDS), ammonium peroxodisulphate, and Tris-hydrochloride were obtained from Sigma (St. Louis, MO, USA). The recombinant murine GDNF was purchased from Pepro Tech (Rocky Hill, NJ, USA). TRIzol reagent, PrimeScript RT Master Mix, and SYBR Green PCR Master Mix reagent kits were obtained from TaKaRa (TaKaRa Biotechnology, Dalian, China). The primers were synthesized by Invitrogen Life Technologies (Carlsbad, CA, USA). The whole protein extraction kits were purchased from KeyGen (KeyGen Biotech. Co. Ltd., Nanjing, China). The goat polyclonal anti-POU3F1 and rabbit polyclonal anti-GFRα1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The rabbit monoclonal anti-AKT (phospho S473) was purchased from Abcam (Cambridge, MA, USA). The mouse monoclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from BioWorld (St. Louis Park, MN, USA). Enhanced Chemiluminescence was obtained from Amersham Biosciences (Uppsala, Sweden).

2.2. *Preparation of YC extract*

The YC consists of 11 traditional Chinese drugs: 13.3%

Yinyanghuo (Herba Epimedii Brevicornus), 13.3% Wangbuliuxing (Semen Vaccariae Segetalis), 13.3% Muli (Concha Ostreae (calcined)), 10% Danggui (Radix Angelicae Sinensis), 10% Huangqi (Radix Astragali Mongolici), 6.7% Shayuanzi (Semen Astragali Complanati), 6.7% Ziheche (Placenta Hominis), 6.7% Huangjing (Rhizoma Polygonati Sibirici), 6.7% Lizhihe (Semen Litchi), 6.7% Shuizhi (Hirudo), and 6.7% Shudihuang (Radix Rehmanniae Preparata). The YC extract was prepared based on the methods described by Kao (*25*) *et al*. and Hu (*26*) *et al*. The content of the YC (3.33 g, equivalent to 10 g of crude drug) was extracted with 333 mL of double distilled water and subsequently subjected to ultrasonic extraction for 45 min. The supernatant was collected and the residue was dissolved and extracted in a similar manner. The two solutions were combined and centrifuged at 13,000 g for 30 min at 4°C to collect the supernatant, which was concentrated to 100 mL with a rotary evaporator at 60°C. The final concentration of the YC extract corresponded to 100 mg/mL of the crude herbal dose. A radioimmunoassay (RIA) confirmed that there was no GDNF in the YC extract, and RIA was performed to avoid the influence of GDNF on the GC-1 spg cells. The pH of the extract was adjusted to 7.0, and the extract was sterilized by filtration on a super clean bench and stored at -80°C for use.

2.3. *Cell culture and treatment*

The mouse GC-1 spermatogonial (spg) cell line was obtained from ATCC (CRL-2053). The GC-1 spg cells were cultured in DMEM medium supplemented with 10% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin, and then incubated in a 5% CO_2 incubator at 37°C. The YC extract was diluted with DMEM without serum to make various concentrations. In the clinical setting, the YC was administered to treat male infertility at a dose of 2 pills three times a day. This dosage was equivalent to 9 g of crude drug. Considering the mean volume of one adult as approximately 0.06 m^3, the distribution of medicine can be estimated as 0.15 mg/mL. Hence, the concentration of 0.1 mg/mL was chosen as the middle dose to feed the cells. Then, this dose was adjusted 10-fold to investigate a range of concentrations corresponding to 0.01, 0.1, and 1 mg/mL of the crude herbal dose.

2.4. *SiRNA transfection and PI3K inhibition of GC-1 spg cells*

The GC-1 spg cells were treated at a density of 2×10^5/well in 6-well plates with 0, 0.01, 0.1, and 1 mg/mL YC extract and 20 ng/mL GDNF (used as positive control), respectively. After 48 h, the cells were collected for messenger RNA and protein analysis to detect the expression of POU3F1 using quantitative real time-polymerase chain reaction (RT-PCR) and Western blots. To explore the underlying signaling mechanism, POU3F1, GFRα1 siRNA, and PI3K inhibitor (LY294002) were carried out. For POU3F1 knockdown, 21-nucleotide siRNA sequences (sense: 5'-CCC UCU ACG GUA ACG UGU UTT -3' and antisense: 3'-TTG GGA GAU GCC AUU GCA CAA -5') targeting mouse POU3F1 sequence (CCC TCT ACG GTA ACG TGT T) were designed using BLOCK-iT RNA interference (RNAi) Designer (Invitrogen, Carlsbad, CA, USA) and synthesized by Invitrogen. For GFRα1 knockdown, 21-nucleotide siRNA sequences (sense: 5'-GCC CUC ACA GGC UUC UGU UTT-3' and antisense: 3'-TTC GGG AGU GUC CGA AGA CAA-5') targeting mouse GFRα1 sequence (GCC CTC ACA GGC TTC TGT T) were also designed and synthesized by Invitrogen (*27*). The Stealth RNAi negative control was obtained from Invitrogen, which was used as a control for monitoring nonsequence-specific effects. Before transfection, GC-1 spg cells were seeded in 6-well plates at a density of 1×10^5/well for 24 h and then POU3F1, GFRα1 siRNA, and Stealth RNAi negative control were transfected into GC-1 spg cells by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). At 24 h after transfection, the cells were treated with 1 mg/mL YC extract for 48 h and then harvested for quantitative RT-PCR and Western blot analyses. For PI3K inhibition, GC-1 spg cells were exposed to 25 μM LY294002 (targeting the ATP-binding site of the PI3K) for abrogating PI3K activation. Two hours later, 1 mg/mL YC extract was added. After 48 h, the cells were harvested for quantitative RT-PCR and Western blot analyses.

2.5. *MTT assay of cell proliferation*

The cells were seeded in 96-well plates and treated with 0 and 1 mg/mL YC extract, 20 ng/mL GDNF, 1 mg/mL YC extract with 25 pmol/mL control siRNA, and 25 pmol/mL POU3F1 siRNA, respectively (n = 5) for 48 h. 20μL of MTT (5 mg/mL) was added to each well and incubated for 4 h before it was discarded. Then the obtained purple-blue MTT formazan precipitate was dissolved in 100μL DMSO. The absorbance (OD) was measured at 490 nm and the proliferation ratio (%) was calculated using the formula: (average OD treatment group/average OD control group-1) × 100%.

2.6. *RNA isolation and quantitative RT-PCR*

Cells at a density of 2×10^5/well were plated in 6-well plates for 48 h. The total RNA was extracted using TRIzol reagent and measured using spectrometry at an OD 260/280. Later, extracted RNAs were reversibly transcribed into cDNA in a total volume of 20 μl with PrimeScript RTMaster Mix. All of the RT-PCR reactions were performed with a CFX96 RT-PCR system (Bio-Rad Laboratories, Hercules, CA, USA) using SYBR Green PCR Master Mix (Bio-Rad

Laboratories, Hercules, CA, USA). GAPDH was used as the internal control. Primer sequences were designed and synthesized by Invitrogen as follows: GAPDH, sense: 5'-AGG TTG TCT CCT GCG ACT TCA-3' and antisense: 5'-GGG TGG TCC AGG GTT TCT TAC T-3'; POU3F1, sense: 5'- TAC CGC GAA GTG CAG AAG C -3' and antisense: 5'- CGT GGG TAG CCA TTG AGG G -3'; and GFRα1, sense: 5'-AGA AGC AGT TTC ACC CAG-3' and antisense: 5' ATC ATC ACC ACC ACC ATC-3'. Reactions were performed at 94°C for 2 min followed by 40 cycles at 94°C for 30 s, 55°C for 15 s, and 70°C for 1 min. The final extension was carried out for 5 min at 72°C. A melting curve analysis was performed to confirm the products. The relative abundance of the target mRNA were calculated using the $2^{-\Delta\Delta Ct}$ method. The data were expressed as the percentage of control (100%)

2.7. *Protein extraction and western blot analysis*

Cells were seeded in 6-well plates at a density of 2×10^5/well for 48 h. The cells were harvested, washed three times with precooled phosphate-buffered saline, and treated with cell lysis buffer for Western blot analysis. After centrifugation at 12,000 g at 4°C for 15 min, the supernatants were collected and stored at -80°C until analysis. The concentrations of protein were measured using the Bio-Rad Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). Proteins were normalized to 60 μg/lane and separated on 12% SDS-polyacrylamide gel electrophoresis, and subsequently transferred to nitrocellulose membranes. After treatment with blocking solution (5% skim milk powder in Tris-buffered saline) at 37°C for 1 h, the membranes were incubated overnight with the primary antibodies goat polyclonal anti-POU3F1 (1:200 dilution), rabbit monoclonal anti-pAKT (1:5000 dilution), rabbit polyclonal anti-GFRα1 (1:400 dilution), or mouse monoclonal anti-GAPDH (1:3000 dilution) at 4°C. After washing with Tris buffered saline with Tween 20 three times, the membranes were incubated with HRP-conjugated secondary antibodies (1:3000 dilution) at 37°C for 1 h and examined using enhanced chemiluminescence. The relative protein levels in each sample were normalized to those of GAPDH to standardize for variations in loading. Densitometric analyses of the scanned immunoblotting images were performed using a Quantity One image system (Bio-Rad Laboratories, Hercules, CA, USA).

2.8. *Statistical analysis*

All data were analyzed using a SPSS (Version 19.0) statistical package and presented as mean ± standard deviation for three independent experiments. One-way analysis of variance was used to analyze the differences between groups, followed by Dunnett's *t*-test. A p value of < 0.05 was considered statistically significant.

3. Results

3.1. *Effects of the YC extract on the expression of POU3F1 mRNA and protein*

As shown in Figure 2A, the expression of POU3F1 mRNA increased significantly after exposure to 0.01, 0.1, and 1 mg/mL YC extract and 20 ng/mL GDNF ($p = 0.083$, $p < 0.001$, $p < 0.001$ and $p < 0.001$ respectively). As shown in Figure 2B, expression of POU3F1 protein also increased evidently under the same conditions. The YC extract enhanced the expression of POU3F1 mRNA and protein in a dose-dependent manner with a maximal effect observed at a 1 mg/mL concentration. Hence, a YC extract dose of 1 mg/mL was selected for further experiments.

3.2. *POU3F1 knockdown blocks YC extract induced proliferation of GC-1 spg cells*

To investigate the transcriptional level mechanism by which YC extract can induce proliferation of GC-1 spg cells, a POU3F1 knockdown was performed.

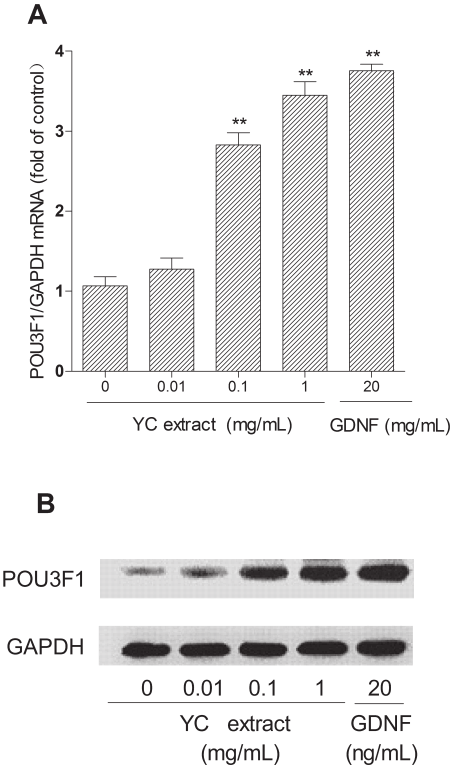

Figure 2. Effects of the YC extract on the expression of POU3F1 mRNA and protein in GC-1 spg cells. The GC-1 spg cells were treated with 0.01, 0.1, and 1 mg/ml YC extract or 20 ng/ml GDNF for 48 h. The expression of mRNA was detected using quantitative RT-PCR. The expression of protein was detected using Western blot analysis. The data are expressed as percentage of control (100%). **$p < 0.01$ compared with control group. Data are presented as means (SD) from three independent experiments and representative bands are shown.

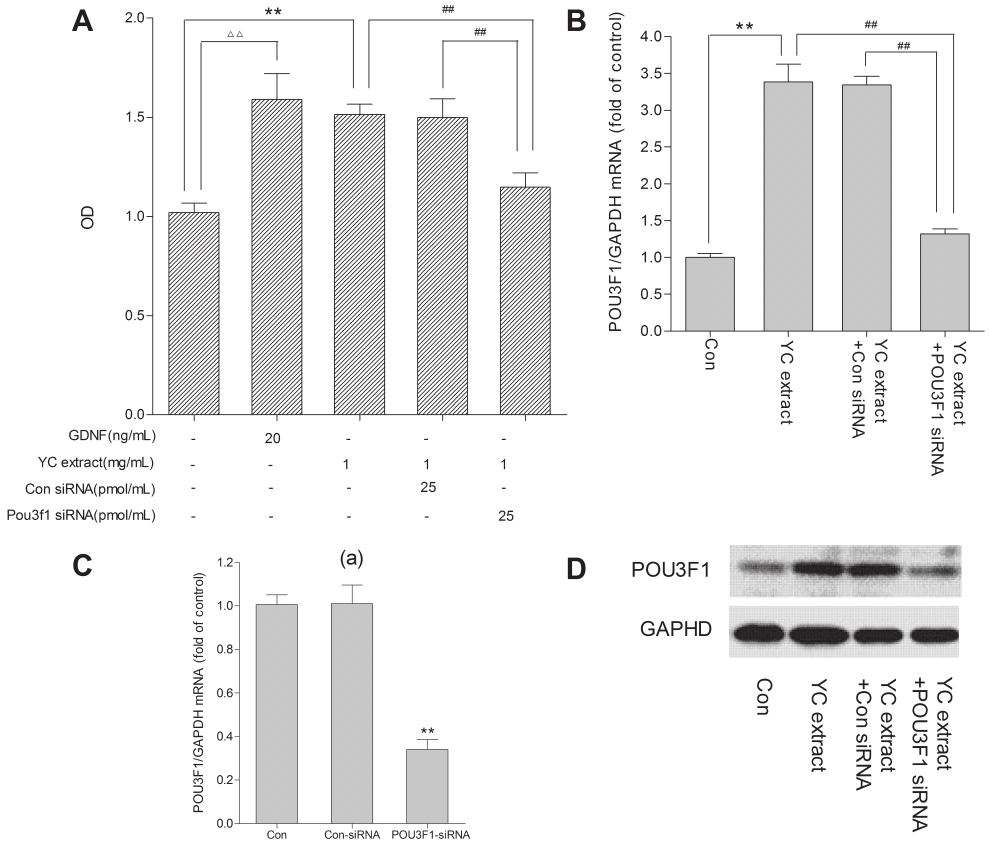

Figure 3. POU3F1 knockdown blocked YC extract induced proliferation of GC-1 spg cells. (A) The GC-1 cells were exposed to control blank, 20 ng/mL GDNF, 1 mg/mL YC extract, and 1 mg/mL YC extract with 25 pmol/mL control siRNA or POU3F1 siRNA. $^{**}p < 0.01$ compared with control group, $^{\Delta\Delta}p < 0.01$ compared with control group, $^{\#\#}p < 0.01$ compared with YC extract and POU3F1 siRNA group, $n = 5$. **(B)**, **(D)** POU3F1 knockdown blocked YC extract induced upregulation of POU3F1 expression. The GC-1 cells were exposed to control blank, 1 mg/mL YC extract, and 1 mg/mL YC extract with 25 pmol/mL control siRNA or POU3F1 siRNA. $^{**}p < 0.01$ compared with control group. $^{\#\#}p < 0.01$ compared with YC extract with POU3F1 siRNA group. **(C)** The expression of POU3F1 mRNA was reduced by 66% in POU3F1 siRNA-treated cultures compared with those treated with nontargeting control siRNA at 36 h after transfection. Data are presented as means (SD) from three independent experiments and representative bands are shown.

As shown in Figure 3A, YC extract and GDNF significantly stimulated cell proliferation ($p < 0.001$ for both). However, POU3F1 siRNA (25 pmol/mL) could evidently reduce the stimulative effect of YC extract by 24% ($p < 0.001$). As shown in Figures 3B and 3D, POU3F1 siRNA could abolish the upregulation of POU3F1 expression at levels of mRNA and protein induced by YC extract with a 61% interference efficiency ($p < 0.001$).

3.3. *PI3K inhibition blocks YC extract induced Up-regulation of POU3F1 expression*

To explore whether the YC extract induced proliferation of GC-1 spg cells *via* PI3K/AKT signaling pathway, LY294002, a PI3K inhibitor, was added 2 h before the YC extract treatment. As shown in Figure 4A, LY294002 could abrogate the upregulatory expression of POU3F1 mRNA induced by YC extract, with a 64% interference efficiency ($p < 0.001$). As shown in Figure 4B, the POU3F1 protein levels also down-regulated remarkably with similarity to POU3F1 mRNA. Meanwhile, the upregulation of pAKT protein expression induced by YC extract, an evidence for PI3K/AKT pathway activation, was also suppressed by LY294002.

3.4. *PI3K inhibition blocks YC extract induced Up-regulation of POU3F1 expression*

Considering the membrane receptor GFRα1's participation in the YC extract mediated pathway, a GFRα1 knockdown experiment was conducted to confirm whether down-regulated GFRα1 had an effect on POU3F1 expression. As shown in Figures 5A and 5D, GFRα1 siRNA significantly inhibited the upregulation of YC extract induced POU3F1 mRNA and protein expression by 58% ($p < 0.001$ for both). Correspondingly, the upregulation of GFRα1 and pAKT expression induced by YC extract were also suppressed by GFRα1 siRNA (Figure 5B and 5D) ($p < 0.001$ for both). So far, an unambiguous signaling pathway

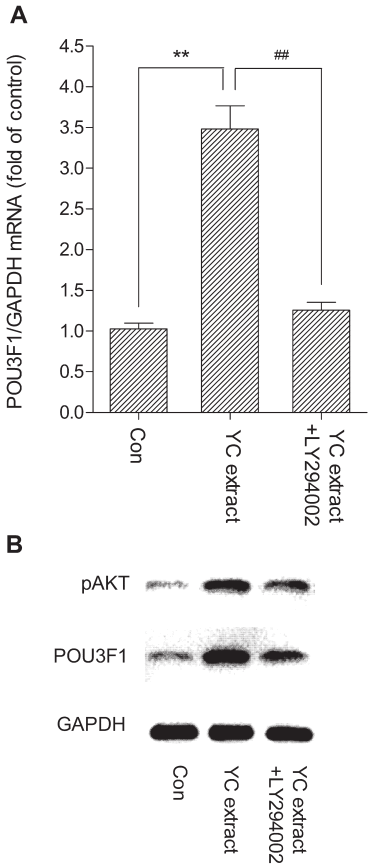

Figure 4. PI3K inhibition blocked the YC extract induced up-regulation of POU3F1 expression. The GC-1 cells were exposed to control blank, 1 mg/mL YC extract, and 1 mg/mL YC extract with 25 μM LY294002. $^{**}p < 0.01$ compared with control group. $^{\#\#}p < 0.01$ compared with YC extract group. Data are presented as means (SD) from three independent experiments and representative bands are shown.

behind the YC extract induced proliferation of GC-1 spg cells at the cytomembrane (GFRα1), cytoplasm (PI3K/AKT), and intra-nuclear transcriptional levels (POU3F1) were confirmed in this study.

4. Discussion

At present, functional assay systems shed new light on studying the SSCs population and enable use of these cells for applications in animal transgenesis and medicine *(28)*. Comprehension of SSCs at the molecular level provides an opportunity to explore TCM mechanism of action on SSCs. Interestingly, the GC-1 spg cell line has served as a convenient tool to critically study pathways regulating the SSCs fate decisions and survival. As stated previously, the YC extract can lead to proliferation of GC-1 spg cells by activating the PI3K/AKT pathway, sharing the same functionality as GDNF, while the downstream signaling mechanism is poorly understood in consideration of POU3F1 as a significant transcription factor regulated by GDNF. In the present study, the role of POU3F1 in YC extract mediated proliferation of SSCs was examined.

The GDNF, secreted by Sertoli cells and peritubular myoid cells, is the major paracrine factor specifically responsible for the maintenance and self-renewal of SSCs *in vivo* (*29,30*). Other signaling pathways like insulin-like growth factor-1 receptor (IGF-1R) (*31*) mediated and fibroblast growth factor 2 (FGF2) (*32*) mediated pathways also play a role. The GDNF binding to GFRα1 induces RET activation that further leads to the activation

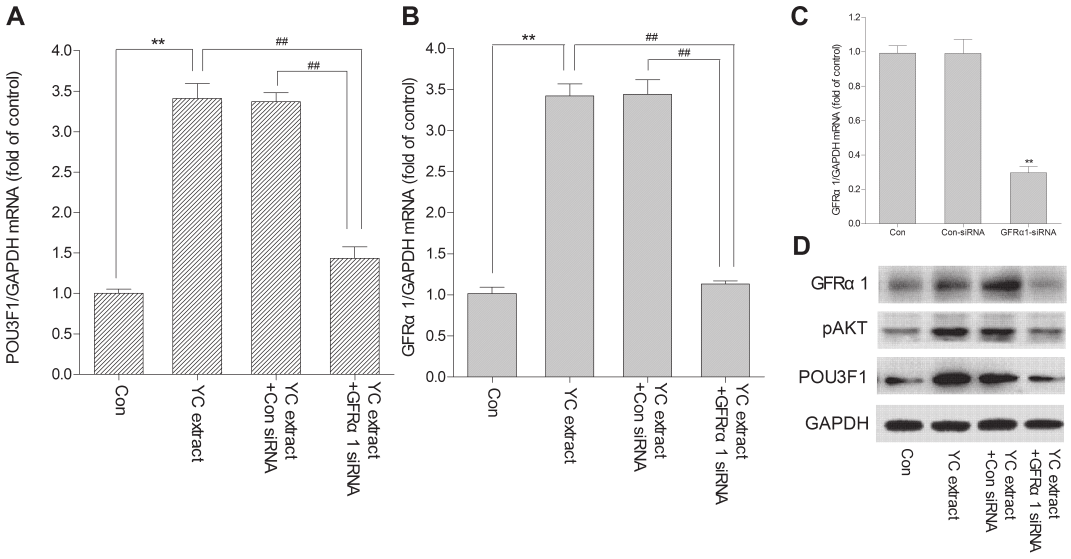

Figure 5. GFRα1 knockdown blocked the YC extract induced up-regulation of POU3F1 and pAKT expression. (A), (B) The GC-1 cells were exposed to control blank, 1 mg/mL YC extract, and 1 mg/mL YC extract with 25 pmol/mL control siRNA or GFRα1 siRNA. $^{**}p < 0.01$ compared with control group. $^{\#\#}p < 0.01$ compared with YC extract with GFRα1 siRNA group. **(A)**, **(D)** GFRα1 knockdown blocked YC extract induced upregulation of POU3F1 expression. **(B)**, **(D)** Correspondingly, the upregulation of GFRα1 and pAKT expression induced by YC extract were also suppressed by GFRα1 siRNA. **(C)**Treatment with GFRα1 siRNA resulted in 70% reduction of gene expression compared with nontargeting control siRNA at 36 h after transfection. Data are presented as means (SD) from three independent experiments and representative bands are shown.

Table 1. Primary active ingredients and functions of Chinese herbs

Chinese herbs	Active ingredients	Functions	*Ref.*
Placenta Hominis	hCG	Promoted the proliferation of SSCs after transplantation.	(*42*)
	GnRH	Promoted the release of FSH and LH by pituitary thereby stimulating spermatogenesis	(*45*)
Herba Epimedii Brevicornus	Total flavonoids	Increased testicular weights, sperm counts and sperm motility. Restored oxidative damage by up-regulating the expression of antioxidant enzymes (SOD3 and GPX1).	(*46*)
	Icariin	Increased testosterone levels by up-regulation the expression of PBR and StAR. Increased FSHR and claudin-11 mRNA expression in Sertoli cells.	(*47*)
Concha Ostreae	Zinc	Promoted the proliferation of SSCs, the progression spermatogenesis and sperm motility.	(*43*)
	Selenium	Promoted the proliferation of SSCs. Protected against oxidative damage to spermatozoa throughout the process of sperm maturation. Selenoproteins served as structural components of mature spermatozoa.	(*44*) (*48*)
Hirudo	Hirudin	Improved testicular microcirculation.	(*49*)
Radix Angelicae Sinensis	Ferulic acid	Improved testicular microcirculation.	

SOD3: superoxide dismutase 3; GPX1: glutathione peroxidase 1; PBR: peripheral type benzodiazepine receptor; StAR: steroidogenic acute regulatory protein; hCG:human chorionic gonadotropin; GnRH: Gonadotropin-releasing hormone; FSH: follicle stimulating hormone; FSHR: follicle stimulating hormone receptor; LH: luteinizing hormone.

of the PI3K/AKT signaling pathway which is required for spermatogonial self-renewal in mice (*11,33*). Further research showed that POU3F1 was an important intrinsic regulator of GDNF-induced survival and self-renewal of mouse SSCs (*10*). Based on the comparable effects of YC extract and GDNF, it was speculated that POU3F1 also played a key role in YC extract's mediated biological effects. In this study we blocked three key signal factors GFRα1 (cytomembrane level), PI3K(cytoplasm level), POU3F1(intra-nuclear transcriptional level) in the GDNF induced signaling pathway to test whether the YC extract went through the same pathway.

To investigate the role of POU3F1 in the proliferation of GC-1 spg cells induced by YC extract, siRNA mediated POU3F1 knockdown was used in this study. As shown in Figure 3, POU3F1 siRNA (25 pmol/mL) almost entirely abolished the proliferative effect of GC-1 cells induced by YC extract. Correspondingly, POU3F1 knockdown also abrogated the elevated expression of POU3F1 mRNA and protein induced by YC extract (Figure 3). On the basis of these results, it was inferred that YC extract exerts biological effects partly *via* POU3F1.

To further explore the YC extract mediated signaling mechanism, the GC-1 cells were treated with 25 μM LY294002 to inhibit the PI3K pathway. In previous studies, it was found that the PI3K pathway played a central role in the GDNF induced self-renewal of SSCs (*33,34*). The binding of GDNF to GFRα1 triggers the PI3K/AKT pathway and eventually leads to self-renewal of SSCs *via* up-regulated expression of POU3F1. As expected, pAKT protein and POU3F1 expression were increased evidently after YC extract treatment, confirming the PI3K/AKT pathway activation (Figure 4). Moreover, LY294002 markedly down-regulated the expression of pAKT and POU3F1, indicating that PI3K/AKT were the explicit signaling molecules of YC extract induced up-regulation of POU3F1 expression.

Furthermore, GFRα1 siRNA was used to interrupt the binding of YC extract to GFRα1, to observe whether GFRα1 expression and downstream pAKT and POU3F1 were restrained. The GFRα1 is expressed in all stages of type A spermatogonia (*35*), and it is a necessary component of the GFRα1/RET complex. The RET alone is unable to bind with GDNF unless it is co-expressed with the GFRα1 receptor. The association of GDNF with RET tyrosine kinase is mediated by GFRα1, and GDNF cannot induce RET autophosphorylation in the cells that lack GFRα1 expression (*27*). A previous study (*24*) revealed that both GFRα1 siRNA and LY294002 could markedly abrogate the stimulative effect of YC extract. As shown in Figure 5, GFRα1 knockdown almost entirely abrogated the elevated expression of GFRα1 mRNA and protein induced by YC extract. Accordingly, the up-regulated pAKT protein, POU3F1 mRNA and protein expression also dropped down after GFRα1 siRNA treatment. Conclusively, it could be inferred that POU3F1, GFRα1, and PI3K played crucial roles in the promotion of GC-1 spg cells self-renewal induced by YC extract.

To date, some medicines have been found with definite effects on the proliferation of SSCs. For example, natural drugs like Petasites Japonicas butanol (*36*), Rhodiola Sachalinensis polysaccharides (*37*), Lycium Bararum polysaccharides (*38*) and Astragalus (*39*), others like growth factors (*40*), follicle stimulating hormone (FSH) (*41*), human chorionic gonadotropin (hCG) (*42*), Zinc (*43*) and Selenium (*44*), all have proliferative effects. In comparison with biomonomers of natural drugs and other chemicals, sophisticated compounds of TCM have various effects made by a variety of chemical components, multi-linked and multi-targeted in the body. As a sophisticated compound, the YC extract contains 11

Chinese medicinal herbs with multitudes of monomers, including hCG, Zinc and Selenium, which show direct evidence of the proliferative effect on SSCs. The primary active ingredients of the YC extract are listed in the Table1. It may promote proliferation of GC-1 spg cells two ways. One way is through the hypothalamic-pituitary-testicular axis that works. Placenta Hominis contains gonadotropin-releasing hormone (GnRH) and hCG. GnRH can promote the release of FSH and luteinizing hormone (LH) by pituitary thereby stimulating spermatogenesis (*45*). hCG exerts the same function as LH (*42*). Another way is through the testicular microenvironment. Total flavonoids of Herba Epimedii Brevicornus can restore oxidative damage in the testis (*46*). Icariin has testosterone mimetic properties and can significantly increase testosterone levels (*47*). Zinc and Selenium are essential trace elements for the maintenance of SSCs, the progression of spermatogenesis, and the regulation of sperm motility (*43,48*). Hirudin and ferulic acid can improve testicular microcirculation and metabolic function (*49*).

5. Conclusions

In summary, it was concluded that YC extract could up-regulate POU3F1 expression, partly *via* GFRα1, by triggering the activation of the PI3K/AKT pathway and finally lead to self-renewal of SSCs. The present study findings provide evidence of molecular biology mechanism of TCM to further guide the clinical treatment of male infertility. In addition, most of the transcription factors (POU3F1, BCL6B, ETV5, ID4, and LHX1) that have been identified as promoting SSCs self-renewal are up-regulated by GDNF (*12*). Since GDNF is crucial for promoting SSCs self-renewal and YC extract possesses similar functionality compared to GDNF, the above mentioned transcription factors could be responsible for coordinating the action of YC extract in SSCs. In future research, it will be meaningful to conduct a microarray analysis on cultures of SSCs induced by YC extract to gain a more comprehensive list of GDNF regulated genes. Furthermore, as YC extract consists of multiple components, further studies will be focused on excavating the primary effective biomonomer.

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