RESEARCH

Baicalin inhibits recruitment of GATA1 to the *HSD3B2* promoter and reverses hyperandrogenism of PCOS

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Abstract

High androgen levels in patients suffering from polycystic ovary syndrome (PCOS) can be effectively reversed if the herb *Scutellaria baicalensis* is included in traditional Chinese medicine prescriptions. To characterize the effects of baicalin, extracted from *S. baicalensis*, on androgen biosynthesis in NCI-H295R cells and on hyperandrogenism in PCOS model rats and to elucidate the underlying mechanisms. The optimum concentration and intervention time for baicalin treatment of NCI-H295R cells were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and ELISA. The functional genes affected by baicalin were studied by gene expression profiling (GEP), and the key genes were identified using a dual luciferase assay, RNA interference technique and genetic mutations. Besides, hyperandrogenic PCOS model rats were induced and confirmed before and after baicalin intervention. As a result, baicalin decreased the testosterone concentrations in a dose- and time-dependent manner in NCI-H295R cells. GEP revealed that 3β-hydroxysteroid dehydrogenase type II (HSD3B2) was the key enzyme of androgen biosynthesis, and baicalin inhibited the expression of *HSD3B2* by regulating the binding of transcription factor GATA-binding factor 1 (GATA1) to the *HSD3B2* promoter. Hyperandrogenic PCOS model rats treated with baicalin significantly reversed the high androgen levels of serum and the abnormal ovarian status, restored the estrous cyclicity and decreased the expression of *HSD3B2* in ovarian. In summary, our data revealed that GATA1 is an important transcription factor activating the *HSD3B2* promoter in steroidogenesis, and baicalin will potentially be an effective therapeutic agent for hyperandrogenism in PCOS by inhibiting the recruitment of GATA1 to the *HSD3B2* promoter in ovarian tissue.

Key Words

- baicalin
- polycystic ovary syndrome
- hyperandrogenism
- *HSD3B2*
- GATA1

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Introduction

Polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders, affecting 6–15% of women in their reproductive age (Fauser *et al.* 2012). Hyperandrogenism, a hallmark of PCOS, is characterized by excessive production of Δ4 steroids such as androstenedione (A2) and testosterone, resulting in

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anovulation, oligomenorrhea and infertility (Ansari 2016). Hyperandrogenism in PCOS patients is of both ovarian and adrenal origin (Yu *et al.* 2017), and it may manifest in mid-childhood as premature exaggerated adrenarche (Dorn *et al.* 2008) and persists beyond the menopausal transition (Markopoulos *et al.* 2011). Numerous studies have revealed that a complex network regulates the androgen synthesis, targeting involved genes and proteins at the transcriptional and post-translational levels. A group of 3β-hydroxysteroid dehydrogenase (HSD3B) enzymes plays a central role early in this network during the biosynthesis of Δ4 androgens (Udhane *et al.* 2013). The microsomal HSD3B of type II (HSD3B2), mainly expressed in the adrenals and gonads (Simard *et al.* 2005), is known as the Δ5–Δ4 isomerase because it converts Δ5 steroids, pregnenolone, 17α-hydroxypregnenolone (17OHPreg) and dehydroepiandrosterone (DHEA) to Δ4 steroids, progesterone, 17α-hydroxyprogesterone (17OHP) and A2. Therefore, HSD3B2 is crucial for the production of sex steroids and plays an essential role in the androgen biosynthesis both in health and disease (Miller & Auchus 2011).

Scutellaria baicalensis, a kind of perennial herb of baicalensis in labiaceae, first recorded in Chinese Shennong Bencaojing (written between 200 and 300 AD) and described in the authentic Compendium of Materia Medica (Bencao Gangmu 1593) (Zhao *et al.* 2016), is popular in traditional Chinese medicine for centuries and has been widely used in obesity, hypertension, depression and detoxication. Modern pharmacological studies show that *S. baicalensis* contains kinds of flavonoids including baicalein, baicalin, wogonin, oroxylin A, skullcapflavone and so on and has various effects including antibacterial, antifungal, antiviral, anti-inflammatory, anti-allergic and antioxidative. Interestingly, the clinical symptoms of hyperandrogenism in PCOS patients have been found to better improve if *S. baicalensis* is added to a traditional Chinese medicine prescription in our clinical practice; however, additional details on the pharmacology of *S. baicalensis* remain obscure. Baicalin (baicalein 7-D-beta-glucuronate), a flavonoid glycoside isolated from *S. baicalensis*, has shown biological activities against several androgen-associated disorders such as prostate cancer (Chan *et al.* 2000), androgenetic alopecia (Kim *et al.* 2014) and acne (Fu *et al.* 2012). So far, no reports have been found about the treatment of hyperandrogenism in PCOS with baicalin.

The aim of this study was to investigate the inhibitory effects and mechanism(s) of baicalin in the regulation of androgen production using NCI-H295R cells and a DHEA-induced rat model of PCOS. Our data may shed light on the pharmacological effects of baicalin and provide a scientific basis for its clinical applications.

Materials and methods

Cell culture and cytotoxicity assay

The NCI-H295R human adrenocortical cell line (CRL-2128; ATCC) is considered to be a well-established model for studying steroidogenesis (Kempná *et al.* 2015) and chosen for the *in vitro* study in our research. The cells were cultured under standard conditions in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 2.5% Nu-serum I and 1% ITS premix (Becton Dickinson and Company). Baicalin (purity ≥95%), purchased from Tokyo Chemical Industry, was dissolved in 0.1% (v/v) DMSO and diluted in a range from 0.1 to 400 μmol/L. The half-maximal inhibitory concentration (IC_{50}) was determined as described previously (Ghidini *et al.* 2015). Besides, the cell supernatant was carefully collected from each well, without disturbing the cells, and the testosterone level was determined using an ELISA kit (R&D Systems) following the manufacturer's instructions. Finally, the most suitable concentration and intervention time for baicalin treatment of NCI-H295R cells were determined.

GEP analysis, real-time PCR and Western blotting

NCI-H295R cells were plated in six-well plates at a density of 1×10^6 cells per well and 24 h later or when the confluence reached approximately 80% were treated with the most suitable concentration of baicalin for an appropriate time. The cells were collected, and the gene expression profile (GEP) analysis was conducted by Invitrogen Co., Ltd. The key genes affected by baicalin were selected and confirmed by real-time PCR (Supplementary Table 1, see section on supplementary data given at the end of this article) and western blotting (Supplementary Table 2) as described previously (Deng *et al.* 2013).

Plasmid constructs, transient transfection and dual luciferase assay

To assess the effect of baicalin on the promoter activities of the key genes, luciferase gene reporter plasmids carrying fragments of the *HSD3B2* gene promoter of different lengths (Supplementary Table 3) were constructed and verified by sequencing. NCI-H295R cells were seeded into 24-well plates at a density of approximately 1×10^5 cells per well

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and incubated in the culture medium. After the confluence reached approximately 90%, the cells were transfected with the pGL3 vector plasmid as a control and with specific promoter–reporter constructs using X-treme GENE-HP (Roche) following the manufacturer's instructions. Six hours later, the cells were washed with ice-cold phosphate-buffered saline and treated with the appropriate concentration of baicalin for 24 h. Finally, the cells were lysed and assayed for luciferase activity using the dual luciferase reporter assay system and protocol from Promega.

Silencing of GATA1 expression, mutation of GATA1-binding site and co-transfection analysis

Based on the promoter deletion analysis and transcription factor-binding site analysis, GATA-binding factor 1 (GATA1) was identified as a transcription factor most relevant to the *HSD3B2* gene expression after cells were incubated with baicalin. To further confirm these results, a shRNA to GATA1 and mutations in the GATA1-binding site in the promoter–reporter constructs (−507 to +194 bp) were designed by Genechem (Shanghai). NCI-H295R cells were transiently co-transfected with specific promoter–reporter constructs or with mutant constructs and either control shRNA or shGATA1. The cells were then incubated with baicalin and assayed using the dual luciferase reporter assay system as mentioned earlier.

Rat model, drug intervention and serum biochemical assessments

According to previous studies (Ikeda *et al.* 2014, Yu *et al.* 2014), hyperandrogenism PCOS rat model was induced by subcutaneous injection of DHEA (0.6 mg/100g/day+0.2 mL of sesame oil) and verified by histological screening of vaginal exfoliated cells and hormonal profiles of the orbital venous blood. Besides, six rats were randomly selected and confirmed by ovarian morphology. The successfully established model rats, with a disordered estrous cycle and hormonal profile, were selected and randomly divided into a model group, baicalin group and spirolactone group. The control group rats (subcutaneously injected with 0.2 mL of sesame oil) and the model group rats were treated orally with the normal saline vehicle at a dose of 10 mL/kg/day. The drug group rats were treated orally with baicalin (dissolved in normal saline) at a dose of 20 mg/kg/day (according to our previous study) or spirolactone (dissolved in normal saline) at a dose of 20 mg/kg/day (Sherajee *et al.* 2013). All rats in the four groups were treated between 9:00 and 10:00 a.m. daily for 4 weeks and then underwent surgery in the anestrus phase of the estrous cycle. For biochemical assessments, the blood was collected from the abdominal aorta of the rats, and serum levels of testosterone, estradiol (E2) and luteinizing hormone (LH), as well as the LH/follicle-stimulating hormone (FSH) ratio were determined using ELISA kits (R&D Systems). All animal procedures were reviewed and approved by the Ethics Committee of Naval Medical University. DHEA (purity ≥99%) and sesame oil (for injection) were purchased from Sigma. Spirolactone (purity >90%) was purchased from J&K.

Hematoxylin and eosin staining of ovarian tissues, real-time PCR and immunohistochemistry analysis

Bilateral ovarian tissues of rats were surgically detached after drug intervention, and their wet weights were measured using an electronic scale. The left-side ovarian tissues were quickly fixed in 10% formalin for hematoxylin and eosin staining and immunohistochemistry (IHC) analysis (Chen *et al.* 2016). The antibodies used in this study are shown in Supplementary Table 2. Six sections of ovarian tissues were randomly selected from each group, and six fields of each ovarian section were captured under a microscope. Two investigators, blinded to the section origin, independently analyzed the sections using the available photographs and calculated the results. The right-side ovarian tissues were immediately stored in liquid nitrogen for real-time PCR analysis. The PCR primer pairs for the analysis were designed and synthesized by Sangon Biotech (Supplementary Table 1).

Statistical analysis

Statistical analysis was performed using the SPSS 21.0 software. Data were first assessed for normality and homogeneity of variance and then presented as the mean ± standard deviation (s.d.). Data were analyzed using a *t*-test or one-way, two-way or repeated-measures ANOVA, followed by Tukey's or Dunnett's post-test. Differences were considered significant at $P<0.05$.

Results

Baicalin reduced testosterone production in NCI-H295R cells by inhibiting HSD3B2 gene expression

The cytotoxicity of baicalin to NCI-H295R cells was analyzed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and the

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IC_{50} value of baicalin was around 275 µmol/L (Fig. 1A). Based on the data, the intervention concentrations and duration of baicalin were set at 200, 100, 50, 25, 12.5, 1 and 0.1 µmol/L and 24, 48 and 72 h respectively. The results showed that cells incubated with baicalin at concentrations of 25 µmol/L for 48 h could significantly reduce the testosterone production (Fig. 1B), and this treatment model (25 µmol/L, 48 h) was determined for subsequent experiments.

GEP analysis showed that a total of 893 genes (555 upregulated and 338 downregulated) were differentially expressed between the baicalin and control (0.1% DMSO) groups. Some of these differentially expressed genes (DEGs) were hierarchically clustered and visualized in a heat map (Fig. 1C). Six genes were randomly selected, including three upregulated genes (*CYP1B1*, *MMP9* and *IL11RA*) and three downregulated genes (*IL1R2*, *CYP4A11* and *IGF1*), and their differential expression was confirmed by real-time PCR (Fig. 1D). After validation of the reliability of the profile data, the biological processes involving DEGs were identified by Gene Ontology (GO) enrichment analysis using the Database for Annotation,

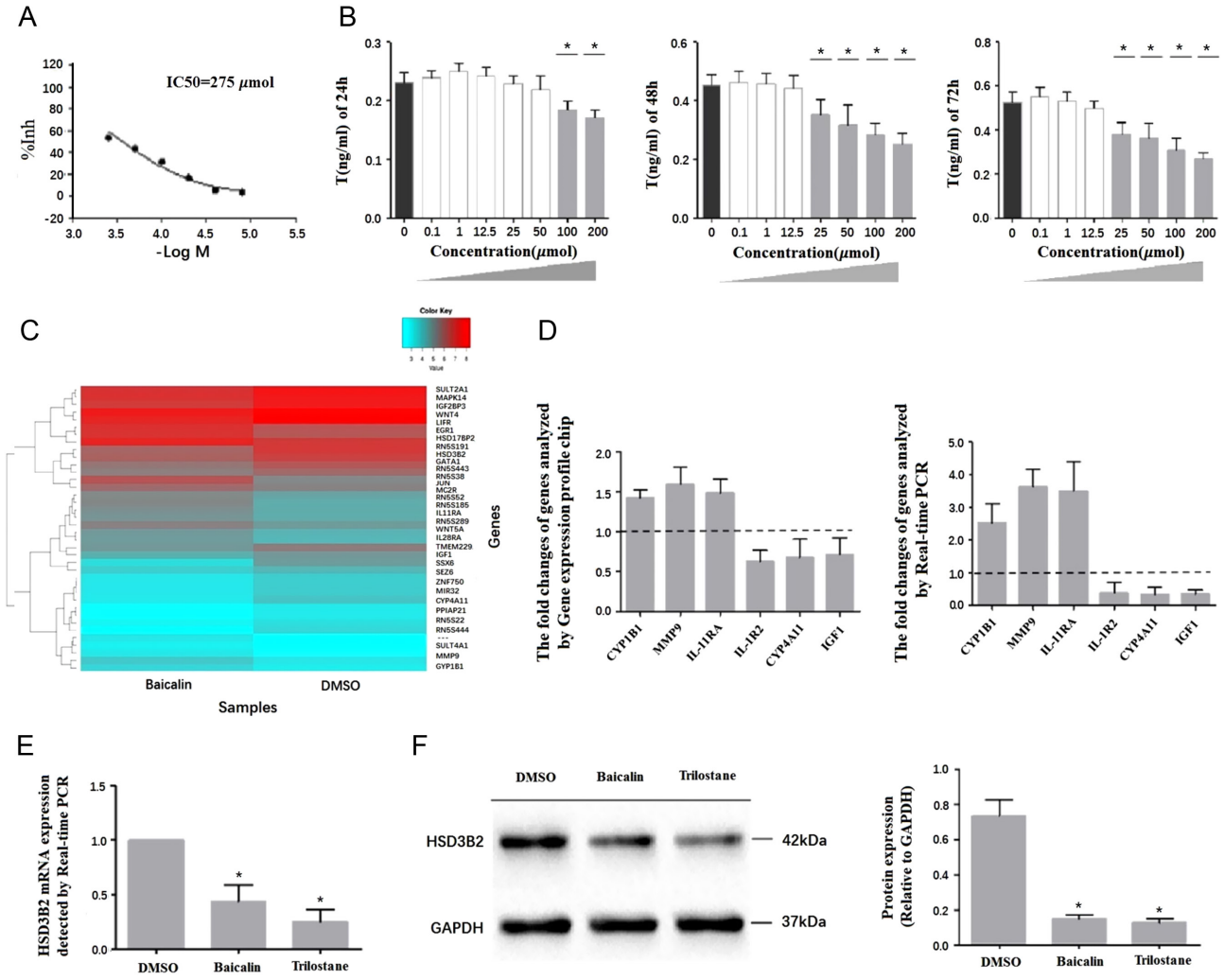

Figure 1

(A) Cytotoxicity of baicalin for NCI-H295R cells, as measured by the MTT assay. The LogIC_{50} value of baicalin was 3.56, and the IC_{50} value was 275.423 µmol/L. (B) Effects of baicalin on testosterone (T) levels in NCI-H295R cells, as measured by ELISA. Cells were exposed to various concentrations (0.1–200 µmol/L) of baicalin or to an equivalent volume of the solvent (0.1% DMSO) alone for 24, 48 and 72 h. (C) A heat map of hierarchically clustered and visualized DEGs, which were upregulated or downregulated in NCI-H295R cells upon baicalin treatment. (D) Validation of microarray data by real-time PCR. Six DEGs were randomly selected by microarray analysis and confirmed by real-time PCR. Data represent the means of triplicate wells and were normalized to the levels of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) expression. (E and F) mRNA and protein levels of HSD3B2, detected by real-time PCR and western blotting, respectively. Trilostane was used as a positive control. *$P < 0.05$ vs the DMSO group. A full colour version of this figure is available at https://doi.org/10.1530/JOE-18-0678.

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Visualization, and Integrated Discovery (DAVID) tool. In total, 121 GO terms were identified for downregulated DEGs, including those associated with steroidogenesis, such as the androgen biosynthetic process, regulation of male gonad development, regulation of the testosterone biosynthetic process, positive regulation of the cortisol biosynthetic process, and so on (Supplementary Fig. 1). According to the enrichment scores, *WNT4* and *HSD3B2* were significantly important genes in the GO term of the androgen biosynthetic process (Supplementary Table 4). Since HSD3B2 has already been proven to be one of the important enzymes, principally expressed in steroidogenic tissues and essential for steroid hormone production, *HSD3B2* was considered the potential target gene for baicalin in androgen production in NCI-H295R cells, which was confirmed by real-time PCR and western blotting. Trilostane (10 μmol/L), a specific inhibitor of HSD3B2 (Nakamura *et al.* 2011), was chosen as a positive control (Fig. 1E and F).

Baicalin repressed promoter activities of HSD3B2 via transcription factor GATA1

To obtain promoter–luciferase reporter constructs, the promoter DNA sequences P1 (−708 to +194 bp), P2 (−507 to +194 bp), P3 (−325 to +194 bp) and P4 (−167 to +194 bp) of the human *HSD3B2* gene (Gene ID: 3248 in the National Center of Biotechnology Information database) were PCR-amplified from genomic DNA using a PrimeSTAR Max premix, cloned into the polylinker of the pGL3 basic vector (Promega) and confirmed by DNA sequencing. According to the dual luciferase reporter assay, the luciferase activity of pGL3-P1 and pGL3-P2 was 10 and 9 times higher than that of the pGL3 basic vector, respectively. However, there were only slight differences in the luciferase activity between the pGL3 basic vector and either pGL3-P3 or pGL3-P4. Interestingly, the luciferase activity of pGL3-P1 and pGL3-P2 significantly decreased, compared with that of pGL3-P3 and pGL3-P4, after baicalin intervention. Therefore, we speculated that the important regulatory elements of the *HSD3B2* promoter, affected by baicalin, were located between −507 and −325 bp (Fig. 2A). Consequently, the DNA sequence from −507 to −325 bp was analyzed using the AliBaba2.1 software (http://www.gene-regulation.com/pub/programs/alibaba2/index.html), and the potential transcriptional regulatory elements, including C/EBPalp, Oct1, HNF1, NF1, GATA1, NF-EM5 and GCN4, were scored with the TRRD software (http://wwwmgs.bionet.nsc.ru/mgs/gnw/trrd/). As a result, GATA1 was identified as the most relevant candidate transcription factor of *HSD3B2* (Supplementary Table 5). The dual luciferase reporter assay showed that the activity significantly decreased after cells were co-transfected with pGL3-P2

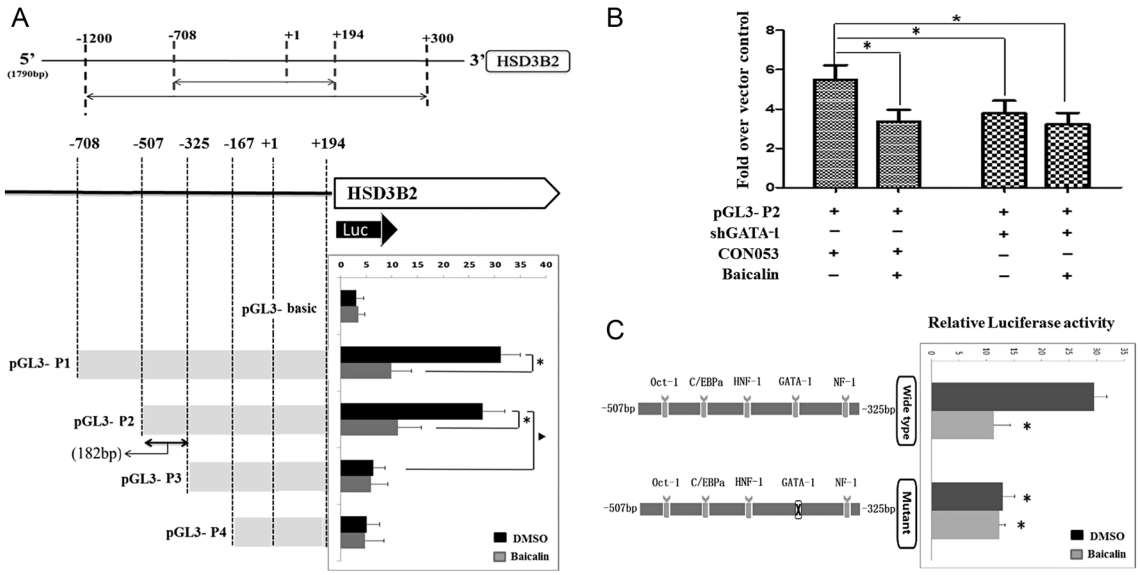

Figure 2

(A) Effects of deletions in the *HSD3B2* promoter region on transcriptional activity of the gene in NCI-H295R cells treated with baicalin. NCI-H295R cells were transfected with luciferase reporter plasmids containing fragments of the upstream regulatory region of *HSD3B2*. (B and C) Activity of pGL3-P2 co-transfected with shGATA1 or mutGATA1 was analyzed by the dual luciferase reporter assay. An empty vector (CON053) was used as a negative control. Data are expressed as fold changes over the vector control and presented as the means ± S.D. of three independent experiments, performed in duplicate. *$P < 0.05$.

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and shGATA1, compared with that of cells co-transfected with pGL3-P2 and CON053 (control of shGATA1; Fig. 2B). This result was further confirmed by a mutGATA1-bs (mutant of GATA1-binding site) reporter assay (Fig. 2C). Moreover, the repression by baicalin was extremely crippled if pGL3-P2 was co-transfected with shGATA1 or mutGATA1-bs. Collectively, these data suggest that baicalin could inhibit the transcriptional activation of the *HSD3B2* promoter by GATA1.

Baicalin improved endocrine abnormalities in PCOS model rats

Based on the classification of vaginal exfoliated cells and the predominant cell types in vaginal smears obtained on ten consecutive days and verified by microscopic analysis, all female Wistar rats ($n=80$, age = 23 days) had a normal estrous cycle of 4–5 days (Fig. 3A) at baseline. After subcutaneous injection of DHEA for 20 days, 46 rats in the DHEA group ($n=70$) were acyclic and endocrine abnormalities according to the classification of vaginal exfoliated cells and the detection of serum sex hormones in orbital vein, that is, the success rate of the PCOS rat model induction by DHEA was 65.71% (46/70). After 4 weeks of the respective treatments, 14 rats in the model group ($n=15$) remained acyclic. Meanwhile, 8 rats in the baicalin group ($n=15$) and 11 rats in the spirolactone group ($n=15$) restored their 4- to 5-day estrous cyclicity, and their recovery rates were 53.33% (8/15) and 73.33% (11/15), that is, significantly higher than the natural recovery rate in

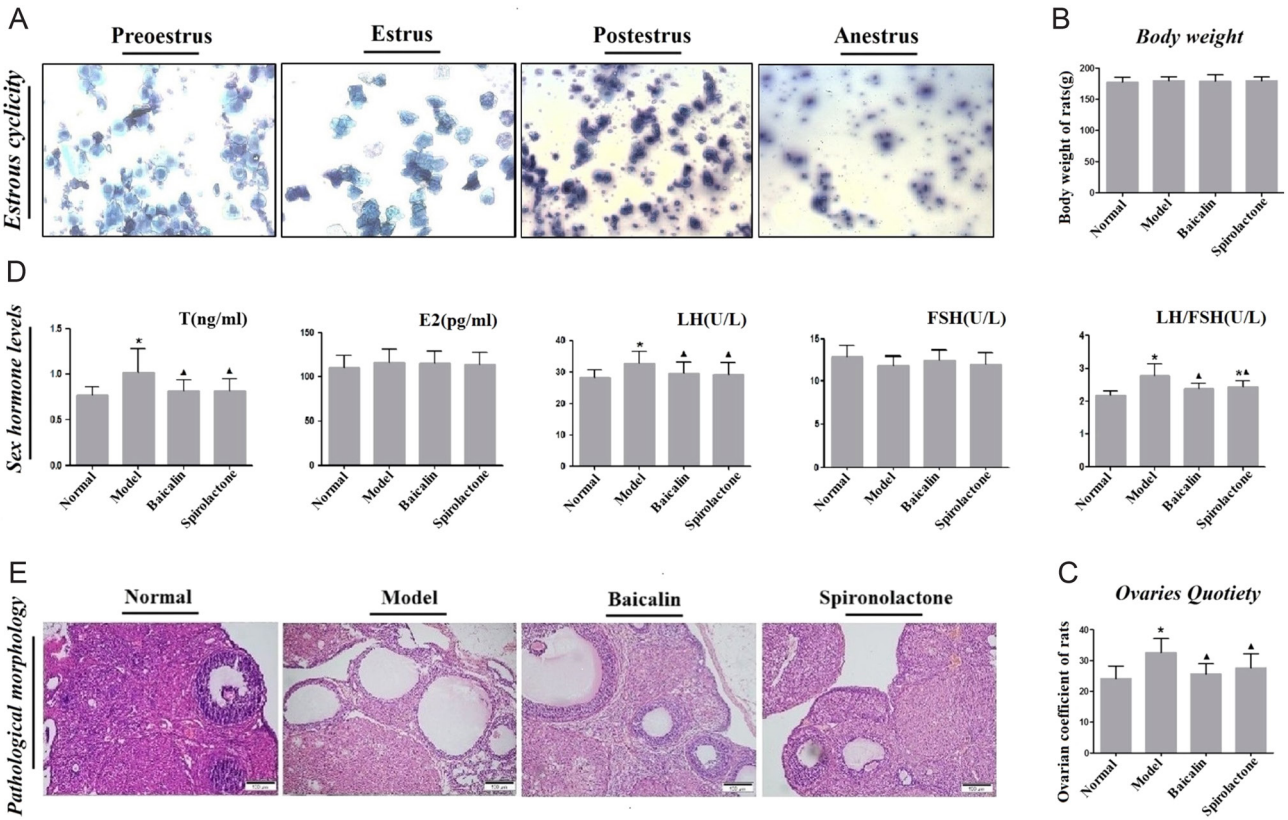

Figure 3
(A) Vaginal exfoliated cells of rats were observed under microscopy. In preoestrus, most cells are nucleated epithelial cells; in estrus, large numbers of keratin epithelial cells are observed; in postestrus, several types of cells are observed, including nucleated epithelial cells, keratin epithelial cells and leukocytes; in anestrus, large numbers of leukocytes and a small amount of mucus are observed. (B and C) Body weights and ovarian quotieties in rats. (D) Sex hormone levels in rats, analyzed by ELISA. *$P<0.05$ vs the normal group; ▲$P<0.05$ vs the model group. (E) Pathological morphology of ovarian tissues analyzed by light microscopy. There were no structural abnormalities in the control rats: the ovarian tissue was pink; follicles and corpora lutea were in varying stages of development; and granulosa cell layers were normal (the number of granulosa cell layers was 6–8). Differences in the model rats were significant: the color of ovarian tissue was generally lighter; the number of cystic follicles (large fluid-filled cysts) increased; granulosa cell layers were abnormal (the number of granulosa cell layers was 2–4 or even less) and the number of corpora lutea dropped sharply. The ovarian pathological morphology was greatly reversed in the drug groups: the color of ovarian tissue and the number of granulosa cell layers showed a certain degree of recovery, and the number of cystic follicles decreased, while that of corpora lutea increased. A full colour version of this figure is available at https://doi.org/10.1530/JOE-18-0678.

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the model group (6.67%, 1/15). Besides, the body weights of all rats were measured, and the ovarian quotiety was calculated as follows: ovarian quotiety=ovarian weight/body weight (mg/100 g). Based on the statistical data, the differences in the body weight among the groups were not significant ($P>0.05$; Fig. 3B). However, the ovarian quotiety was significantly higher in the model group than in the normal group and in the drug groups (Fig. 3C). In addition, the serum levels of testosterone and LH, as well as the LH/FSH ratios were effectively reversed after baicalin and spirolactone interventions (Fig. 3D), and the numbers of cystic follicles, corpora lutea and granulosa cell layers were greatly improved compared with those in the model group (Fig. 3E).

Baicalin inhibited HSD3B2 expression in ovarian tissues of PCOS rats

To further confirm our findings, the expression of *HSD3B2* mRNA and protein in ovarian tissues of the PCOS rats was determined by real-time PCR and IHC (Fig. 4A). The results showed that the expression of HSD3B2 was obviously downregulated by baicalin and spirolactone compared with that in the model group, which was consistent with the *in vitro* experimental results (Fig. 4B and C).

Discussion

It is well known that PCOS, which is characterized by accumulation in ovaries of incompletely developed follicles, due to anovulation, is a highly prevalent heterogeneous syndrome of the menstrual cycle and an endocrine dysfunction with clinical and/or biochemical hyperandrogenism, according to the Rotterdam criteria (Sirmans & Pate 2013). However, the pathophysiological mechanisms underlying PCOS and the exact physiological regulation of androgen biosynthesis remain obscure.

Research studies have advanced our understanding of the process and indicated that androgen biosynthesis in humans primarily occurs in steroidogenic tissues, predominantly in the zona reticularis of the adrenal cortex as well as in the theca or Leydig cells of the gonads (Kempná *et al.* 2015). All steroids are produced from cholesterol, and the process depends on a well-described cascade of steroid enzymes, which are specifically regulated to fulfill diverse biological functions. Cholesterol is transported into mitochondria through the action of steroidogenic acute regulatory protein (StAR), where it is converted into pregnenolone by the cytochrome P450 side-chain cleavage enzyme (CYP11A1), supported by ferredoxin reductase. Pregnenolone may then be converted to progesterone by the HSD3B2 enzyme or may undergo 17α-hydroxylation

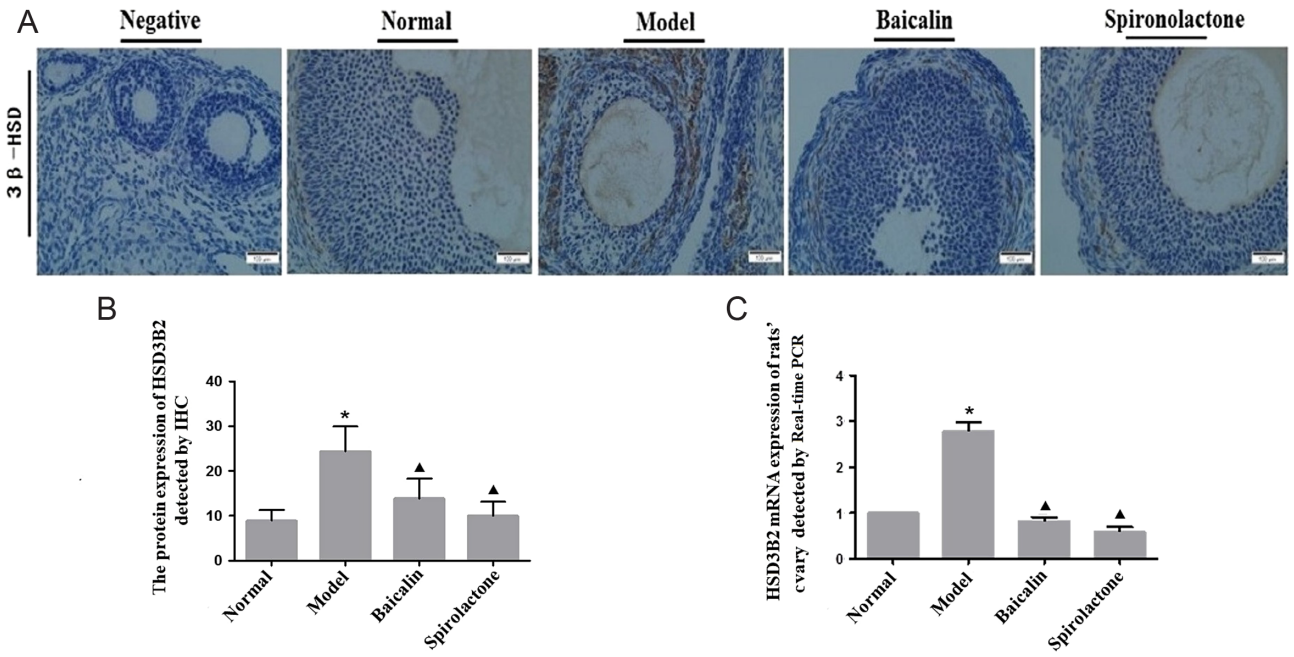

Figure 4
Expression of the HSD3B2 protein and mRNA in rat ovarian tissue was determined by IHC and real-time PCR, respectively. (A) The negative control group was used to account for nonspecific expression, and HSD3B2 was primarily located in theca cells (shade of brown). (B and C) *$P<0.05$ vs the normal group; ▲$P<0.05$ vs the model group. A full colour version of this figure is available at https://doi.org/10.1530/JOE-18-0678.

to 17OHPreg by the CYP17A1 enzyme. 17OHPreg may then be converted stepwise to 17OHP by HSD3B2 or to DHEA by the 17,20-lyase activity of CYP17A1, followed by the conversion to DHEAS by sulfotransferase SULT2A1 or to Δ4A by HSD3B2. Overall, it can be concluded that the CYP17A1 and HSD3B2 enzymes play essential roles in the biosynthesis of all classes of active steroid hormones, although some other enzymes such as CYP21A2, CYB5 and AKR1C3 (HSD17B5) participate in the initial steps.

Previous studies have focused on the role of the CYP17A1 enzyme, which seems to be related to qualitative regulation of androgen biosynthesis. The CYP17A1 enzyme has both hydroxylase and lyase activities (Miller 2012). The reports have shown that phosphorylation of CYP17 at serine or threonine residues is one of the post-translational events enhancing the CYP17 lyase activity (Tee & Miller 2013). Unlike the post-translational regulation of CYP17 lyase activity, specific regulation of HSD3Bs is largely unknown, although no less important. HSD3Bs are membrane-bound enzymes catalyzing two reactions, the conversion of the hydroxyl group to a keto group on carbon 3 and the isomerization of the double bond from the B ring (Δ5 steroid) to the A ring (Δ4 steroid) (Miller & Auchus 2011). The human HSD3B family consists of two members, namely, HSD3B type I (HSD3B1) and HSD3B type II (HSD3B2). HSD3B1 is mainly expressed in the placenta, mammary glands and prostate, as well as in peripheral tissues such as the skin and adipose tissue (Gingras *et al.* 1999). HSD3B2 is mainly expressed in adrenals and gonads and acts as a Δ5–Δ4 isomerase. Reports have shown that the deficiency of HSD3B2, caused by genetic mutations, leads to adrenal hyperplasia, which manifests as a primary adrenal insufficiency and a sexual development disorder (Welzel *et al.* 2008). Inversely, overexpression of HSD3B2 in ovarian tissues or the adrenal cortex leads to excessive androgen synthesis, which manifests as PCOS (Kempná *et al.* 2015). Interestingly, as revealed in our study, specific overexpression of HSD3B2 could be found in ovaries of the PCOS model rats and in steroidogenic cells by GEP analysis and was confirmed by real-time PCR and western blotting.

Here, it is necessary to explain the animal models and steroidogenic cell lines used in our study. Considering the ethical limitations in human studies, animal models that have many mimic features of PCOS are crucial resources to investigate this syndrome. At present, there are several methods for the preparation of PCOS animal models. For example, DHEA-induced PCOS, DHT-induced PCOS, TP (testosterone propionate)-induced PCOS, EV (estradiol valerate)-induced PCOS, letrozole-induced PCOS and so on. So far, the exact animal models that fully reflect the characteristics of PCOS have not been established. According to the literatures (Noroozzadeh *et al.* 2017) and our previous experimental results, we finally chose DHEA-induced PCOS, for which has a stable elevated serum levels of T and indicating the hyperandrogenic phenotype of PCOS. We know that it is very difficult to obtain ovarian tissue from PCOS patients by way of surgery due to the risk of damage to ovary function, and the ovarium-related cell lines used in the current researches are mostly related to ovarian cancer which have little to do with the regulation of steroid hormone synthesis of PCOS. Instead, human adrenocortical carcinoma NCI-H295R has a hyperandrogenic steroid profile and produces more androgen when grown without serum and insulin under starvation condition; therefore, NCI-H295R cell line was adopted in the experiments (Lee *et al.* 2016, Pyun *et al.* 2018).

Importantly, both our *in vivo* and *in vitro* studies showed that HSD3B2 and its specific regulation seem to play an important role in the pathogenesis of PCOS. Nevertheless, so far little is known about the exact regulation of HSD3B2. Previous studies have shown that transcription of *HSD3B2* can be activated by specific transcription factors such as Nur77 (nerve growth factor IB, NGFIB family), members of the Stat family, GATA elements, and so on. It has been suggested that Nur77 regulates the *HSD3B2* transcription in the granulosa as well as in Leydig and adrenal cells through a nuclear binding response element site (Havelock *et al.* 2005) and is essential for the HSD3B2 expression in the fetal adrenal steroidogenesis, from DHEA to cortisol production, during the time window of the development of external genitalia for safeguarding the female phenotype in girls (Goto *et al.* 2006). Stats are a family of cytoplasmic transcription factors, which are activated by a number of extracellular protein ligands, including cytokines, growth factors and prolactin. Thus, the prolactin-stimulated Stat5 activates the human *HSD3B2* promoter (Simard *et al.* 2005). The GATA family of proteins is a new group of transcription factors, which have emerged as important regulators of steroidogenesis. The human *HSD3B2* promoter can be activated by GATA4 and GATA6 and acts in concert with the nuclear receptor SF1 and liver receptor homolog 1 (LRH1) (Martin *et al.* 2005). This suggests that GATA factors are key regulators of HSD3B2 and that deregulated GATA expression and activity may be relevant to pathological

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processes associated with aberrant HSD3B2 expression, such as the adrenal insufficiency and PCOS (Simard *et al.* 2005).

The GATA family consists of six transcription factors (GATA1–6), which share conserved zinc finger motifs in the DNA-binding domains, recognize a consensus sequence (T/A)GATA(A/G) and are involved in a variety of physiological and pathological processes (Lentjes *et al.* 2016). GATA1, the first recognized member of the GATA family, was originally identified as a transcription factor exclusively required for the cell-specific expression of globin genes and other erythroid lineage-specific genes (Manna *et al.* 2002). Although an increasing number of studies are being published describing the expression and function of the *GATA1* gene during hematopoietic development of erythroid and megakaryocytic cell lineages, few reports have been published about its related roles in steroidogenic biosynthesis. Some studies have shown that the promoters of several cyclic AMP (cAMP)-regulated genes such as *StAR*, *CYP17*, *CYP11A1* and aromatase lack the 'classical' cAMP-response elements and that GATA1 functions as a downstream effector of cAMP signaling, which is a vital intracellular hormonal pathway (Zhang *et al.* 2002). Some researchers (Qamar *et al.* 2009) have demonstrated that the gene expression of the androgen receptor corepressor of 19 kDa (ARR19) in testicular Leydig cells is regulated by LH/cAMP signaling via the control of GATA1 expression, resulting in the control of testicular steroidogenesis. Similarly, in our study, the 5′ deletions and sequence analysis of the *HSD3B2* promoter revealed the presence of GATA1-binding motifs. Further analyses showed that the expression and transcriptional activity of *HSD3B2* could be markedly reduced if GATA1 was downregulated or its binding sites were disrupted. Thus, we speculated that GATA1 is one of the most important transcription factors for the activation of the *HSD3B2* promoter.

This study is originated from clinical findings; that is, the clinical symptoms of hyperandrogenism in PCOS patients could be better improved if *S. baicalensis* is added to the traditional Chinese medicine prescription. Thus, we propose a hypothesis that baicalin might have an inhibitory effect on the synthesis of androgens by regulating the expression or biological activities of certain genes or proteins and has the potential to be an effective therapeutic agent for hyperandrogenism in PCOS. A series of experimental studies provide novel evidence that HSD3B type II 2 (HSD3B2) gene transcription is activated by the transcription factor GATA1 which is recruited to the HSD3B2 gene promoter in the human adrenocortical carcinoma NCI-H295R cell line. Using gene expression profiling, we also identified GATA1 as one of the key genes affected by a traditional Chinese herbal medicine, baicalin. As well, baicailin treatment disrupts GATA1 binding to the HSD3B2 gene promoter, inhibited HSD3B2 expression and decreased testosterone production by NCI-H295R cells. Furthermore, in a rat model of PCOS, baicalin treatment decreased HSD3B2 in the ovaries, decreased serum testosterone, reversed endocrine abnormalities and restored estrous cyclicity. Therefore, we concluded that baicalin has potential as a therapeutic agent for the treatment of hyperandrogenism in PCOS. Frankly, limitation of this study is that data were obtained from NCI-H295R cell line and DHEA-induced PCOS rat models, that means these data maybe not fit humans exactly; moreover, some questions need to be elucidated including side effects of baicalin and potential pitfalls or risks of using this supplement in clinic. Compared with spironolactone, baicalin is a flavonoid compound extracted from natural plants herbs, and we are confident that it is safer and more accessible.

Supplementary data

This is linked to the online version of the paper at https://doi.org/10.1530/JOE-18-0678.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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