PTPN5 promotes follicle-stimulating hormone secretion through regulating intracellular calcium homeostasis

Hongmei Wang1 | Siyuan Bu1 | Jiajian Tang1 | Yi Li2 | Chunhua Liu3 | Junhong Dong4

1Department of Pharmacology, School of Medicine, Southeast University, Nanjing, China
2Department of Joint Surgery, Shandong Provincial Hospital Affiliated to Shandong First Medical University, Jinan, China
3Department of Physiology, Shandong First Medical University, Taian, China
4Department of Biochemistry, School of Basic Medicine, Weifang Medical University, Weifang, China

Correspondence
Chunhua Liu, Department of Physiology, Shandong First Medical University, Taian, Shandong 271000, China. Email: chhliu@sdfmu.edu.cn
Junhong Dong, Department of Biochemistry, School of Basic Medicine, Weifang Medical University, Weifang, Shandong 261021, China. Email: djh196@163.com

Abstract
Protein tyrosine phosphatase non-receptor type 5 (PTPN5), also called striatal-enriched protein tyrosine phosphatase (STEP), is highly expressed in neurons of the basal ganglia, hippocampus, cortex, and related structures, also in the pituitary. Gonadotropins are the key regulator of the reproduction in mammals. In this study, PTPN5 is detected to express in murine pituitary in a developmental manner. Moreover, the expression of PTPN5 in the pituitary is heavily reduced after ovary removal. Follicle-stimulating hormone (FSH) secretion in gonadotropes is regulated by PTPN5 via binding GnRH to GnRH-R. Two parallel signaling pathways, Gs-protein kinase A (PKA)-PTPN5 and Gq-phospholipases C (PLC)-p38 MAPK-PTPN5, cooperatively regulate GnRH-induced FSH secretion. We also show that influx of Ca2+ activates the Ca2+-dependent phosphatase calcineurin, leading to the phosphorylation and activation of PTPN5. The intracellular release of Ca2+ is reduced via TC2153. In conclusion, blocking or knocking out of PTPN5 reduces the release of FSH in whole pituitary. Mechanically, PTPN5 regulates gonadotropes' function through regulating intracellular calcium homeostasis.

KEYWORDS
calcium channel, FSH secretion, GnRH, OVX, PTPN5

Abbreviations: ER, estrogen receptor; EREs, estrogen response elements; ERK, extracellular signal-regulated kinase; FSH, follicle-stimulating hormone; GAPDH, reduced glyceraldehyde phosphate dehydrogenase; GnRH, gonadotropin-releasing hormone; GnRH-R, gonadotropin-releasing hormone receptor; KIM, kinase-interactive motif; LH, luteinizing hormone; MAPK, mitogen-activated protein kinase; O VX, ovariectomized; PKA, protein kinase A; PLC, phospholipase C; PTP, protein tyrosine phosphatase; PTPN5, protein tyrosine phosphatase non-receptor type 5; PTPN5-KO, PTPN5 knockout; RNA-seq, RNA sequence; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; STEP, striatal-enriched protein tyrosine phosphatase; TSH, thyroid-stimulating hormone.

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## INTRODUCTION

Protein phosphorylation plays a central role in cell signaling. A delicate balance exists between the level of phosphorylation caused by protein tyrosine or serine/threonine kinases and the opposing actions of protein tyrosine or serine/threonine phosphatases. Protein tyrosine phosphatase non-receptor type 5 (PTPN5), also called striatal-enriched protein tyrosine phosphatase (STEP), is highly expressed in neurons of the basal ganglia, hippocampus, cortex, and related structures, also in the pituitary.1

The pituitary is an important endocrine system organ in the body. There are five types of hormones secreted by the pituitary gland: growth hormone, prolactin, adrenocorticotropic hormone, thyrotropin, and gonadotropin.2 Follicle-stimulating hormone (FSH), a glycoprotein polypeptide hormone, is produced by the anterior pituitary gland, and is ubiquitously considered to function in regulation of sex hormone synthesis in the gonads.3 Many studies established that FSH levels become elevated combined with a decrease of estrogen in menopause.4 Estrogen regulates the gene expression through genomic signaling pathways and non-genomic signaling pathways. Estrogen can enter the cell membrane and interact with estrogen receptor (ER), which can mediate non-genomic signaling pathways on the cell membrane and genomic signaling pathways in the cytoplasm. In genomic signaling pathways, estrogen activates ER to bind estrogen response elements (EREs) in the DNA-regulated region, mediating the transcription of target genes.5,6 To date, several studies have explored the roles of PTPN5 in physiological and pathophysiological processes in the brain. Studies also suggest that FSH levels may be more highly associated with health outcomes of postmenopausal women, compared with estrogen levels.3 However, the effects of PTPN5 on FSH levels of menopausal and postmenopausal women have not been reported.

The aim of the present study is to examine the role of PTPN5 in the regulation of FSH in the pituitary. Female mice are ovariectomized to eliminate estrogen production and the inhibitory feedback effect of estrogen on pituitary FSH production and release, in order to simulate menopausal women.7 In the gonadotropes, FSH and luteinizing hormone (LH) secretion on gonadotropin-releasing hormone (GnRH) stimulation suggests the need for synchronization of the cellular secretory machinery.1,8 Meanwhile GnRH activates intracellular signaling pathways causing membrane depolarization and a rapid change in intracellular Ca2+ concentration, which results in FSH and LH secretion.9 Considering that estrogen is required for FSH and LH secretion, and ERα can upregulate genes, especially PTPN5,10 we hypothesize that PTPN5 primes the pituitary to regulate FSH and LH secretion which ovariectomized (OVX) mice are used to simulate menopausal women.

## MATERIALS AND METHODS

### 2.1 Animals and ethics statement

Adult male *Ptnp5*−/− mice were purchased from The Jackson Laboratory (stock #016556; Bar Harbor, ME, USA) and extensively backcrossed on the C57BL/6 background for over 10 generations. Animals were housed in cages lined with sawdust under a standard 12/12 hours light/dark cycle (lights on at 08:00 AM) with food and water available ad libitum. *Ptnp5*−/− (KO) mice and their *Ptnp5*+/+ (WT) littermates were randomly divided into two groups. The 12-week-old female mice underwent either sham operation (control group, n = 9-11) or bilateral ovariectomy (OVX group, n = 9-11) while under anesthesia with inhalation gas isoflurane.11,12 Four weeks after surgery, mice in the OVX groups were sacrificed for experiment. Age-matched 16-week-old females were studied in the followed experiments. All experimental procedures were approved and performed in accordance with the ethic regulations and the animal welfare committees of Southeast University (20170302006). All efforts were made to minimize animal suffering and to reduce the number of animals used.

### 2.2 In vitro pituitary stimulation

Sixteen-week-old animals were killed using CO2. Mice intact pituitary glands were removed and placed in 2 mL of DMEM with a protease inhibitor cocktail on 6-well plate. All female mice were killed during the diestrus phase of the cycle, as determined by visual inspection. All incubations were carried out at 37°C, 5% CO2, with constant shaking. The pituitary glands were pre-treated with or without 10 nM TC2153 (SML1299, Sigma-Aldrich), which was the selective inhibitor of PTPN5,13 and TC-2153 demonstrated psychotropic activity and low level of acute toxicity.14 After 60 minutes, the medium was replaced with fresh medium with 100 nM GnRH (L7134, Sigma-Aldrich) to active GnRH receptor on gonadotropes. Fifty microliters of samples was taken at 30, 60, and 120 minutes, and pituitary glands were also collected following stimulation to measure total hormone levels.15

All hormone measurements were performed with a Milliplex MAP mice pituitary magnetic bead panel (RPTMAG-86K; Millipore, Billerica, MA) on a Luminex Magpix (Austin, TX) with Milliplex Analyst software.

### 2.3 Ovariectomy

For ovary removal surgery, 12-week-old female *Pttnp5*−/− mice and their *Pttnp5*+/+ littermates (breeding in Animal Center of Southeast University) were administered with inhalation anesthesia (Isoflorane, RWD, Shenzhen, China) and subjected to
either a sham operation (WT or PTPN5) or bilateral ovariectomy (WT-OVX or PTPN5-OVX). Complete OVX was evaluated by visual inspection of the ovaries after their extraction. Four weeks later, mice of Sham group and OVX group were used for further experiments. All female mice were killed during the dioestrus phase of the cycle, as determined by visual inspection.

2.4 | Quantitative real-time PCR and transcriptome analysis

Total RNA was extracted from the mouse pituitary (WT, PTPN5, WT-OVX, or PTPN5-OVX) using a standard TRIzol RNA isolation method (Invitrogen). The cDNA synthesis and PCR amplification were performed with the ReverTra Ace qPCR RT Kit (TOYOBO FEQ-101), according to the manufacturer’s protocols. The real-time PCR was conducted in the LightCycler quantitative PCR (qPCR) apparatus (BIO-RAD) using the FastStart Universal SYBR Green Master (Roche). The expression value was normalized to Gapdh in the same sample and then normalized to the control. The sequences of the primer pairs are provided in Table 1.

The expression patterns of PTPN family were extracted from a published murine gonadotroph RNA sequencing data (Molecular Plasticity of Male and Female Murine Gonadotropes Revealed by mRNA Sequencing, supplemental Table 1). This published RNA-seq data were obtained from libraries prepared from murine gonadotropes enriched from juvenile and adult mice (including different female hormonal sages). We considered all PTPN family members with FPKM > 1 to be expressed. The heatmap of the expression patterns of PTPN family was generated using the MATLAB clustergram script.

2.5 | Isolation of primary cells, cell culture, and transfection

Dissociated pituitary cells were prepared at 16 week from female C57BL/6 wild-type PTPN5-KO and OVX mice, as described previously. These cells were grown in DMEM with 10% fetal calf serum (all from Gibco by Life Technologies, Paisley, UK), and maintained at 37°C and 5% CO₂. Cells were cultured in grid coverslip for further experiment. The cells were analyzed as gonadotropes which were activated by GnRH and the cells were FSH/LH-positive cells, and were used for statistical analysis in each group of experiments. About 100 positive cells of 3-5 mice were used in each group.

RC-4BC cell line was also cultured in the same medium with dissociated pituitary cells. For PTPN5 overexpression experiments, human full-length coding region of PTPN5 was amplified from liver cDNA library using the primers: 5′-A ATTATGAGGGAGCCAGGAGTGAGAG-3′ and 5′-TTCT GGGGACTGGGACACGTGTCTTTC-3′, and cloned into pcDNA3.1 vector. The pcDNA3.1-HA-vector, pcDNA3.1-HA-PTPN5-WT, S245A, T255A, and S268A were transfected into RC-4BC cells with Lipofectamine 3000 (Invitrogen, 100022234). For PTPN5 knockdown experiment, a pair of oligonucleotides, 5′ UUC UUU GAA AUC CCC AUG ATT 3′ (sense) and 5′ UCA UGG GGA UUU CAA AGA ATT 3′ (anti-sense), was prepared to silence PTPN5 meanwhile another pair of oligonucleotides, siRNA-GFP was used as a negative control. Briefly, RC-4BC cells, plated on 6-well culture dishes, after 48 hours, were treated with GnRH or different inhibitors for the indicated time to analyze FSH secretion and calcium signal.

2.6 | Measurements of cytoplasmic Ca²⁺ by Fura-2

Cells were cultured on coated grid coverslips and loaded with 5 μM Fura-2 AM (Invitrogen by Thermo Fisher Scientific, Eugene, Oregon, USA) for 45 minutes at 37°C in the dark. For every 2 seconds, Fura-2 was alternately excited (0.5 Hz) at 340 and 380 nm for 20 ms, and the emitted fluorescence was recorded. After background correction, ratio images were calculated from 340 to 380 nm images. The details were described in the study by Belkacemi et al.

| TABLE 1 | RT-PCR primers |
|-----------------|-----------------|-----------------|-----------------|
| **Names** | **Forward primer (5′ to 3′)** | **Reverse primer (5′ to 3′)** | **Amplified product (bps)** | **Accession** |
| Ptpn5 | GCACTGTGGGCCGACTTTTG | CTGCACGGTGATCTCCACG | 149 | NM_013643.2 |
| Ptpn13 | ACCATGAATCAGACTCATCCACT | TGAAGAAACAATGCTCATCTTT | 143 | NM_011204.2 |
| Ptpn1 | GGAACCTGGGCCGCTATTTC | CAAAAGGGCTGACATCTCGGT | 117 | NM_011201.3 |
| Ptpn18 | AGTCGCCACTAGGGACTTG | CTTTAATGTCGGTAACTACG | 100 | NM_011206.2 |
| Ptpn22 | CAGCAACTCTGAAAAGACCC | AGGATAGATTTTGTCGGCCTTG | 114 | NM_008979.2 |
| Ptpn12 | ATGGAGCAACTGGAGACTTCCTG | TCTCAATGCGATGCTGCCCTG | 108 | NM_001356590.1 |
| Gapdh | TACGGGGTCAGTACCTAGCA | GAGGGCTGACTCCGTATT | 128 | NM_008084.3 |
Then pituitary cells were incubated with guinea pig and rabbit polyclonal α-FSH, and LH antibodies for immunofluorescence which were provided by the National Hormone and Peptide Program (Torrance, CA). For immunofluorescence, the secondary antibodies were α-guinea pig Alexa Fluor 488 and α-rabbit Alexa Fluor 647 (1:800) purchased from Life Technologies (Madison, WI). Secondary α-rabbit- or α-mouse-conjugated horseradish peroxidase (H + L) antibodies, used for immunoblot analysis (diluted 1:1000 or 1:10 000), were purchased from Synaptic Systems.

2.7 | Electrophysiology

Electrophysiological signals were recorded with an EPC-10 amplifier (HEKA Electronic, Germany) in the whole-cell patch clamp configuration. Patch pipettes had a resistance of 4-5 MΩ with intracellular solution contained (in mmol/L): 140 KCl, 1 MgCl2, 10 HEPES, pH 7.2 with KOH. The extracellular solution contained (in mmol/L): 120 NaCl, 2 CaCl2, 2 MgCl2, 4.7 KCl, 10 Glucose, 10 HEPES, pH 7.4 with NaOH. The holding and recording potential were set to −60 mV for GnRH-induced outward current.

2.8 | Single-cell RT-multiplex PCR

For single-cell RT-PCR, we applied the following procedure: After whole-cell patch clamping, the single pituitary cell which was response to GnRH was harvested by applying negative pressure to the patch pipette, then the pipette tip containing the cell was broken into a reaction tube containing 8.5 µL of buffer solution (Maxima First Strand cDNA Synthesis Kit, Thermo Fisher Scientific). After freezing for at least 1 hour at −20°C, the enzyme mix was added and the cDNA synthesis was performed according to the manufacturer’s guide. Primer sequences were given in Table 2.

2.9 | Protein extraction and western blot analysis

RC-4BC cells (1.5 × 105 cells/mL, 1 mL) were cultured in six-well culture plates for 24 hours. Subsequently, cells were transfected with mock RNAI and 1 × 107 IU/mL PTPN5-RNAI lentiviral vector for 48 hours. These cells were used to extract the total protein with lysis buffer (PMSF: RIPA = 1:100) and the concentration of protein was measured with the BCA protein assay kit (Beyotime, Shanghai, China). Thirty-five micrograms of protein was separated by 10% SDS-PAGE gels. After electrophoresis, transmembrane, and blockage, the PVDF membranes (Millipore, USA) were incubated with primary antibodies overnight at 4°C anti-HA: 1:400, anti-PTPN5: 1:400 (Santa Cruz, CA, USA), and anti-β-Actin: 1:2000 (Biosynthesis, Beijing, China). Then, the membranes were incubated with the secondary antibody (1:2000) at room temperature for 1 hour. Finally, the immunoblots were developed using ECL reagents (Santa Cruz, CA, USA) and detected after exposure to X-ray film. Densitometer readings were carried out to quantify the immunoreactive bands.

2.10 | Statistical analysis

All statistical analyses were performed with GraphPad Prism software version 6.07 (RRID: SCR_002798, www.graphpad.com/company/, USA). Results were expressed as mean values ± standard error of the mean (SEM). The differences between two groups were tested by independent unpaired two-tailed Student’s t tests. One-way ANOVA or two-way ANOVA was applied to analyze multiple groups as indicated in the figure legends. Differences were considered significant at *# P < .05 and **, ##, $$P < .01. The sequence alignments were performed using the T-coffee multiple sequence alignment program (www.expasy.org/, Switzerland).

3 | RESULTS

3.1 | PTPN5 is expressed in adult female mouse pituitary and its expression is reduced after OVX

PTPN5 is very important in the central nervous system. To figure out whether PTPN5 is expressed in the pituitary, we first analyze the expression in the developmental pituitary by RT-qPCR (Figure 1A-D). Our results revealed that PTPN5

| Names | Forward primer (5’ to 3’ ) | Reverse primer (5’ to 3’) | Amplified product (bps) | Accession |
|-------|---------------------------|---------------------------|-------------------------|-----------|
| Lhβ   | AGAATGGAGAGCGCTCCAGGG     | CCATGCTAGGACATGACCGG      | 187                     | NM_008497.2 |
| Fshβ  | ATTTCAACATTTAGCTTGATT     | AGAAAACACTAGGAACCCTT     | 169                     | NM_008045.3 |
| Tshβ  | AGAGTGACGGCATAACAGGTG     | GCAAGAGAAAAAGACGGA       | 108                     | NM_009432.2 |
| Gapdh | TACGGGTGCAACGTAGCTCA      | GAGGGCTGCAGTCCGTATT      | 128                     | NM_008084.3 |
was expressed in the mouse pituitary in a developmental level, with barely detectable levels in 2-week-old and 4-week old female pituitaries. The mRNA level of *Pttn5* showed the highest expression at 16-week-old female pituitary compared with other ages. Western blot also showed significant difference of PTPN5 expression in pituitary between the pups and the adult female mice (Figure 1E). For male mice pituitaries, there were barely detected significant difference in developmental ages (Figure 1C). To further investigate which cell types express PTPN5 in the pituitary, we extracted a published
RNA-seq dataset which examined the transcriptome of murine gonadotropes from different hormonal stages. We found *Ptpn5, Ptpn1, and Ptpn12* were expressed in gonadotropes (Figure S1). Interestingly, *Ptpn5* was highly expressed in adult female gonadotropes, revealing a gender difference and developmental manner. Moreover, among the adult female gonadotropes, *Ptpn5* showed the highest expression in proestrus gonadotropes and the lowest in lactating gonadotropes, indicating that the expression pattern of *Ptpn5* in gonadotropes was regulated by hormonal status. We next performed ovary removal on mice and then measured the mRNA level of *Ptpn5* in the pituitary. After OVX, the mRNA expression of *Ptpn5* decreased sharply compared with 16-week mice (Figure 1B). Western blot also showed significant difference of *PTPN5* protein expression in pituitary between adult and OVX female mice, with no band detected in the pituitary from *Ptpn5* knockout mice (Figure 1E). There was no *Ptpn5* expression in pituitary between adult and OVX female mice. Statistical analysis was performed by one-way ANOVA, **P < .01** compared with 2-week-old mice.

#### 3.2 TC2153 treatment reduced FSH secretion in female but not PTPN5 knockout or OVX mice

Since *Ptpn5* was expressed in the pituitary and its expression was regulated by gonadotropin, we decided to investigate whether *Ptpn5* played a role in the pituitary. The pituitary gland exerted the role as an endocrine organ, by secreting a variety of hormones into the circulation system to affect the targeted organs. We adapted a whole-organ culture system to investigate the hormone secreting ability of the pituitary. We stimulated the pituitary with GnRH under different conditions to figure out whether *Ptpn5* played a role in gonadotropins release. To determine whether *Ptpn5*-regulated FSH or LH release from gonadotropes, hormone secretion from whole dissected pituitary glands (Figure 2A), of 16-week female wild-type (WT) mice, *Ptpn5* knockout (KO) mice, or ovariectomized (OVX) mice, was monitored with stimulated the addition of gonadotropin-releasing hormone (GnRH). Secretion of FSH was stimulated by the addition of GnRH (100 nM) and TC2153 (10 nM) at different time points. As shown in Figure 2B, FSH secretion was significantly reduced under TC2153 treatment (an inhibitor of PTPN5), at 60 and 120 minutes. Meanwhile *Ptpn5*-KO mice also showed a reduced FSH level at the same time points compared with WT mice (Figure 2B). However, we did not find that LH level was significantly changed under both conditions (Figure 2C). Interestingly, we found that FSH or LH was unaffected under all conditions in OVX of WT and PTPN5-KO mice (Figure 2B,H). Total hormone levels in whole pituitary glands were detected, and we found that there was no significant difference in four groups of adult female mice (Figure 2E-G). These data revealed that the role of *Ptpn5* in FSH secretion was periodic specific. TC2153 treatment did not affect either FSH or LH secretion in OVX mice under any conditions tested (Figure 2H,I). Furthermore, the release of another hormone, TSH was unaffected by TC2153 treatment in the GnRH-stimulated condition at all time points, in both WT and PTPN5-KO mice (Figure 2D). For total hormone levels of pituitary, FSH, LH, and TSH were showed in similar levels in four groups of OVX mice (Figure 2J,K,M). Totally, the induction of FSH secretion in response to GnRH stimulation in vitro pituitary culture was reduced in PTPN5-KO adult female mice. Furthermore, there was no increase in FSH secretion in response to GnRH treatment in the presence of OVX mice between WT and PTPN5-KO mice. Additionally, FSH secretion was decreased in WT adult female mice on pre-treated TC2153 and GnRH stimulation.

#### 3.3 Knockout or inhibition of PTPN5 reduced cytosolic Ca$^{2+}$ levels and the current peak in gonadotropes from adult female mice under GnRH application

Canonically, the stimulation of GnRH induces the activation of the cyclic adenosine monophosphate (cAMP)/PKA pathway and PLC/PKC pathway and an increase of cytosolic Ca$^{2+}$
levels in gonadotropes. To evaluate the cellular function of PTPN5 in gonadotropes, we tested whether blocking of PTPN5 had effect on the intracellular calcium increase induced by GnRH in gonadotropes.

To investigate the possibility that TC2153 stimulation resulted in a falling of intracellular calcium, we utilized the fluorescence video-imaging technique with the Ca**2+**-sensitive probe Fura-2 in gonadotropes (Figure 3A). Then,
we found that the calcium increased only upon 10 nm GnRH, as shown in Figure 3B, we found a strong intracellular calcium increase, followed by calcium oscillations in gonadotropes after GnRH treatment. Interestingly, the cytoplasmic Ca$$^{2+}$$ increase was significantly reduced when the gonadotropes were preincubated with TC2153 (10 nM) for 10 minutes (Figure 3C). Moreover, the Ca$$^{2+}$$ increase was also significantly reduced in gonadotropes form PTPN5-KO mice upon GnRH stimulation (Figure 3D), and this reduction was not influenced by preincubation of TC2153 (Figure 3E). A Ca$$^{2+}$$ increase was significantly reduced in gonadotropes of PTPN5-KO mice upon GnRH stimulation, and not inhibited with GnRH-TC2153 treatment as depicted by the exemplifying traces (Figure 3D,E) and by the bar graph showing the average amplitudes of the Ca$$^{2+}$$ signals (Figure 3F,G). Resting cytoplasmic [Ca$$^{2+}$$] prior to agonist application was not significantly different after GnRH or TC2153 treatment, but GnRH-induced Ca$$^{2+}$$ increase was significantly lower in gonadotropes of PTPN5-KO adult female mice compared with WT adult female mice. We found that the presence of TC2153 counteracted the effect of GnRH on the intracellular Ca$$^{2+}$$ increase in gonadotropes of WT adult female mice, suggesting that activation of PTPN5 by GnRH involved calcium-dependent pathway.

### 3.4 Inhibition of PTPN5 KO and TC2153 reduced the current peak responding to GnRH in gonadotropes of adult female mice

To understand the contributions of the intrinsic properties of PTPN5 to burst firing, we performed whole-cell current-clamp recordings on dissociated gonadotropes which were confirmed by the response of GnRH simulation and single-cell PCR in the presence of TC2153 to antagonize effects attributable to the activation of GnRH receptor.

The frequency plots did not show any difference between cycle stages on different treatment. These data suggested that PTPN5-regulated calcium current properties to affect FSH secretion in adult female mice. Gonadotropes exhibited the distinct firing pattern in response to −60 mV for GnRH-induced outward current. Representative examples under control conditions were shown in Figure 3H. We averaged the instantaneous firing frequency of each cell type on each cycle stage and plotted this as a function of spike interval number. We calculated the size of the peak and found that the current peak was decreased with TC2153 treatment compared with only GnRH treatment in adult female mice (Figure 3H,I). The same experiment was applied to PTPN5-KO adult female mice, and we found different results in WT female mice. The current peak was significantly lower under 10 nM GnRH treatment in gonadotropes of PTPN5-KO adult female mice compared with WT adult female mice (Figure 3J). Meanwhile the current peak was not inhibited under 10 nM TC2153 (Figure 3K). The average amplitudes of the electrophysiological signals were shown as the bar graph (Figure 3L). The frequency plots did not show any difference between cycle stages on different treatments. These data suggested that PTPN5 regulated calcium current properties to affect FSH secretion in adult female mice. Taken together, these results suggested that PTPN5 is involved in the GnRH-induced calcium pathway in gonadotropes.

### 3.5 Inhibition of PTPN5-TC2153 induced no changes in [Ca$$^{2+}$$] in gonadotropes of ovariectomized female mice with GnRH treatment

Next, we explored the effects of PTPN5 in gonadotropes of ovariectomized female mice with GnRH treatment, in the same concentration range, on intracellular calcium homeostasis, a key parameter in the control of gonadotropes function. TC2153-induced GnRH-dependent changes under intracellular calcium concentration in adult female mice (Figure 3B,C). After we detected the calcium signal, we did immunofluorescence to confirm the cell type (Figure 4A). Again, we analyzed the calcium increase with 10 nM GnRH of gonadotropes in OVX female mice (Figure 4B). There was still a strong intracellular calcium buildup, followed by calcium oscillations. When challenged with 10 minutes long exposure to TC2153 (10 nM), gonadotropes showed a similar
cytoplasmic Ca\textsuperscript{2+} increase (Figure 4C). When treated with the same condition in PTPN5-KO adult female mice, gonadotropes also showed a similar cytoplasmic Ca\textsuperscript{2+} increase between GnRH and GnRH-TC2153 groups (Figure 4D,E). Ca\textsuperscript{2+} is released from intracellular stores, mainly the endoplasmic reticulum (ER), and the cytoplasmic Ca\textsuperscript{2+} increases. This increase was monitored by Fura-2 and characterized as peak amplitude and area under the trace in gonadotropes.
mice showed less sensitive to TC2153 than adult mice. Under this condition, gonadotropes of ovariectomized female mice exhibited different rebound patterns based on cycle stage led us to examine potential underlying mechanisms in greater detail. We first tested the electrophysiological response to GnRH on gonadotropes at different stages could exhibit different rebound patterns based on cycle stage led us to examine potential underlying mechanisms in greater detail. We first tested the electrophysiological response to GnRH on gonadotropes of WT adult female mice. C. The responses of the same cells measured with Fura-2 in the presence of addition of 10 nM GnRH from gonadotropes of WT adult female mice. C, The responses of the same cells measured with Fura-2 in the presence of addition of 10 nM GnRH and 10 nM TC2153 from gonadotropes of WT adult female mice. D, Ca\(^{2+}\) response in gonadotropes of PTPN5-KO adult female mice with 10 nM GnRH stimulation. E, Ca\(^{2+}\) response in gonadotropes of PTPN5-KO adult female mice with and 10 nM TC2153 treatment and 10 nM GnRH simulation. F and G, Summarized Δratios from experiments like the ones presented from B to E, calculated as areas under the curve and peak amplitude. The data were analyzed using two-way ANOVA, *P < .05 and **P < .01 compared with corresponding time points of WT. H, A representative current-clamp recording under 10 nM GnRH-induced depolarization. The resting membrane potential was ~60 mV from gonadotropes of WT adult female mice. I, A decreased current-clamp recording after 10 nM TC2153 treatment when stimulating with 10 nM GnRH. Inhibition of PTPN5 reduced the current peak response to GnRH in gonadotropes. J and K, Similar experiment as above in the presence of pre-treated 10 nM TC2153 and then 10 nM GnRH stimulation from gonadotropes of PTPN5-KO adult female mice. L, Summary of the significant depolarizing effect of TC2153 in the presence of GnRH compared with H. Data were shown as mean ± SEM. The number of cells was obtained from three wild-type and three PTPN5-KO mice. P values were calculated using two-way ANOVA, ***P < .01 compared with WT.

(Figure 4F,G). TC2153 did not lead to significantly reduce on cytoplasmic Ca\(^{2+}\) in OVX adult female mice. GnRH-stimulated GnRH receptor in gonadotropes, followed by IP3-dependent Ca\(^{2+}\) release. This Ca\(^{2+}\) release which was stimulated by TC2153 was similar to the curve only of GnRH treatment on both WT and PTPN5-KO OVX mice (Figure 4D,E). The Ca\(^{2+}\)-induced fluorescence was plotted and the area under the curve was analyzed in Figure 4F,G. Under this condition, gonadotropes of ovariectomized female mice showed less sensitive to TC2153 than adult mice.

### 3.6 Inhibition of TC2153 had no effect on current peak response to GnRH in gonadotropes of ovariectomized female mice

The observation that gonadotropes at different stages could exhibit different rebound patterns based on cycle stage led us to examine potential underlying mechanisms in greater detail. We first tested the electrophysiological response to GnRH on gonadotropes at different stages could exhibit different rebound patterns based on cycle stage led us to examine potential underlying mechanisms in greater detail. We first tested the electrophysiological response to GnRH on gonadotropes of WT adult female mice (Figure 4H). Then 10 nM TC2153 was applied in the presence of GnRH (Figure 4I). TC2153 application did not result in significant depolarization of gonadotropes in the presence of GnRH compared with Figure 4H, and the cells did not fully recover to baseline membrane potential for the duration of the recording. The scatter plots were analyzed statistically that TC2153 had no effect on electrophysiological signals in OVX female mice (Figure 4I). RT-qPCR and western blot in OVX mice showed that the expression levels of Ptpn5 mRNA and protein were significantly decreased or disappeared. These results confirmed the regulatory effect of PTPN5 on calcium and electrical signals.

### 3.7 Regulation of FSH secretion by two parallel signaling pathways

To gain further insight into the molecular events of PTPN5 that contributed to the regulation of FSH secretion, we determined to test whether PTPN5 regulates FSH secretion in RC-4BC cell line. According to the current research progress, FSH secretion with GnRH stimulation are regulated through protein kinase C/MAPK pathways, calcium/calmodulin-dependent kinase II (CaMK II) pathway, calcineurin/NFAT pathway, and cAMP/PKA/CREB pathway. PKA (protein kinase A) has the function to phosphorylate S245 site of PTPN5; T255 and S268 of PTPN5 is phosphorylated by ERK or p38 MAPK to increase its activity. In Figure 5A, to study the molecular mechanisms, we constructed WT, S245A, T255A, S268A, and C496S mutants of PTPN5 (Figure 5A and Sup. Figure 2A), meanwhile amino acid sequence alignment showed the conserved pattern of these three sites in KIM- and KIS-containing PTPs (Figure 5B). We first overexpressed PTPN5-WT in RC-4BC cells (Sup. Figure 2B). RC-4BC cells were preincubated with or without 10 nM TC2153 for 30 minutes and then stimulated with 10 nM GnRH. We found that FSH levels were increased at 60 and 120 minutes after GnRH application. Furthermore, TC2153 incubation abolished the effects of PTPN5 overexpression on FSH secretion (Figure 5C). We next knocked down the expression of PTPN5 by transferring cell line with oligomeric siRNA of Ptpn5 (siPTPN5). As shown in Figure 5D and Supporting Information Figure S2C, the expression of PTPN5 was reduced by up to 60% of basal expression in RC-4BC cell line, 48 hours after transfection. Knocking down the expression of PTPN5 decreased FSH secretion to approximately 50% compared with the control siGFP-treated group at 60 and 120 minutes (Figure 5D).

We next used specific Gq and Gs antagonists to dissect the contribution of each pathway to FSH secretion. The application of the PKA antagonist H89, the PLC antagonist U73122, or the P38 MAPK antagonist significantly reduced FSH secretion, but did not abolish it. Among them, the PLC antagonist U73122 decreased FSH secretion predominantly in the pituitaries of WT mice (Figure 5E,F). However, PTPN5 knockout mice did not show significant inhibitory effects under different inhibitors (Figure 5G,H). To further confirm these data, RC-4BC cell line was also incubated with...
Inhibition of PTPN5 did not change the Intracellular Ca$^{2+}$ and current peak to GnRH in gonadotropes from WT or PTPN5-KO ovariectomized female mice. A, Double-label immunofluorescence revealed FSH and LH immunoreactivity localized in the gonadotropes of the pituitary after calcium image. Arrows indicate location of FSH and LH-positive cell; scale bars 50 µm. B, The responses of the same cells measured with Fura-2 in the presence of addition of 10 nM GnRH from gonadotropes of WT-OVX adult female mice. C, The responses of the same cells measured with Fura-2 in the presence of addition of 10 nM GnRH and 10 nM TC2153 from gonadotropes of WT-OVX adult female mice. D, Ca$^{2+}$ response in gonadotropes of PTPN5-KO-OVX mice with 10 nM GnRH simulation. E, Ca$^{2+}$ response in gonadotropes of PTPN5-KO-OVX mice with and 10 nM TC2153 treatment and 10 nM GnRH simulation. F and G, Summarized Δratios from experiments like the ones presented from B to E, calculated as areas under the curve and peak amplitude. $P$ values were calculated using two-way ANOVA. H, A representative current-clamp recording under 10 nM GnRH-induced depolarization. The resting membrane potential was −60 mV from gonadotropes of WT-OVX mice. I, A similar current-clamp recording after 10 nM TC2153 treatment when stimulating with 10 nM GnRH. Inhibition of PTPN5 uneffected the current peak response to GnRH in gonadotropes of OVX mice. J, Summary of the significant depolarizing effect of TC2153 in the presence of GnRH compared with H. Data were shown as mean ± SEM. The number of cells was obtained from three OVX mice. $P$ values were calculated using student’s $t$ test.
these inhibitors, and similar phenomenon had been observed (Figure 5I). As we know, PKA (protein kinase A) functions to phosphorylate S245 site of PTPN5; T255 and S268 of PTPN5 are phosphorylated by ERK or p38 MAPK to increase its activity.\textsuperscript{28,30} The GnRH-induced increase in FSH level was also detected in basic cultivation at 120 minutes (Figure 5J).
Phosphorylation at the serine residue in the KIM domain of PTPN5 (Ser245, Figure 5A) was mutated to S245A, which reduced its activity. T255A and S268A in the KIS domain of PTPN5 affected their own phosphorylation via ERK and p38 MAPK. We also investigated FSH secretion with overexpression mutations of PTPN5. Compared with PTPN5-WT group, S245A group had no effect on FSH secretion, while T255A and S268A mutations made FSH level lower in the medium. T255A and S268A mutations inhibited PTPN5 activity to reduce FSH secretion (Figure 5E,I). Therefore, we concluded that FSH secretion with GnRH stimulation was mainly regulated by Gq-PLC-ERK/p38 MAPK pathway via phosphorylation of PTPN5-T255 and S268 sites, and Gs-PKA pathway also regulated FSH release by targeting other sites of PTPN5.

The previous data showed that siPTPN5 or TC2153 treatment downregulated FSH secretion via reducing calcium release. Furthermore, we analyzed intracellular calcium influx in RC-4BC cell line, and measured the intracellular calcium concentration. As shown in Figure 6A, A Ca\(^{2+}\) increase was detected under GnRH stimulation, and was sharply reduced in RC-4BC upon U73122 incubation as depicted by the exemplifying traces (Figure 6B). The intracellular calcium concentration under siPTPN5 treatment was decreased compared with control group (Figure 6A,C), and also was significantly reduced by U73122 incubation in siPTPN5/RC-4BC cells (Figure 6D). U73122 was an inhibitor of intracellular calcium concentration. The average area and average amplitudes of the Ca\(^{2+}\) signals were shown in Figure 6E,F. Finally, we constructed a model depicting the role of PTPN5 in the regulation of FSH release (Figure 6G). Loss of PTPN5 function in gonadotropes led to a decrease in FSH secretion. Phosphorylation at T255 and S268 sites of PTPN5 enhanced the activity of PTPN5, resulting in higher cytosolic Ca\(^{2+}\) levels, and then increased FSH release. These results suggested that alteration in Ca\(^{2+}\) levels, PTPN5 activation, and phosphorylation impacted onto the function of gonadotropes and increased FSH secretion.

4 | DISCUSSION

The tyrosine phosphatase PTPN5 is enriched in basal ganglia, but widely distributed in the brain. Recent reports on the function of PTPN5 reveals that PTPN5 plays an important role in the central nervous system. High levels of PTPN5 are present in human postmortem samples and animal models of Alzheimer’s disease, Parkinson’s disease, schizophrenia, and fragile X syndrome. Low levels of PTPN5 activity are present in additional disorders that include ischemia, Huntington’s chorea, alcohol abuse, and stress disorders. Yoav Kohn et al found PTPN5 affected normal cognitive function and contributed to aspects of the neuropathology of schizophrenia. And Kim et al reported that PTPN5 was expressed in pituitary. In this paper, we addressed the question whether PTPN5 was also present in gonadotropes to influence hormone release, following the idea that a modulation of the phosphorylation state of multiple substrates might affect hormone secretion. In this study, we demonstrated that GnRH-induced endocrine physiological activity which was regulated by the activity of PTPN5. The PTPN5 inhibitor, 8-(trifluoromethyl)-1,2,3,4,5-benzopentathiepin-6-amino hydrochloride...
(TC-2153) resulted in decreased FSH secretion in gonadotropes of adult female mice, while it had no effect on FSH secretion in gonadotropes of ovariectomized female mice which were used to simulate menopausal women. For LH level, it looked like that there was no significant difference between GnRH-stimulated group and GnRH-TC2153 condition group in normal adult or ovariectomized female mice. Tomomi Karigo et al showed that there were differential regulation
The effect of PTPN5 on calcium responses to GnRH in RC-4BC cell line. A, The effect of GnRH on calcium oscillations in RC-4BC cell. GnRH stimulated a sharp increase in \( \text{Ca}^{2+} \). B, The application of U73122 significantly decreased calcium signal. C, siPTPN5 inhibited \( \text{Ca}^{2+} \) oscillations evoked by GnRH in the same cell. D, The application of U73122 significantly decreased calcium signal in siPTPN5 RC-4BC cells. E and F, Summarized \( \Delta \) ratios from experiments like the ones presented from A to D, calculated as areas under the curve and peak amplitude. Statistical analysis was performed by one-way ANOVA, *** \( P < .001 \) compared with control group or siPTPN5 group; ** \( P < .01 \) compared with control group. G, Model depicting the role of PTPN5 in the regulation of FSH release. GnRH activates two signaling pathways: the Gs-PKA pathway and the Gq-PLC-p38 MAPK/ERK-pathway, which then effect PTPN5's function, eventually regulate calcium response to modulate FSH secretion.

The details of FSH release are still not fully understood. Previous findings have shown that PTPN5 plays an important role at nerve terminals in the regulation of \( \text{Ca}^{2+} \) homeostasis and neurotransmitter release.\textsuperscript{24} The similar phenomenon on PTPN5-KO mice with TC2153 treatment or OVX surgery suggested that although LH was regulated by the estrous cycle, it may play a small role or no role in generating rebound bursts under our experimental conditions.

The details of FSH release are still not fully understood. The specific expression of PTPN5 in gonadotropes suggests a role for PTPN5 in the signaling pathway mediated by GnRH-regulated system. This change in PTPN5 expression pattern suggests that PTPN5 interacts with PKA or p38 MAPK in estrous cycle, as well as at the postsynaptic site in the adult. Therefore, it is plausible to speculate that modulation of PTPN5 activation would have serious consequences on hormone secretion of pituitary. Thus, some changes in PTPN5 activity or PTPN5 expression mediated by developmental ages may result in FSH alterations in gonadotropes via PKA, p38 MAPK, or ERK regulation. This observation leads to an attractive hypothesis for a possible role of PTPN5 in reproduction and development, and it is conceivable that the function of PTPN5 would be distinctly altered on the stage of reproduction and development.

FSH is one of the family member of glycoprotein hormones. It is released from the pituitary gland, and then bind to FSH receptor on the female ovary, which plays an important role in the development of female secondary sexual characteristics, promoting growth, and maturation of granulosa cells and estrogen secretion. Accordingly, FSH is closely related to the last stage of follicular maturation and it plays a crucial role in female fertility.\textsuperscript{41} In addition, FSH receptors have also been reported to exist in the testis and it can promote spermatogenesis. When FSH binds to receptors on Sertoli cells in the testis, it promotes the proliferation and differentiation of Sertoli cells and ensures the normal quantity and quality of sperm.\textsuperscript{31,42} Totally, FSH plays an important role not only in the reproductive development of females, but also in males. Clinical medical use of FSH is mainly several recombinant and biosimilar FSH molecules. FSH preparations identical to endogenous human FSH can promote follicular development and has certain therapeutic effects on female sterility and assisted reproduction. In men, it is used to promote testicular growth, spermatogenesis, and to treat oligoasthenozoospermia.\textsuperscript{43-45} However, although it is common to use FSH and its biosimilar preparation to treat infertility, in practice, due to the short biological half-life of FSH, only 1 day,\textsuperscript{46} some FSH preparations on the market require patients to receive subcutaneous injection every day, which brings a lot of inconvenience to patients’ daily life. Although there are a small number of long-acting FSH agents on the market,

mechanisms for LH and FSH release by GnRH which could explain the phenomenon of LH release on GnRH-TC2153 condition.\textsuperscript{35} The similar phenomenon on PTPN5-KO mice with TC2153 treatment or OVX surgery suggested that Ca\textsuperscript{2+} homeostasis is likely regulated by PTPN5 and PTPN5 inhibitor-TC2153 regulates calcium current properties to affect FSH secretion. Thus, PTPN5 is involved in the mechanism of FSH secretion and it is a promising molecular target for reproductive development drugs of new generation. In gonadotropes of mice, PTPN5 expression was rhythmically varied with periodicity (Figure 1). However, no significant differences in the expression levels of other pituitary-enriched protein phosphatases were observed among groups. Furthermore, we found that selected Ca\textsuperscript{2+} channels expression levels were significantly correlated with estrous cycle in individual mice.\textsuperscript{17} Interestingly, we observed a dynamic change in PTPN5 expression, showing relatively stronger expression in gonadotropes in the adult, then barely detectable expression level in mice underwent OVX, which is a surgical model of menopause. The dynamic change in PTPN5 expression was positively correlated with the expression of calcium channel. These data also suggested that there was an important interaction between PTPN5 and elevated intracellular calcium.

It is now largely accepted that calcium signal contributes to different physiological responses in neuronal cells such as neuronal cell death and development as well as synaptic plasticity and that modulating calcium influx in the CNS can result in different neurophysiological responses.\textsuperscript{36,37} In addition, calcium signal can also play a key role in endocrine system like catecholamine release in adrenal chromaffin cells, glucagon secretion in human pancreatic alpha cells, and insulin secretion in beta cells.\textsuperscript{38-40} In this context, PKA, p38 MAPK, or ERK can regulate PTPN5 activity, such as via T255 and S268, which is critical as a physiological regulator that regulates calcium signal to affect FSH secretion in gonadotropes at a variety of biodevelopment cycle environments. The specific expression of PTPN5 in gonadotropes suggests a role for PTPN5 in the signaling pathway mediated by GnRH-regulated system. This change in PTPN5 expression pattern suggests that PTPN5 interacts with PKA or p38 MAPK in estrous cycle, as well as at the postsynaptic site in the adult. Therefore, it is plausible to speculate that modulation of PTPN5 activation would have serious consequences on hormone secretion of pituitary. Thus, some changes in PTPN5 activity or PTPN5 expression mediated by developmental ages may result in FSH alterations in gonadotropes via PKA, p38 MAPK, or ERK regulation. This observation leads to an attractive hypothesis for a possible role of PTPN5 in reproduction and development, and it is conceivable that the function of PTPN5 would be distinctly altered on the stage of reproduction and development.
corifollitropin alfa may overstimulate oocytes, resulting in cycle cancelation. 47

As an upstream target, PTPN5-targeted drugs can indirectly affect ovarian function by regulating endogenous FSH release. Therefore, the research on the regulatory mechanism of PTPN5 can provide more possibilities for the future drug development targeting female and male fertility. In addition, because the biological half-life of FSH is only 1 day, PTPN5-targeted drugs can better simulate the release process of FSH, which has higher clinical application value, compared with exogenous injection of FSH. Through experiments, it is known that PTPN5 affects the release of FSH through phosphorylation in pituitary cells, but the action site and specific mechanism of PTPN5 remain to be further explored. It is necessary to make clear that whether FSH affects not only the function of ovaries and testes but also other organs. The New England Journal of Medicine reported that FSH receptor was specifically expressed by endothelial cells in tumors of all grades, including prostate, breast, colon, pancreas, urinary bladder, kidney, lung, liver, stomach, testis, and ovary. 48 We speculate that PTPN5 may also play a role in the treatment of cancer patients. However, the effects of PTPN5 on these organs have not been clearly defined, and the specific mechanisms need to be further explored. In conclusion, PTPN5 has far-reaching significance for the treatment of infertility and also in the cancer.

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CONFLICT OF INTEREST
The authors declare that there is no duality of interest associated with this manuscript.

AUTHOR CONTRIBUTIONS
H. Wang and J. Dong designed the research and wrote the manuscript; H. Wang, S. Bu, and J. Tang performed the research; H. Wang, S. Bu, J. Tang, and Y. Li analyzed the results and data; H. Wang and C. Liu contributed research materials.

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SUPPORTING INFORMATION
Additional Supporting Information may be found online in the Supporting Information section.

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