Original Article

Reproductive Sciences
2016, Vol. 23(11) 1565-1574
© The Author(s) 2016
Reprints and permission:
sagepub.com/journalsPermissions.nav
DOI: 10.1177/1933719116648215
rs.sagepub.com

SPOP Regulates Endometrial Stromal Cell Decidualization in Mice

Na Liu, MD[1], Xin Liu, MD[1], Qiubo Yu, PhD[2], Xuemei Chen, PhD[1], Yubin Ding, PhD[1], Junlin He, MD[1], Rufei Gao, PhD[1], Yingxiong Wang, MD[1], and Xueqing Liu, MD[1]

Abstract

Ubiquitination is a regulatory mechanism that occurs after protein translation. To date, few studies have reported on ubiquitination during embryo implantation. We used real-time quantitative polymerase chain reaction, immunohistochemistry, and Western blotting analyses to analyze the expression of speckle-type pox virus and zinc finger (POZ) protein (SPOP; an adapter of E3 ligases of ubiquitination) in mouse uteri during early pregnancy and pseudopregnancy using an artificially induced decidualization model and a steroid hormone–processing model. At the same time, we established an artificially induced decidualization in vitro model. We observed that SPOP regulates endometrial stromal cell decidualization in mice and that hormones regulate the expression of SPOP. This study suggests that ubiquitination may be involved in embryonic implantation.

Keywords

SPOP, decidualization, hormones, ubiquitination

Introduction

A bidirectional interaction of the blastocyst with the maternal endometrium is a key for successful pregnancy. The special stromal cells surrounding the implanting embryo undergo proliferation and subsequently differentiate into decidualization cells in the endometrium, which support embryo growth and maintain early pregnancy. Stromal cell decidualization is regulated by the ovarian steroid hormones estrogen (E2) and progesterone (P4). Under the influence of those steroids, the stroma expresses genes important for blastocyst implantation.[1-3] In other words, steroid hormones regulate differential expression of different genes in which stromal cell plays a key role.

Based on the results from next-generation sequencing and bioinformatics analyses, we found that the expression of speckle-type POZ protein (*Spop*) was significantly different in the D5 implantation site (IS) compared to the D5 inter-IS (IIS). SPOP is a highly conserved 42-kDa protein. It includes 3 domains and has 374 residues. The domains contain an N-terminal MATH domain (residues 28-166) that recruits substrates, a C-terminal nuclear localization sequence (residues 365-374), and an internal BTB domain (residues 190-297) that binds Cul3.[4-8] The main function of *Spop* is involved in ubiquitination. Ubiquitination has been confirmed as a regulatory mechanism that occurs after protein translation. Ubiquitination can regulate cell proliferation, differentiation, and apoptosis by degrading target protein.[9] Ubiquitination occurs through 3 classes of enzymes termed E1 (activating enzyme), E2 (transport enzyme), and E3 (ligase). Among these, E3 ubiquitin ligase determines the selection of specific substrates.[10,11] The SPOP is an E3 ubiquitin ligase adaptor, and it plays an important role in the selectivity of E3. Therefore, SPOP is significant for ubiquitination and the subsequent proliferation or differentiation by ubiquitin regulation. Until now, research about the expression and function of ubiquitin-related proteins in the endometrium is limited; for example, p27 and its ubiquitin ligase Skp2 expression in endometrium of in vitro fertilization patients with repeated hormonal stimulation, modulation of ubiquitin system genes in human endometrial cell line infected with mycobacterium tuberculosis, neural precursor cell expressed developmentally down-regulated protein 4 (NEDD4) ubiquitin ligase is a putative oncogene in endometrial cancer, and so on.[12-14] At the same time, some studies found SPOP plays a role in steroid receptor activation. In addition, it regulates Hedgehog signaling pathway that affects development of embryos after combining Gli2 and Gli3. Furthermore, SPOP promotes the phosphorylation of PI3K

[1] Laboratory of Reproductive Biology, School of Public Health, Chongqing Medical University, Yuzhong District, Chongqing, People's Republic of China
[2] Molecular Medical Laboratory, Chongqing Medical University, Yuzhong District, Chongqing, People's Republic of China

Corresponding Author:
Xueqing Liu, Laboratory of Reproductive Biology, School of Public Health, Chongqing Medical University, No. 1 Yixueyuan Rd, Chongqing, 400016, People's Republic of China.
Email: cqmu_liuxueqing@126.com

signaling pathway, which plays an important role in embryo implantation.[15-24]

In order to understand the role of ubiquitin-related genes in stromal cell decidualization, we designed the following experiments to study *Spop* expression and function in endometrial stromal cell decidualization during early pregnancy in mice.

Materials and Methods

Mice and Treatments

Eight- to 10-week-old female and male Kunming mice were selected for the study. All of the mice were housed in the Animal Facility of Chongqing Medical University (Certification No.: SCXK [YU] 20120127). The animal procedures were performed according to the Ethics Committee of Chongqing Medical University. The mice in this study were maintained in a nonspecific pathogen-free room in the experimental animal center of Chongqing Medical University. All of the animals involved in the experiments were fed with laboratory chow and water under a constant photoperiod (14:10-hour light–dark cycle). The female mice were caged with males in 1:3 ratios, and the appearance of the vaginal plug was designated as day 1.

The pregnant mice were randomly assigned to 7 groups (D1-D7), containing 8 mice per group. Additionally, female mice were caged with vasectomized males (2 weeks after vasectomy) at a 1:3 ratio to induce pseudopregnancy models, and the appearance of the vaginal plug was designated as pseudopregnancy day 1 (PD1).[25] The pseudopregnant mice were also randomly assigned to 7 groups (PD1-PD7), containing 8 mice per group. After being anesthetized with 0.2 mL of 10% chloral hydrate, the females in each group were killed between 09:00 and 09:30 AM. Part of the endometrium tissue was collected and stored in liquid nitrogen for real-time quantitative polymerase chain reaction (RT-qPCR) and Western blotting (WB) analyses, and the rest of the uteri tissue was fixed in 4% paraformaldehyde for immunohistochemistry (IHC). The tissue of 8 females in each group was divided into 3 tissue pools containing the uteri of 2 or 3 mice to meet the needs for RT-qPCR and WB. The method for collecting the ISs and IISs tissues was described in a previous report.[26] The ISs, on day 5 of the pregnancy, were identified by intravenous injection through tail vein of 0.1 mL of 1% Chicago blue (Sigma, St Louis, Missouri).

Artificial decidualization was induced by intraluminal injections of corn oil (10 mL/mouse) in day 4 mice in the morning during the pseudopregnancy, whereas the contralateral horn without any infusion served as the control. The females were killed at day 8 of the pseudopregnancy, and the decidual reaction was confirmed by an increased uterine weight and histological examination. The uteri were processed for IHC analysis.

Steroid Hormone Processing Model

To test the ovarian hormonal influence on uterine SPOP expression, females were ovariectomized and rested for 7 days[27-29] and were subsequently injected with oil (0.1 mL/mouse), E2 (100 ng/mouse), P4 (2 mg/mouse), or a combination of E2 and P4. A portion of the females were subcutaneously injected with Fulvestrant (ICI 182 780; 0.5 mg/mouse; Tocris Cookson, Inc, Ballwin, Missouri) 2 hours before the E2 injection and with RU486 (progesterone [PR] antagonist, 2 mg/mouse, M8046; Sigma) 2 hours before the P4 injection, or a combination of ICI and RU486. The females were killed, and the uteri were collected 8 hours after each treatment.

Primary Uterine Stromal Cell Culture and In Vitro Decidualization

Murine primary uterine stromal cells (mESCs, mouse endometrium stromal cells) were isolated and cultured as described previously with some modifications.[30,31] Uterine horns from D4 pseudopregnant mice were split longitudinally to expose the uterine lumen and were placed into a sterile Petri dish. Then, they were cut into small pieces (3-4 mm). After washing thoroughly with Hanks' balanced salt solution (HBSS; BOSTER, China), the uterine tissues were placed in 5-mL HBSS containing 1% (wt/vol) trypsin (BOSTER) and 6 mg/mL dispase (Roche, Indianapolis, Indiana) for 1 hour at 4°C followed by 1 hour at room temperature and then 10 minutes at 37°C. Following the digestion steps, the tissues were gently pipetted up and down multiple times to dislodge the sheet of luminal epithelial cells using a 1-mL pipette, and the supernatant containing the epithelial cells was discarded. The remaining tissues were rinsed 3 times with HBSS and incubated in 2-mL HBSS containing 0.15-mg/mL collagenase I (Invitrogen, Carlsbad, California) at 37°C for 30 minutes.[31] Following the digestion steps, the tissues were gently pipetted up and down multiple times to dislodge the sheet of luminal epithelial cells using a 1-mL pipette, and the supernatant containing the epithelial cells was discarded. The remaining tissues were rinsed 3 times with HBSS and incubated in 2-mL HBSS containing 0.15-mg/mL collagenase I (Invitrogen) at 37°C for 30 minutes. The digested cells (mESCs) were then vigorously shaken and passed through a 70-μm nylon filter to eliminate clumps of epithelial cells and then were centrifuged, and the pellet was washed twice with HBSS. The mESCs were resuspended in complete medium consisting of Dulbecco Modified Eagle medium (DMEM)-nutrient mixture F-12 Ham (DMEM-F12, Sigma) with 10% heat-inactivated fetal bovine serum (FBS; Biological Industries, Inc, Israel). The cells were plated at a density of 2×10^5 cells per 6-well cell culture plate in phenol red-free culture medium (DMEM/Hams F-12, 1:1) with 10% charcoal-stripped FBS and 10-U/mL penicillin–streptomycin solution (Co222; Beyotime). After an initial incubation for 1 hour, the unattached cells were removed by several washes with fresh phenol red-free culture medium (DMEM/Ham F-12, 1:1) and cell culture continued after the addition of fresh phenol red-free culture medium (DMEM/Ham's F-12, 1:1) containing 10% charcoal-stripped FBS, estradiol-17β (E2,10 nmol/L), and P4 (1 mmol/L) and 10-U/mL penicillin–streptomycin solution to induce decidualization.

Immunohistochemistry

Immunohistochemistry was performed using 5-μm paraffin-embedded sections. The tissues were fixed in 4% paraformaldehyde, dehydrated via graded ethanol solutions, and embedded in paraffin.[32] The procedure for the IHC analysis was described previously.[33] The tissue sections were dewaxed in xylene and rehydrated in descending concentrations of ethanol, followed by antigen retrieval and cooling to room temperature. The following experiments were performed using Histostain-Plus Kits (Zhongshan Golden Bridge Biotechnology, China), and the chromogenic reaction was performed with 3, 30-diaminobenzidine (Zhongshan Golden Bridge Biotechnology) according to the manufacturer's protocol. The primary antibody used in this study was a rabbit monoclonal anti-SPOP antibody at a 1:300 dilution (Abcam, United Kingdom). As a negative control, the primary antibodies were replaced with phosphate-buffered saline. The sections were counterstained with hematoxylin and mounted with resinene. The IHC images were captured on an Olympus microscope (BX40, Olympus, Japan).

Real-Time RT-PCR Analysis

Total RNA was extracted from the mice endometrial tissues with TRIzol reagent (TaKaRa, China) and was reverse transcribed into cDNA using the PrimeScript RT Reagent Kit (TaKaRa, China) according to the manufacturer's instructions. The specific primers for *Spop*, *Cyclin D3*, *decidual/trophoblast prolactin-related protein (Dtprp)*, and *β-actin* for qPCR were designed and synthesized by Sangon Biotech Co. Ltd. (Shanghai, China). The sequences of the primers for qPCR are shown in Table 1. The PCR reaction was performed on a Bio-Rad iQ 5 Real-Time PCR Detection System using SYBR Green Mastermix (Takara, China). The experiments were performed in triplicate for each sample, and the 2−ΔΔCt method was used to calculate the relative expression of *Spop*, *Cyclin D3,* and *Dtprp* in the endometrium on different pregnancy days with *β-actin* as the internal control.

Western Blotting Analyses

Protein extracts from the endometrium of the mice from each group were dissolved in RIPA lysis buffer (Beyotime Biotechnology, China), containing proteinase inhibitor. The protein concentrations were determined using the bicinchoninic acid method according to the manufacturer's instructions (Beyotime Biotechnology, China). The lysates were boiled in 5× sodium dodecyl sulfate (SDS) sample loading buffer for 10 min. Equal amounts of total protein (50 g) were separated using 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes (Bio-Rad Laboratories). The membranes were blocked in 5% skim milk for 1 hour at RT followed by incubation with the appropriate primary antibodies diluted in blocking buffer (4°C, overnight) as previously described. Immunoblotting was performed using a rabbit polyclonal SPOP antibody at a 1:300 dilution (Abcam, United Kingdom). A mouse monoclonal β-actin antibody at a 1:2000 dilution (Sigma) was used to ensure equal loading of the samples. After 3 washes with phosphate buffer saline tween-20 (PBST) (15 minutes each), the membranes were incubated with a goat anti-rabbit IgG and a goat anti-mouse IgG secondary antibody corresponding to the source of the primary antibodies for 1 hour at RT. After 3 washes with PBST (15 minutes each), the immunoreactive bands were visualized using ChemiDoc XRS+ (Bio-Rad) and chemiluminescence reagents (Millipore, WBKLS0500, Billerica, MA). The image collection and the densitometry analysis were performed using Quantity One version 4.6.2 analysis software (Bio-Rad Laboratories).

Table 1. Primer Sequences for qPCR.

Gene sequence of primers 5′ to 3′
Spop forward GTAAACCCGAAAGGGCTAGATG
Reverse CGAACTTCACTCTTTGGACAG
CyclinD3 forward CGAGCCTCCTACTTCCAGTG
Reverse GGACAGGTAGCGATCCAGGT
Dtprp forward AGCCAGAAATCACTGCCACT
Reverse TGATCCATGCACCCATAAAA
β-Actin forward TGGAATCCTGTGGCATCCATGAAAC
Reverse TAAAACGCAGCTCAGTAACAGTCCG

Abbreviations: qPCR, quantitative polymerase chain reaction.

Statistical Analysis

All of the experiments were replicated at least 3 times. The data were analyzed using the Statistical Package for the SPSS 13.0 (Chicago). The values are expressed as the mean ± SD. A Student's *t* test was used to analyze the relative expression of messenger RNA (mRNA) and protein between the ISs and the IISs. The relative expression of mRNA and protein during early pregnancy and pseudopregnancy was determined using 1-way analysis of variance (ANOVA). Shapiro-Wilk was used to confirm data were normally distributed. Least significant difference (LSD) was performed after ANOVA. The protein expression of SPOP was determined using Kruskal-Wallis H test. The differences were considered significant if $P < .05$.

Results

Expression of SPOP in Mouse Uteri During Early Pregnancy

In this study, we tested the protein expression of *Spop* in mice uteri during early pregnancy by RT-PCR, immunobloting, and IHC analyses. We found the expression levels of *Spop* mRNA were always lower from D1 to D3 than on other days, but its mRNA levels were significantly increased from D4, and this trend continued to D6 then it reduced on D7 ($P < .05$; Figure 1A). Next, we examined the protein expression of SPOP by WB, and the results were consistent with the mRNA results ($P < .05$; Figure 1B). The SPOP protein levels at the IS were significantly higher than at the IIS on D5 ($P < .05$;

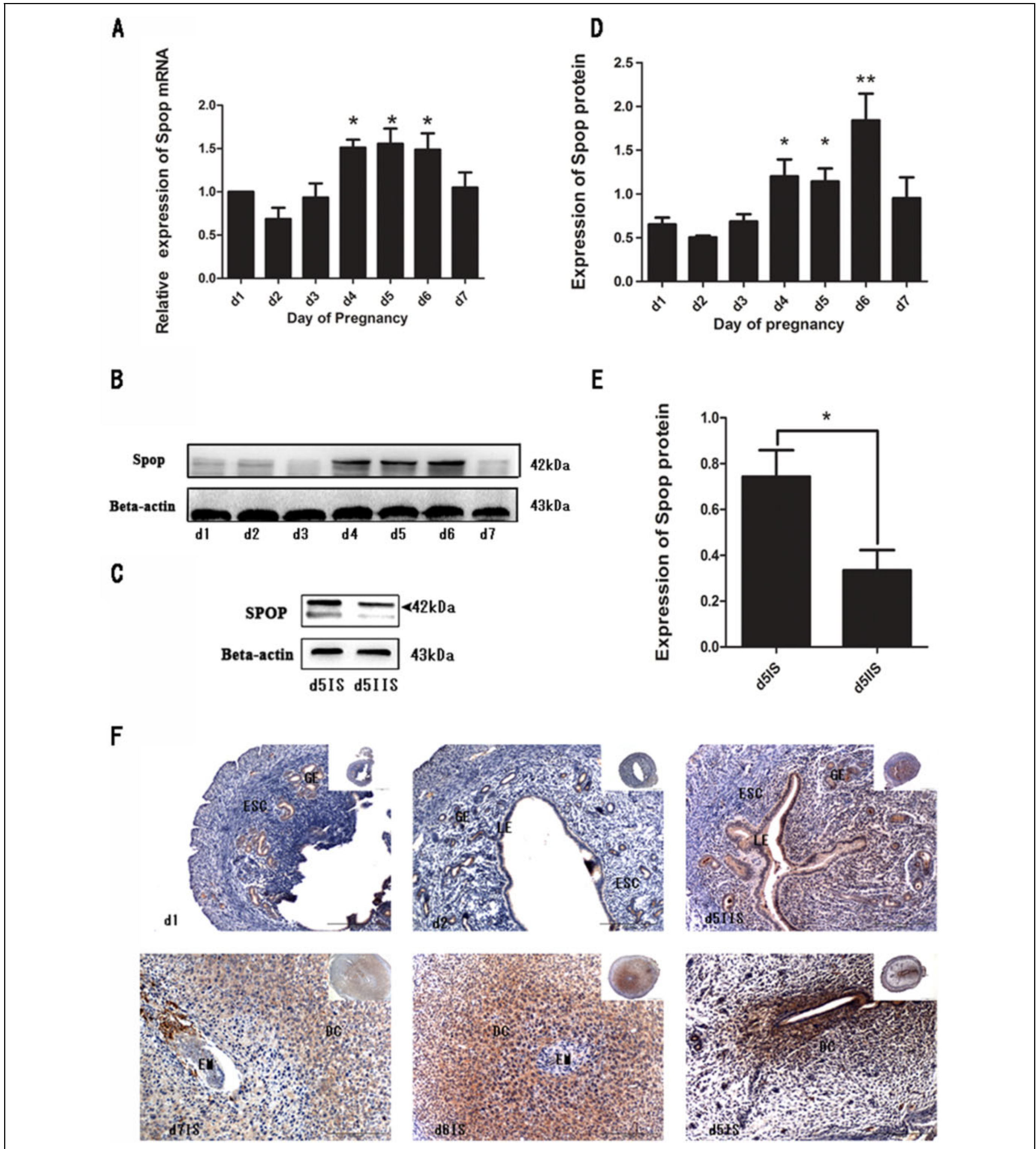


Figure 1. A, RT-PCR analysis of the *Spop* mRNA on d1 to d7 during mice pregnancy (N = 56). B, Western blot analysis of the SPOP protein (N = 56). C SPOP protein dynamic expression in d5 IS and d5 IIS. D and E, Statistical analysis of the Western blot results of the d5IS and d5IIS (**P* < .05). F, SPOP protein present in pregnant mice by IHC. Ge indicates glandular epithelium; Le, luminal epithelium; ESC, endometrium stromal cell; DC, decidualization cell; EM: embryo; RT-PCR, real-time polymerase chain reaction; mRNA, messenger RNA; IS, implantation site; IIS, inter-IS; SPOP, speckle-type POZ protein.

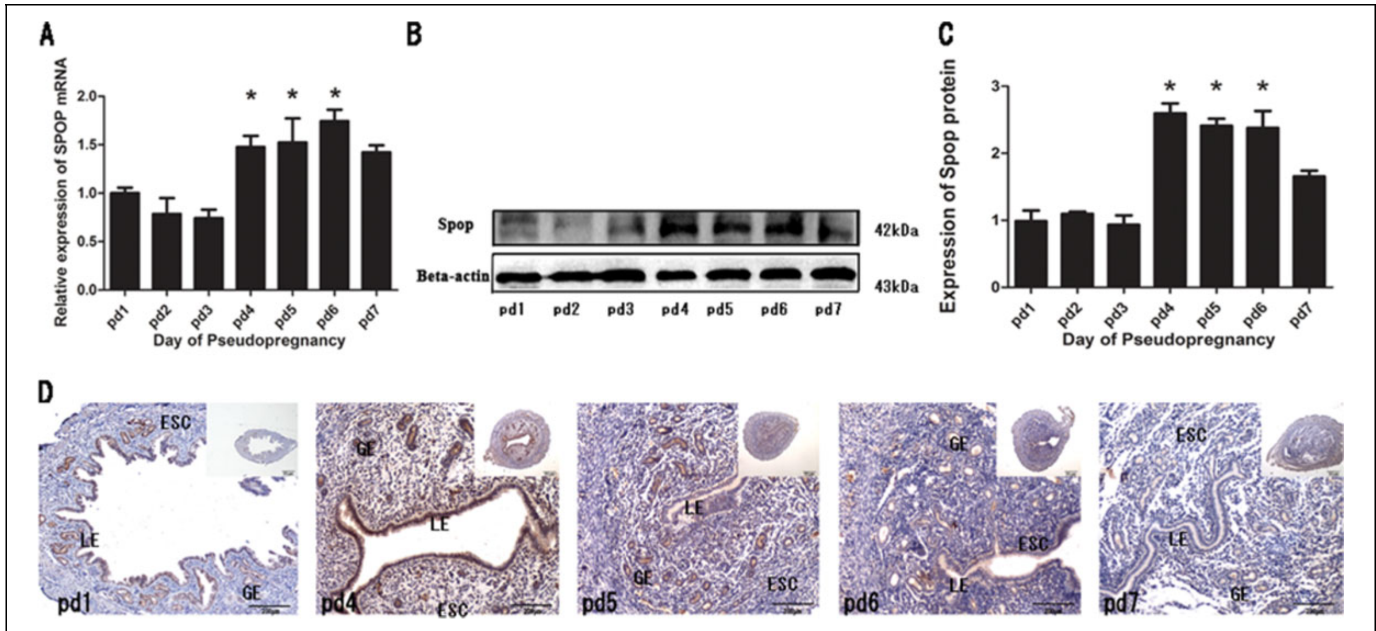


Figure 2. A, Expression of *Spop* mRNA in the endometrium of pseudopregnant mice was measured by RT-PCR (N = 56). B, Expression of SPOP protein in the endometrium of pseudopregnant mice was measured by Western blot (N = 56). C, Optical density values of SPOP protein was analyzed (*$P < .05$). D, SPOP protein present in pseudopregnant mice by IHC. Ge indicates glandular epithelium; Le, luminal epithelium; ESC, endometrium stromal cell; IHC, immunohistochemistry; mRNA, messenger RNA; SPOP, speckle-type POZ protein.

Figure 1C). Figure 1D and E is the quantitative figure of Figure 1B and 1C, respectively. The localization of SPOP protein in the endometrium on days 1 and 4 to 7 of pregnancy by IHC is shown in Figure 1F. As the results show, SPOP expression was observed in the glandular and luminal epithelium on D1 and D4 of pregnancy. With the onset of embryo attachment, SPOP was extensively expressed in the primary decidual and second decidual zone, surrounding the implanting blastocyst on D5 and D6, respectively. Finally, SPOP expression was significantly reduced on D7.

Expression of SPOP in the Mouse Uteri During Early Pseudopregnancy

We examined the expression of *Spop* during early pseudopregnancy using real-time RT-PCR, WB, and IHC analyses. The levels of *Spop* mRNA were like waves from PD1 to PD7 ($P < .05$; Figure 2A). The expression of *Spop* mRNA was at a low level on PD1 to PD3, and it significantly increased on PD4, which was sustained to PD6 and reduced on D7. Next, the expression level of SPOP protein during early pseudopregnancy was tested by WB ($P < .05$; Figure 2B). Figure 2C is the quantitative figure of Figure 2B. The results show that the protein expression levels were consistent with the results obtained by RT-PCR. To investigate SPOP localization in the endometrium during early pseudopregnancy, we used IHC to detect SPOP (Figure 2D). The IHC results suggest that SPOP is expressed in virtually all of the glandular and luminal epithelium in the days of early pseudopregnancy. Specifically, the SPOP expression was different compared with early pregnancy on D5 and D6 (Figure 2D). In pseudopregnancy, the protein expression of SPOP remained low in the endometrial stromal cells on PD5 and PD6.

Expression of SPOP Is Regulated by E2 and P4 in Mouse Uteri

It is well known that the principal hormones that directly influence uterine receptivity are ovarian E2 and P4. We already confirmed that SPOP is involved in stromal cell decidualization. To explore the influence of E2 and P4 on uterine SPOP expression, we employed an ovariectomized mouse model injected with E2, P4, or E2 plus P4 and the corresponding inhibitors (ICI, RU486). Western blot analyses showed that the expression of SPOP was significantly stronger after treatment with hormones, and it was reduced when the hormones were in the presence of the inhibitors (Figure 3A and B; $P < .05$). The IHC analyses also revealed that SPOP protein levels were significantly higher with the hormones compared to the control groups (Figure 3C). However, after treatment with hormones and inhibitors, the protein expression of SPOP disappeared. Therefore, we conclude that E2 and P4 promote the expression of SPOP in mice uteri.

Expression of SPOP in Artificially Induced Decidualization

First, we successfully established a model to artificially induce decidualization and examined the mRNA levels of *Dtprp* (mouse decidualization markers) by RT-PCR ($P < .05$; Figure 4A). We found that the expression of SPOP in the artificially

Figure 3. A, Expression of SPOP protein in different treatment groups. B, Optical density values of SPOP protein were analyzed (**P* < .05, compared with the hormone group; #*P* < .05, compared with the control group). C, Location of SPOP protein in the endometrium of mice treated with E2 or/and P4 as detected by IHC. Con indicates blank contrast; oil, corn oil decidualization; P4, progesterone; E2, estrogen; E2P4, estrogen combine progesterone; E2 + ICI, estrogen and antagonist fulvestrant; P4 + RU486, progesterone and antagonist mifepristone; E2 + P4 + RU486 + ICI: estrogen and progesterone antagonize fulvestrant and mifepristone; SPOP, speckle-type POZ protein.

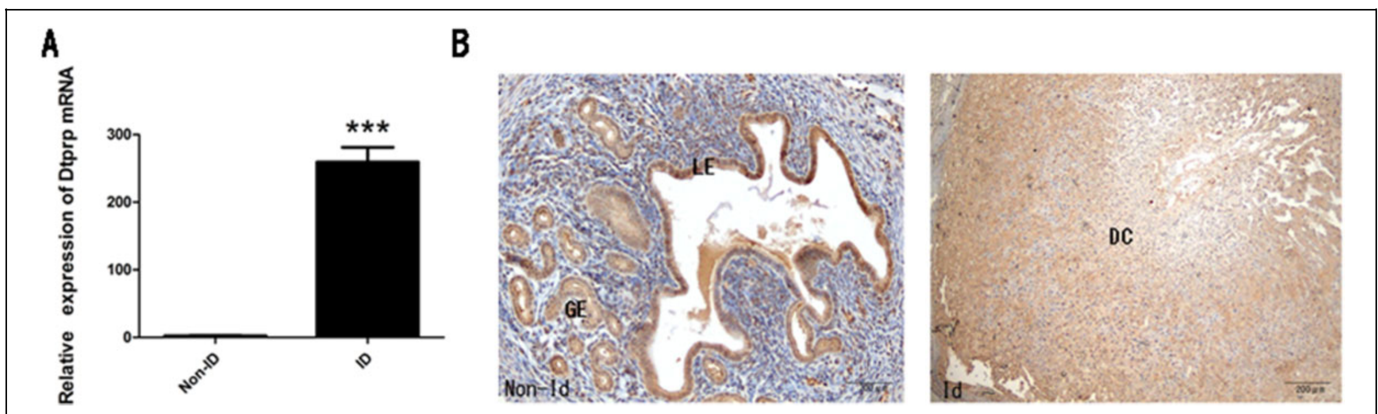


Figure 4. A, Expression of *Dtprp* mRNA by RT-PCR (**P* < .05). B, Location of SPOP protein in the endometrium of artificially decidualized mice as detected by IHC. Non-Id indicates nondecidualized endometrium; ID, artificial decidualization endometrium; mRNA, messenger RNA; RT-PCR, real-time polymerase chain reaction; SPOP, speckle-type POZ protein.

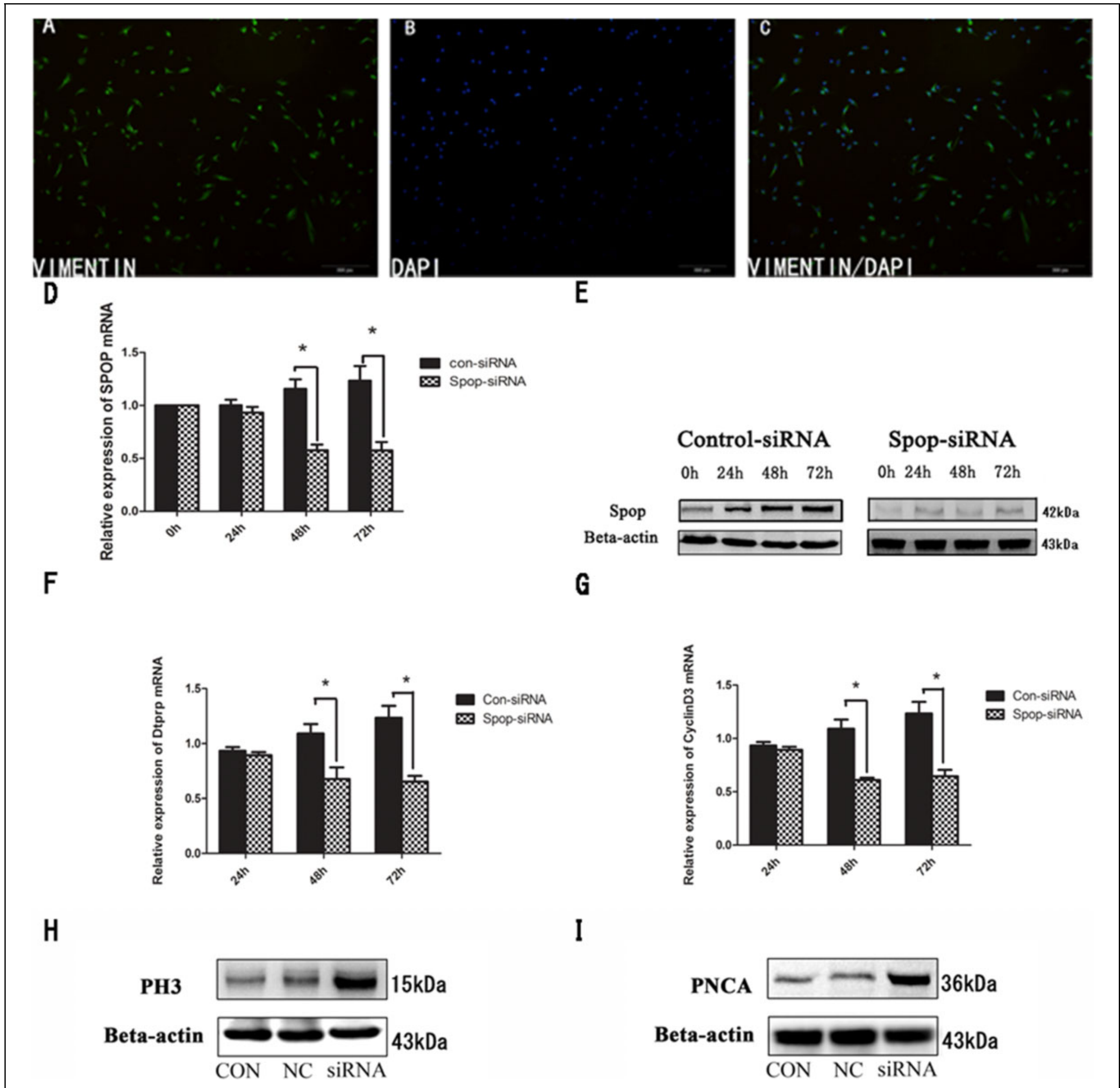


Figure 5. A-C, Immunocytochemical analysis of vimentin protein in primary stromal cells (DAPI, 4′,6-diamidino-2-phenylindole). D, RT-PCR to detect SPOP mRNA. E, Western blot to measure SPOP protein expression after siRNA transfection (24, 48, and 72 hours). F, *Dtprp* mRNA expression after siRNA transfection. G, *CyclinD3* mRNA expression after siRNA transfection (*$P < .05$). H, Western blot to measure *phospho-histone 3 (PH3)* expression after siRNA transfection. I, Western blot to measure *proliferating cell nuclear antigen (PNCA)* expression after siRNA transfection. mRNA indicates messenger RNA; RT-PCR, real-time polymerase chain reaction; SPOP, speckle-type POZ protein; siRNA, small interference RNA.

induced decidualization model was higher than that in control (Figure 4B). The SPOP localization in the control group was mainly in the glandular and luminal epithelium. However, it was widely expressed in decidualization cells in artificially induced decidualization model. This finding indicated that SPOP is involved in stromal cell decidualization.

Downregulation of SPOP Expression Affects Stromal Cell Decidualization by Increasing Proliferation

We established a primary uterine stromal cell (mESC) culture system and used vimentin immunofluorescence staining to identify the purity of the isolated uterine stromal cells

(Figure 5A-C). Next, we designed and selected a small interference RNA (siRNA) to knockdown *Spop* in the mESCs. As shown in Figure 5D-E, siRNA significantly reduced *Spop* expression at both the protein and mRNA levels in mESCs ($P < .05$). Next, we employed mESCs in an in vitro decidualization model to further investigate the function of *Spop* during decidualization.

We used RT-PCR to examine the expression of decidualization marker genes, including *CyclinD3* and *Dtprp*, in mESCs that were cotransfected with the specific siRNAs, E2 and P4 at 48 and 72 hours. At the same time, we transfected cells with a control siRNA as a negative control experimental group. As shown in Figure 5F-G, the results demonstrate that at 48 hours and 72 hours after treatment, the mRNA expression levels of *CyclinD3* and *Dtprp* in the test group were significantly lower than in the control group ($P < .05$). Next, we used WB to measure the expression of proliferation marker genes (*phospho-histone 3 [PH3]*, *proliferating cell nuclear antigen [PNCA]*) at 48 hours after treatment. As shown in Figure 5H-I, the results demonstrate the protein expression of PH3 and PNCA in the treatment group were significantly higher than in the control group. Therefore, we determined that *Spop* regulates decidualization in mice uterine stromal cells by inhibiting the overproliferation.

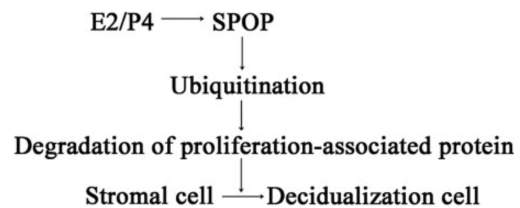
Discussion

Our study confirmed that the expression of *Spop* in the endometrium of early pregnant mice appeared in a waveform with dynamic change. *Spop* expression in D1 to D3 was lower than on other days, but it was significantly increased at the key time points (D4, D5, and D6), and it later declined at D7. At the same time, the expression of *Spop* in pseudopregnancy resembles the pregnancy results, but the expression sites were mainly limited to the epithelial and luminal epithelium. The expression of SPOP is not induced by a competent blastocyst. Because E2 and P4 are the most important factors in embryo implantation and stromal cells begin an extensive proliferation when rising P4 is superimposed with preimplantation E2 after day 4,[34] we surmised that E2 and P4 regulate the expression of SPOP. The IHC results of the SPOP expression in the ovariectomized mouse model confirmed that inhibiting E2 and P4 reduced the expression of SPOP.

Combined, many studies have demonstrated that E2 and P4 can regulate endometrial stromal cell decidualization. Decidualization in mouse is a transient process that begins to develop at the time of blastocyst attachment on day 4.5 of the pregnancy.[3,35] Next, during the subsequent 3 days of pregnancy, the decidualization cells surrounding the site of embryo attachment proliferate and differentiate extensively, eventually becoming larger.[36-38] Therefore, during pregnancy, D4 to D7 is a highly important time period for stromal cells to become decidualization cells in order to support the developing blastocyst. Thus, we questioned whether SPOP affects stromal cell decidualization. Our study found that SPOP expression significantly increased not only during D4 to D6 but also at the sites of expression from the epithelial/luminal epithelium to the stromal cells, starting at D5. Therefore, SPOP may play a role in stromal cell decidualization.

To confirm that SPOP is involved in stromal cell decidualization, we established in vivo and in vitro artificially induced decidualization mouse models. SPOP expression was highly upregulated in the decidual tissue or the decidual cells. After using siRNA to knockdown *Spop* in the in vitro experiments, the markers of decidual cells were significantly reduced, and cell proliferation rose. These results show that SPOP expression may influence decidualization by regulating stromal cell proliferation. Many studies have confirmed the SPOP or a complex of SPOP with a substrate inhibits cell proliferation by ubiquitination.[5,39-45] We considered that SPOP-mediated ubiquitination involved in degradation of the proliferation-associated proteins to make stromal cells exit from the cell cycle and enter a differentiation program that allows their transition to decidualization cells.[46] SPOP plays a role in inhibiting the overproliferation of endometrial stromal cells, and thus it may promote normal pregnancy.

Many studies demonstrated that the stromal cell decidualization is essential for embryo implantation and normal pregnancy in mice.[1,34,36] Our study indicated E2 and P4 influence stromal cell decidualization by regulating SPOP-mediated ubiquitination in mice. So we speculated that it may participate in the process of embryo implantation. In order to further prove its effect on embryo implantation, it is necessary to employ *SPOP* knockout mouse model. Simultaneously, the role of SPOP in human endometrium also needs to be confirmed. Considering that SPOP as an important ligase mediating ubiquitination and the importance of ubiquitination, we consider that the further study will help to understand the role of ubiquitin in embryo implantation.



Acknowledgments

The authors would like to thank all of the members in our research group for their technical support.

Author Contributions

Na Liu and Xin Liu contributed equally to this work and should be considered the cofirst authors.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by the Science and Technology Commission of

Chongqing, Chongqing, China (Certification No:CSTC2012gg-yyjs10007).

References

1. Zhang S, Lin H, Kong S, et al. Physiological and molecular determinants of embryo implantation. *Mol Aspects Med*. 2013; 34(5):939-980.
2. Carson DD, Bagchi I, Dey SK, et al. Embryo implantation. *Dev Biol*. 2000;223(2):217-237.
3. Filant J, Spencer TE. Endometrial glands are essential for blastocyst implantation and decidualization in the mouse uterus. *Biol Reprod*. 2013;88(4):93.
4. Geng C, He B, Xu L, et al. Prostate cancer-associated mutations in speckle-type POZ protein (SPOP) regulate steroid receptor coactivator 3 protein turnover. *Proc Natl Acad Sci U S A*. 2013; 110(17):6997-7002.
5. Zeng C, Wang Y, Lu Q, et al. SPOP suppresses tumorigenesis by regulating Hedgehog/Gli2 signaling pathway in gastric cancer. *J Exp Clin Cancer Res*. 2014;33:75.
6. Zhuang M, Calabrese MF, Liu J, et al. Structures of SPOP-substrate complexes: insights into molecular architectures of BTB-Cul3 ubiquitin ligases. *Mol Cell*. 2009;36(1):39-50.
7. Furukawa M, He YJ, Borchers C, Xiong Y. Targeting of protein ubiquitination by BTB-Cullin 3-Roc1 ubiquitin ligases. *Nat Cell Biol*. 2003;5(11):1001-1007.
8. Pintard L, Willis JH, Willems A, et al. The BTB protein MEL-26 is a substrate-specific adaptor of the CUL-3 ubiquitin-ligase. *Nature*. 2003;425(6955):311-316.
9. Hershko A, Ciechanover A, Varshavsky A. Basic medical research award. The ubiquitin system. *Nat Med*. 2000;6(10): 1073-1081.
10. Pei XH, Bai F, Li Z, et al. Cytoplasmic CUL9/PARC ubiquitin ligase is a tumor suppressor and promotes p53-dependent apoptosis. *Cancer Res*. 2011;71(8):2969-2977.
11. Petroski MD, Deshaies RJ. Function and regulation of cullin-RING ubiquitin ligases. *Nat Rev Mol Cell Biol*. 2005;6(1): 9-20.
12. Lahav-Baratz S, Koifman M, Sabo E, Auslender R, Dirnfeld M. p27 and its ubiquitin ligase Skp2 expression in endometrium of IVF patients with repeated hormonal stimulation. *Reprod Biomed Online*. 2016;32(3):308-315.
13. Meenu S, Thiagarajan S, Ramalingam S, Michael A, Ramalingam S. Modulation of host ubiquitin system genes in human endometrial cell line infected with Mycobacterium tuberculosis. *Med Microbiol Immunol*. 2016;205(2):163-171.
14. Zhang Y, Goodfellow R, Li Y, et al. NEDD4 ubiquitin ligase is a putative oncogene in endometrial cancer that activates IGF-1R/PI3K/Akt signaling. *Gynecol Oncol*. 2015;139(1):127-133.
15. Wang C, Pan Y, Wang B. Suppressor of fused and Spop regulate the stability, processing and function of Gli2 and Gli3 full-length activators but not their repressors. *Development*. 2010;137(12): 2001-2009.
16. Von Ohlen T, Lessing D, Nusse R, Hooper JE. Hedgehog signaling regulates transcription through cubitus interruptus, a sequence-specific DNA binding protein. *Proc Natl Acad Sci U S A*. 1997;94(6):2404-2409.
17. Harfe BD, Scherz PJ, Nissim S, Tian H, McMahon AP, Tabin CJ. Evidence for an expansion-based temporal Shh gradient in specifying vertebrate digit identities. *Cell*. 2004;118(4):517-528.
18. Liu L, Wang Y, Yu Q. The PI3K/Akt signaling pathway exerts effects on the implantation of mouse embryos by regulating the expression of RhoA. *Int J Mol Med*. 2014;33(5):1089-1096.
19. Zeng X, Mao X, Huang Z, Wang F, Wu G, Qiao S. Arginine enhances embryo implantation in rats through PI3K/PKB/mTOR/NO signaling pathway during early pregnancy. *Reproduction*. 2013;145(1):1-7.
20. Yin X, Pavone ME, Lu Z, Wei J, Kim JJ. Increased activation of the PI3K/AKT pathway compromises decidualization of stromal cells from endometriosis. *J Clin Endocrinol Metab*. 2012;97(1): E35-E43.
21. Takahashi I, Kameoka Y, Hashimoto K. MacroH2A1.2 binds the nuclear protein Spop. *Biochim Biophys Acta*. 2002;1591(1-3): 63-68.
22. Liu A, Desai BM, Stoffers DA. Identification of PCIF1, a POZ domain protein that inhibits PDX-1 (MODY4) transcriptional activity. *Mol Cell Biol*. 2004;24(10):4372-4383.
23. Li C, Ao J, Fu J, et al. Tumor-suppressor role for the SPOP ubiquitin ligase in signal-dependent proteolysis of the oncogenic co-activator SRC-3/AIB1. *Oncogene*. 2011;30(42):4350-4364.
24. Zhang P, Gao K, Jin X, et al. Endometrial cancer-associated mutants of SPOP are defective in regulating estrogen receptor-alpha protein turnover. *Cell Death Dis*. 2015;6:e1687.
25. Chen X, He J, Ding Y, et al. The role of MTOR in mouse uterus during embryo implantation. *Reproduction*. 2009;138(2): 351-356.
26. Ma XH, Hu SJ, Ni H, et al. Serial analysis of gene expression in mouse uterus at the implantation site. *J Biol Chem*. 2006;281(14): 9351-9360.
27. Kapur S, Tamada H, Dey SK, et al. Expression of insulin-like growth factor-I (IGF-I) and its receptor in the peri-implantation mouse uterus, and cell-specific regulation of IGF-I gene expression by estradiol and progesterone. *Biol Reprod*. 1992;46(2):208-219.
28. Cooke PS, Buchanan DL, Young P, et al. Stromal estrogen receptors mediate mitogenic effects of estradiol on uterine epithelium. *Proc Natl Acad Sci U S A*. 1997;94(12):6535-6540.
29. Castracane VD, Jordan VC. The effect of estrogen and progesterone on uterine prostaglandin biosynthesis in the ovariectomized rat. *Biol Reprod*. 1975;13(5):587-596.
30. Kover K, Liang L, Andrews GK, Dey SK. Differential expression and regulation of cytokine genes in the mouse uterus. *Endocrinology*. 1995;136(4):1666-1673.
31. Tan Y, Li M, Cox S, et al. HB-EGF directs stromal cell polyploidy and decidualization via cyclin D3 during implantation. *Dev Biol*. 2004;265(1):181-195.
32. He H, Kong S, Liu F, et al. Rbbp7 Is Required for Uterine Stromal Decidualization in Mice. *Biol Reprod*. 2015;93(1):13.
33. He H, Teng H, Zhou T, et al. Unravelling the proteome of adult rhesus monkey ovaries. *Mol Biosyst*. 2014;10(3):653-662.
34. Cha J, Sun X, Dey SK. Mechanisms of implantation: strategies for successful pregnancy. *Nat Med*. 2012;18(12):1754-1767.
35. Li Q, Kannan A, Wang W, et al. Bone morphogenetic protein 2 functions via a conserved signaling pathway involving Wnt4 to

regulate uterine decidualization in the mouse and the human. *J Biol Chem*. 2007;282(43):31725-31732.
36. Ramathal CY, Bagchi IC, Taylor RN, Bagchi MK. Endometrial decidualization: of mice and men. *Semin Reprod Med*. 2010; 28(1):17-26.
37. Ansell JD, Barlow PW, McLaren A. Binucleate and polyploid cells in the decidua of the mouse. *J Embryol Exp Morphol*. 1974;31(1):223-227.
38. Wang H, Dey SK. Roadmap to embryo implantation: clues from mouse models. *Nat Rev Genet*. 2006;7(3):185-199.
39. Huang Y, Tan N, Jia D, et al. Speckle-type POZ protein is negatively associated with malignancies and inhibits cell proliferation and migration in liver cancer. *Tumour Biol*. 2015;36(12):9753-9761.
40. Xu J, Wang F, Jiang H, Jiang Y, Chen J, Qin J. Properties and clinical relevance of speckle-type POZ protein in human colorectal cancer. *J Gastrointest Surg*. 2015;19(8):1484-1496.
41. Di Marcotullio L, Ferretti E, Greco A, De Smaele E, Screpanti I, Gulino A. Multiple ubiquitin-dependent processing pathways regulate hedgehog/gli signaling: implications for cell development and tumorigenesis. *Cell Cycle*. 2007;6(4):390-393.
42. Duan S, Pagano M. SPOP mutations or ERG rearrangements result in enhanced levels of ERG to promote cell invasion in prostate cancer. *Mol Cell*. 2015;59(6):883-884.
43. Chen Z, Zhou Y, Zhang Z, Song J. Towards more accurate prediction of ubiquitination sites: a comprehensive review of current methods, tools and features. *Brief Bioinform*. 2015;16(4): 640-657.
44. Sigal LH. Basic science for the clinician 40: ubiquitin, programmed protein degradation, and the proteasome. *J Clin Rheumatol*. 2006;12(5):255-258.
45. Yang Y, Staudt LM. Protein ubiquitination in lymphoid malignancies. *Immunol Rev*. 2015;263(1):240-256.
46. Nallasamy S, Li Q, Bagchi MK, Bagchi IC. Msx homeobox genes critically regulate embryo implantation by controlling paracrine signaling between uterine stroma and epithelium. *PLoS Genet*. 2012;8(2):e1002500.