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Inhibition of TPPP3 attenuates β -catenin/NF- κ B/COX-2 signaling in endometrial stromal cells and impairs decidualization

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Abstract

Embryo implantation and decidualization are critical events that occur during early pregnancy. Decidualization is synchronized by the crosstalk of progesterone and the cAMP signaling pathway. Previously, we confirmed the role of TPPP3 during embryo implantation in mice, but the underlying role and mechanism of TPPP3 in decidualization has not yet been understood. The current study was aimed to investigate the role of TPPP3 in decidualization *in vivo* and *in vitro*. For *in vivo* experiments, decidual reaction was artificially induced in the uteri of BALB/c mice. TPPP3 was found to be highly expressed during decidualization, whereas in the uteri receiving TPPP3 siRNA, decidualization was suppressed and the expression of β -catenin and decidual marker prolactin was reduced. In human endometrium, TPPP3 protein was found to be predominantly expressed in the mid-secretory phase (LH+7). In the primary culture of human endometrial stromal cells (hESCs), TPPP3 siRNA knockdown inhibited stromal-to-decidual cell transition and decreased the expression of the decidualization markers prolactin and *IGFBP-1*. Immunofluorescence and immunoblotting experiments revealed that TPPP3 siRNA knockdown suppressed the expression of β -catenin, NF- κ B and COX-2 in hESCs during decidualization. TPPP3 inhibition also decreased NF- κ B nuclear accumulation in hESCs and suppressed NF- κ B transcriptional promoter activity. COX-2 expression was significantly decreased in the presence of a selective NF- κ B inhibitor (QNZ) implicating that NF- κ B is involved in COX-2 expression in hESCs undergoing decidualization. TUNEL assay and FACS analysis revealed that TPPP3 knockdown induced apoptosis and caused loss of mitochondrial membrane potential in hESCs. The study suggested that TPPP3 plays a significant role in decidualization and its inhibition leads to the suppression of β -catenin/NF- κ B/COX-2 signaling along with the induction of mitochondria-dependent apoptosis.

Key Words

- ▶ decidualization
- ▶ TPPP3
- ▶ NF- κ B
- ▶ COX-2
- ▶ apoptosis

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Introduction

Decidualization of endometrial stromal cells (ESCs) is the hallmark of tissue remodeling which supports embryo implantation and proper placental development

(Gellersen & Brosens 2014, Yu *et al.* 2017, Liu *et al.* 2017). Decidualization is initiated during the mid-luteal phase of each cycle in response to the postovulatory

rise in progesterone and increasing endometrial cAMP levels (Gellersen & Brosens 2003, Grasso *et al.* 2014, Peter Durairaj *et al.* 2017). ESCs play a critical role in the implantation process, not only by relaying hormonal signals to the overlying surface epithelium but also by dramatic morphologic and functional differentiation of the ESCs (Cooke *et al.* 1997, Li *et al.* 2011, Weimar *et al.* 2013). The decidual tissue provides the important secretory factors for nourishing the developing embryo before the maturation of placenta and forms an immune tolerance environment for the allograft embryo (Garrido-Gomez *et al.* 2011, Krieg *et al.* 2012, Su *et al.* 2015). During decidualization, cytoskeletal remodeling drives the morphologic transformation of ESCs into decidual cells (Yen *et al.* 2017). Dysregulated β -catenin activity in the stroma affects stromal decidualization in mice, and the inhibition of β -catenin activation results in the loss of the differentiation potential of hESCs (Herington *et al.* 2007, Patterson *et al.* 2017). In human endometrium, NF- κ B levels are typically elevated during the premenstrual phase and also during early pregnancy, which may regulate the molecules vital for implantation (King *et al.* 2001, Sakowicz 2018). Isolated stromal cells under conditions mimicking progesterone and estradiol withdrawal displayed activated NF- κ B (Sugino *et al.* 2004). Reports show that NF- κ B stimulates COX-2 expression in a variety of cells including ESCs (Nakao *et al.* 2002, Tsai *et al.* 2002, Sugino *et al.* 2004). Aside from that, COX2-deficient mice showed a decidualization failure (Lim *et al.* 1997). In humans, defective endometrial stromal proliferation and differentiation is linked to endometriosis and recurrent pregnancy loss (RPL), pre-eclampsia, intrauterine growth restriction and unexplained infertility in the clinical setting (Achache & Revel 2006, Laird *et al.* 2006, Arck & Hecher 2013, Garrido-Gomez *et al.* 2017).

TPPP3 (tubulin polymerization-promoting protein 3), a member of the TPPP family, is reported to induce tubulin polymerization and microtubule bundling (Vincze *et al.* 2006). Knockdown of TPPP3 inhibited cell proliferation, induced cell apoptosis and cell cycle arrest *in vitro*, and suppressed tumor growth (Zhou *et al.* 2010a,b, Li *et al.* 2016). Some reports have also defined the involvement of TPPP3 in mares' reproductive system (Klein *et al.* 2010, Hayes *et al.* 2012). In our previous study, a decreased expression of TPPP3 in the mid-secretory phase of an infertile endometrium was observed (Manohar *et al.* 2014a). More recently, we have demonstrated the functional role of TPPP3 during embryo implantation in mice and its relation with β -catenin (Shukla *et al.* 2018). However, the function of TPPP3 in the process of

decidualization remains unclear. Therefore, this study was aimed to explore the functional significance of TPPP3 in the decidualization process and the signaling mechanism involved during endometrial stromal cell differentiation.

Materials and methods

Antibodies

Antibodies for TPPP3 (sc-244482), β -catenin (sc-7663), COX-2 (sc-376861), Cytokeratin (sc-57004), Vimentin (sc-32322), Prolactin (sc-271758), IGFBP-1 (sc-25257) and β -actin (sc-1616) were procured from Santa Cruz Biotechnology. Antibodies for NF- κ B p65 (#8242), Bax (#2772), BCL-2 (#15071), Cleaved caspase-9 (#9508) and Cleaved caspase-3 (#9661) were procured from Cell Signaling Technology.

Subjects and sample collection

Endometrial tissues were collected in the operating room of the Department of Obstetrics and Gynecology, King George's Medical University (KGMU), Lucknow, UP, India. Human endometrial biopsies were collected in the operating rooms during the receptive phase (LH+7 i.e. 7 days after the LH surge occurred) from fertile and infertile women with unexplained infertility, aged 25–35 years ($n=5$). Normal endometrial biopsies (15 different cases, $n=15$) were collected from the patients undergoing hysterectomy due to uterine prolapse. The patients aged between 25 and 40 years with regular menstrual cycle, who had not received hormone therapy, were considered for this study. A specific informed consent was obtained from each patient, and the study was approved by the Human Ethics Committee of KGMU, Lucknow (#80th ECM II-A/P6 and #59th ECM IIA/P2).

Artificially induced *in vivo* deciduoma and knockdown of TPPP3 in mice

As previously described (Peng *et al.* 2015), 2-month-old female mice were bilaterally ovariectomized. After 2 weeks, the mice were injected daily with 100 ng of E_2 for 3 days, followed by 2 days of rest. The mice were injected daily with 1 mg P_4 and 6.7 ng E_2 for 3 days. One uterine horn was scratched with a needle to induce decidualization and then scrambled siRNA or TPPP3 siRNA was transfected using Lipofectamine RNAiMAX. As a control, the other uterine horn was not traumatized. The mice were

continuously injected with P₄ and E₂ for another 5 days (Fig. 1A). Mice were euthanized on day 26 and uterine tissue was excised and processed for extraction of protein (number of animals per group=5). All animal procedures were carried out as per the guidelines provided by the Institutional Animal Ethics, Use and Care Committee. Prior approval was obtained from the Institutional Animal Ethics Committee (IAEC) of CSIR-Central Drug Research Institute, Lucknow, India for animal experimentation (#IAEC/2017/F-305).

In vitro decidualization of human ESCs

Briefly, the endometrial tissue samples were washed with DMEM-F12 (Sigma-Aldrich) containing 50 mg/mL Penicillin Streptomycin (Sigma-Aldrich) and were minced to <1 mm³. Tissues were incubated in fresh DMEM-F12 medium (4 mL) containing 2 mg/mL collagenase (Sigma-Aldrich) for 60 min at 37°C. After enzymatic digestion, stromal cells were separated from epithelial aggregates by passing them through a 40 µm nylon cell strainer (BD Biosciences). After culture for three passages, decidualization was induced by incubating the cells in phenol red-free DMEM/F12 medium containing 2% charcoal-stripped FBS (Hyclone, Logan, UT, USA),

100 U/mL penicillin, 100 mg/mL streptomycin with db-cAMP and 1 × 10⁻⁶ M medroxy progesterone acetate (MPA). Following the stimulation of a differentiated phenotype, the decidual markers prolactin and IGFBP-1 were evaluated (Grinius *et al.* 2006, Frolova *et al.* 2011, Peter Durairaj *et al.* 2017). The cells were harvested within 36 h after steroid withdrawal at 12-h intervals.

Transient transfection and transactivation assay

For experiments involving siRNA transfection, human ESCs (hESCs) were seeded and allowed to attain the confluency of 70–80%. These cells were transiently transfected with TPPP3 siRNA (30 nM) or with negative control, that is, scrambled siRNA (random sequence without any homology with mammalian gene) (Santa Cruz Biotechnology) using Lipofectamine RNAiMAX reagent (Invitrogen). The hESCs were transfected on day 0 and day 6 after MPA and db-cAMP treatment and were cultured for 9 days with medium changes every three days (Shindoh *et al.* 2014) (Fig. 2D). TPPP3 knockdown efficiency was measured by immunoblotting.

In the transactivation assay, all plasmids were prepared using QIAGEN plasmid DNA preparation kits. The cells were then transfected with 500 ng of pNF-kB-luc

Figure 1

Effect of TPPP3 knockdown during artificial decidualization in mice. (A) Representation of stimulated decidualization procedures. (B) Gross morphology of un-stimulated (UH) or stimulated (SH) uterine horn. (C) Protein expressions of TPPP3, β-catenin and decidual marker prolactin were examined by Western blotting (left panel). Densitometric quantitation of protein expression levels is shown as fold changes (right panel). Data are presented as mean ± S.E.M. *P* values: ^a*P* < 0.001, ^b*P* < 0.01, ^c*P* < 0.05 and ^d*P* > 0.05 vs un-stimulated or non-decidual horn (number of animals per group=5). A full color version of this figure is available at <https://doi.org/10.1530/JOE-18-0459>.

Figure 2

TPPP3 knockdown in hESCs seized stromal to decidual cells transformation. (A) Immunohistochemical staining of TPPP3 in the mid-secretory phase endometrium samples from fertile women and unexplained infertile patients (LH+7). Nonspecific rabbit IgG was used as a negative control. Brown represents positive staining. (B) Immunostaining with vimentin, a stromal cell biomarker and cytokeratin, an epithelial cell biomarker. Vimentin was visualized as green. Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). (C) The TPPP3 was significantly increased during decidualization i.e. in the presence of MPA and db-cAMP which was suppressed in the cells transfected with TPPP3 siRNA. Data are presented as mean $\pm$ s.e.m. P values: $^aP < 0.001$, $^bP < 0.01$, $^cP < 0.05$ and $^dP > 0.05$ vs hESCs. (D) hESCs were transfected with TPPP3 siRNA or scrambled siRNA (control) on day 1 and day 6 and were cultured with db-cAMP and MPA for 9 days with medium changes at every 3 days. (E) TPPP3 knockdown in hESCs suppressed the mRNA level of decidualization markers prolactin and *IGFBP-1*. (F) Representative micrographs demonstrating the fluorescein isothiocyanate-labeled phalloidin to label F-actin filaments, and immunofluorescence was used to analyze the morphological transformation of hESCs during *in vitro* decidualization. (G) Co-localization of β -catenin and TPPP3 during *in vitro* decidualization. Each experiment was performed three times with three tissue samples.

(Stratagene, La Jolla, CA, USA) using Lipofectamine RNAiMAX transfection reagent (Invitrogen) as per manufacturer's protocol. To normalize for transfection efficiencies, 200 ng pRL-SV40-luc (Promega) were co-transfected. After overnight transfection, medium was changed and the cells were transfected with scrambled siRNA or TPPP3 siRNA. After 24 h, the cells were lysed with lysis buffer. Luciferase activity was measured using Dual Luciferase Assay System (Promega) according to the manufacturer's protocol to detect the transcriptional activity of the transfected promoter. The firefly luciferase activity for each group was normalized with the transfection efficiency determined by Renilla luciferase activity (Popli *et al.* 2015). Each experiment was performed three times with three different tissue samples.

Western blot analysis

Protein was extracted from the whole uterine tissue (*in vivo* experiments) or the cells (*in vitro* experiment). Briefly, the uterine tissue was homogenized in an ice-cold RIPA lysis buffer (150 mM NaCl, 1.0% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) (Sigma-Aldrich) and was supplemented with a protease inhibitor cocktail (PIC) (Sigma-Aldrich) for 5 h at 4°C and then kept overnight at -20°C. The protein was obtained as a supernatant by centrifuging at 12,000 *g* for 60 min at 4°C. Protein estimation was done using Bradford reagent (Sigma-Aldrich). Equal amounts of protein (20 µg) were separated by SDS-PAGE and transferred to an immunoblot PVDF membrane. The membrane was blocked for 2 h in 5% skimmed milk and incubated with the primary antibody overnight at 4°C. Antibody binding was detected by using enhanced chemiluminescence detection system (Bio Rad ChemiDoc XRS⁺). After developing, the membrane was stripped and re-probed with β -actin antibody. Densitometry of band density was performed using Quantity-One software (v.4.5.1). The band density volume of each group was measured and normalized using β -actin as an internal control (Manohar *et al.* 2014b, Kaushal *et al.* 2017). Fold change was calculated in relation to control group (taken as 1). Each experiment was performed three times with three different tissue samples.

Immunohistochemistry

Immunohistochemistry was performed to localize the expression of TPPP3 in a normal mid-secretory phase (LH+7) endometrium and an unexplained infertile mid-secretory phase (LH+7) endometrium in women using

ABC staining kit (Santa Cruz Biotechnology). Formalin-fixed tissues were dehydrated and thereafter embedded in paraffin wax. Paraffin sections of 5 µm were cut from the tissues. After de-paraffinization and rehydration, antigen unmasking was performed by heating the sections in 10 mM sodium citrate buffer (pH 6.0) at 95°C for 10 min, and they were allowed to cool for 30 min. Avidin-biotin peroxidase immunostaining was performed as per the manufacturer's protocol. Briefly, the sections were treated with 0.5% H₂O₂ in deionized water for 15 min to block the endogenous peroxidase activity and were blocked with 3% normal goat serum for 2 h at 25°C. The sections were then incubated with primary TPPP3 antibody (1:100 dilutions) in a humid chamber overnight at 4°C. On the following day, the sections were incubated with biotinylated goat anti-rabbit IgG antibody at 37°C for 30 min. Next, they were stained with 3,3'-diaminobenzidine (DAB) and counterstained with hematoxylin. In control sections, nonspecific rabbit IgG was used instead of primary antibody (Shukla *et al.* 2018). The experiment was performed three times with three different tissue samples.

Total uterine RNA extraction, first-strand cDNA synthesis and real-time PCR

Total RNA from cells was extracted using TRIzol reagent following the manufacturer's instructions. Briefly, the isolated RNA was treated with RNase-free DNase to remove any residual genomic DNA. The concentration of RNA was measured by using NanoDrop (Thermo Fisher Scientific). First strand of DNA (cDNA) was prepared from total RNA (1 µg) at each stage using high-capacity cDNA reverse transcription kit, according to the manufacturer's procedure (Thermo Fisher Scientific). Quantification of genes using RT-PCR was performed with a Light Cycler (Roche Life Science). Quantitative PCR analyses were performed using appropriate primers: Prolactin (Human): Left 5'-GAACAGATTGAAATTGAATTGAACA-3', Right 5'-GCATATTACAATAGCTTCTCCTCCA-3'; *IGFBP-1* (Human): Left 5'-TCAAAAATGGAAGGAGCCCT-3', Right 5'-AATCCATTCTTGTTCAGTTT-3'; *GAPDH* (Human): Left 5'-AGCCACATCGCTCAGACAC-3', Right 5'-AATACGACCAAATCCGTTGACT-3'. Expression of the investigated gene was normalized to the steady expression of a housekeeping gene GAPDH. Comparative cycle threshold (2^{-ΔΔCt}) method was used for relative quantification. The real-time PCR system was programmed according to the manufacturer's instructions. The experiment was performed three times with three tissue samples. (Shukla *et al.* 2018).

Immunofluorescence imaging by confocal microscopy

HESCs were fixed in methanol and acetone in 1:1 ratio at 4°C and permeabilized with 0.1% Triton X-100 and mixed with microtubule-stabilizing buffer (100 mM PIPES, 1 mM MgCl₂, 5 mM EGTA, pH 6.8) (Palazzo *et al.* 2011). The HESCs were then washed with PBS and blocked with 1% BSA and incubated with TPPP3, β -catenin, NF- κ B, COX-2, vimentin or cytokeratin antibody overnight followed by 1-h incubation with a fluorescence-tagged secondary anti-rabbit/mouse/goat antibody, and then counterstained with DAPI for 10 min. In another experiment, cells were incubated with fluorescein isothiocyanate-labeled phalloidin at 4°C overnight to stain the F-actin filaments. On the following day, cell nuclei were stained with DAPI (Garrido-Gomez *et al.* 2017). Images were captured at 63 $\times$ using Carl Zeiss LSM 510 META microscope, and they analyzed using LSM Image-Examiner Software to detect the fluorescence and DAPI emissions (Shukla *et al.* 2015). Control IgG as a negative control showed no detectable immunofluorescence. The experiment was performed three times.

Annexin-v/propidium iodide labeling and flow cytometry assay for apoptosis

The hESCs (2 $\times$ 10⁵ cells/mL) were cultured in six-well plates and transfected with scrambled or TPPP3 siRNA for 48 h according to the previously described siRNA knockdown protocol. After trypsinization, the cells were probed with FITC-conjugated Annexin-V and PI for 15 min. Fluorescence staining profile was determined through FACSscan and Cell-Quest software (Shukla *et al.* 2018). The experiment was performed three times.

Terminal deoxynucleotidyltransferase-mediated nick end labeling assay

TUNEL staining was performed using 'In situ apoptosis detection kit' (Shiga Prefecture, Japan) as per manufacturer's protocol. Briefly, cells were incubated for 90 min at 37°C with terminal deoxynucleotidyltransferase (TdT) incubation buffer. The negative control slide was incubated without the TdT enzyme. The reaction was terminated by washing with PBS, and the slide was examined under fluorescence microscope (Nikon Eclipse 80i, Shinagawa-Ku, Tokyo, Japan) (Kaushal *et al.* 2018). The experiment was performed three times with three tissue samples.

Measurement of mitochondrial membrane potential

In brief, hormonally induced human endometrial decidual cells were transfected with scrambled or TPPP3 siRNA for 48 h according to the previously described siRNA knockdown protocol and harvested by trypsinization. Cells were incubated with 2 mL of medium containing JC-1 dye (1 μ g mL⁻¹) for 15 min at 37°C. JC-1, a cationic carbocyanine dye that accumulates in mitochondria, was used as an indicator of mitochondrial membrane potential. The stained human decidual cells were washed with PBS and subjected to flow cytometry analyses as per standard protocol using FL1 and FL2 channels (Shukla *et al.* 2015). The experiment was performed three times with three tissue samples.

Statistical analysis

Statistical analysis was performed using GraphPad Prism v6.0. All statistical tests are described in their respective figure legends. Briefly, data are presented as mean $\pm$ S.E.M. for at least three separate determinations for each experiment. One-way ANOVA in combination with Tukey test was done to compare the multiple groups (three to four groups), and the Student's 't' test was performed for comparing two groups. *P* values less than 0.05 were considered as significant.

Results

Transient knockdown of TPPP3 is impaired in artificial decidualization

To understand the functional role of TPPP3 in the regulation of decidualization, we first examined the TPPP3 protein expression in the mouse uterus during artificial decidualization (Fig. 1A and B). In the immunoblotting experiment, an increased expression of TPPP3 (~2.2-fold) was observed in the uteri of mice receiving mechanical stimulation compared to those of un-stimulated horn (Fig. 1C). The protein expression of TPPP3 and β -catenin decreased by ~2-fold (*P* < 0.001) and the decidual marker prolactin decreased by ~3-fold (*P* < 0.001) in TPPP3 siRNA-transfected horn than in scrambled siRNA-transfected horn during artificial decidualization in mice (Fig. 1C).

TPPP3 is expressed in human endometrium during the mid-secretory phase (LH + 7)

To investigate the expression of TPPP3 during human endometrial decidualization, we evaluated the TPPP3

protein expression by immunohistochemistry in the mid-secretory phase (LH+7) of menstrual cycle. We found a higher expression of TPPP3 in the cytoplasmic region of ESCs in a mid-secretory phase endometrium from fertile subjects compared to that from infertile subjects (Fig. 2A).

Transient knockdown of TPPP3 in hESCs inhibits stromal-to-decidual cell transition *in vitro* and suppresses decidualization markers

Immunohistochemical result showed the expression of TPPP3 in the mid-secretory phase in endometrial stroma. Further, to investigate whether TPPP3 is involved in decidualization, we performed *in vitro* decidualization experiment using hESCs. The homogeneity of the hESCs preparation was confirmed by the expression of vimentin (stromal cell marker) determined using immunofluorescence (Fig. 2B). The immunoblot analysis showed the increased protein expression of TPPP3 in decidual cells compared to control hESCs (~2.7 fold) ($P < 0.001$) (Fig. 2C). To investigate the role of TPPP3 in decidualization, hESCs were transfected with TPPP3 siRNA or scrambled siRNA on days 1 and 6 during long-term culture (Fig. 2D). TPPP3 knockdown caused a reduction in TPPP3 by ~0.6 times ($P < 0.001$) (Fig. 2C). The gene expression of decidual markers prolactin and *IGFBP-1* was decreased by ~3-fold ($P < 0.001$) and ~2.5-fold ($P < 0.001$), respectively, in TPPP3 siRNA-transfected hESCs compared to scrambled siRNA-transfected hESCs (Fig. 2E). To assess the morphological changes in hESCs after TPPP3 knockdown, we further examined its effect on organization of the F-actin cytoskeleton. As shown in Fig. 2E, decidualized hESCs displayed polygonal cell morphologies with random distribution of F-actin filaments compared with non-decidualized hESCs. When endogenous TPPP3 was knocked down, stromal cells maintained a long fibroblast-like phenotype (Fig. 2F). These results confirmed that TPPP3 siRNA effectively suppressed decidualization in hESCs.

Functional blockage of TPPP3 inhibits the expression of TPPP3 and β -catenin in hESCs: co-localization study

In our previous report, we confirmed that TPPP3 regulates β -catenin expression during peri-implantation period in mice (Shukla *et al.* 2018). To corroborate the putative interaction of TPPP3 and β -catenin in decidual cells, a double immunofluorescence detection to analyze their co-localization, was performed (Fig. 2G). TPPP3 and β -catenin showed a clear co-localization in the cytoplasm of decidualized hESCs. Immunofluorescence (IF) staining

for TPPP3 protein was strong and predominantly localized into the nucleus and cytoplasm of the hESCs transfected with scrambled siRNA or in hESCs without siRNA, whereas the staining of TPPP3 and β -catenin was reduced in TPPP3 siRNA-transfected hESCs during decidualization (Fig. 2G).

TPPP3 inhibition blocks NF- κ B p65 nuclear translocation with diminished expression of COX-2

The β -catenin signaling is known to modulate the inflammatory responses through communication with NF- κ B (Nejak-Bowen *et al.* 2013). Therefore, we assessed the effect of TPPP3 knockdown on NF- κ B expression. TPPP3 knockdown suppressed NF- κ B expression (by ~0.6 times) compared to that in scrambled siRNA-transfected cells during *in vitro* decidualization. In the immunoblotting experiment, we found an increase in the expression of NF- κ B by ~2.5-fold in decidual cells compared to that in hESCs ($P < 0.001$) (Fig. 3A).

Next, we assessed whether TPPP3 knockdown inhibits nuclear translocation of NF- κ B p65 during hESCs decidualization. As demonstrated by IF, TPPP3 knockdown inhibited the translocation of p65 (ser 536), a typical subunit of NF- κ B, into nucleus which was almost completely inhibited by QNZ (a selective NF- κ B activation inhibitor i.e., 6-amino-4-(4-phenoxyphenylethylamino) quinazoline) (Wagley *et al.* 2013, Popli *et al.* 2015) (Fig. 3B). Furthermore, to examine whether TPPP3 inhibition can reduce NF- κ B activation in decidual cells, NF- κ B-binding sites promoter luciferase reporter construct (pNF- κ B luc) was transfected in decidual cells, and the luciferase activity was assessed. The data showed that knockdown with TPPP3 siRNA reduced luciferase activity more than that with scrambled siRNA-transfected cells (Fig. 3C).

COX-2 has been shown to contain several NF- κ B-binding sites in its promoter region (Pedram *et al.* 2002, Schmedtje *et al.* 1997). COX-2 is responsible for inflammation, and the COX-2 deficient mice showed decidualization failure (Lim *et al.* 1997). Thus, we determined whether NF- κ B activation induced the COX-2 expression in hESCs. The results of immunoblotting and IF experiments confirmed that the expression of COX-2 was significantly decreased in TPPP3 siRNA-transfected hESCs compared to scrambled siRNA-transfected hESCs during *in vitro* decidualization (Fig. 4A and B). The densitometric analysis showed that TPPP3 knockdown caused more reduction in COX-2 (by ~0.5 times) than scrambled siRNA-transfected hESCs ($P < 0.001$). These results indicated that TPPP3 knockdown inhibits NF- κ B/COX-2 in decidualized hESCs.

Figure 3

Protein expression and nuclear translocation of NF-κB p65 during *in vitro* decidualization. (A) TPPP3 knockdown suppressed NF-κB p65 protein expression. (B) TPPP3 knockdown in decidual cells inhibits NF-κB p65 nuclear translocation. Representative micrographs demonstrating the distribution of NF-κB p65 are shown. Cell images were grasped using a confocal fluorescence microscope (×63). (C) Transcriptional activation of the NF-κB promoter in decidual cells analyzed after TPPP3 knockdown and then transiently transfected with pNF-κB-luc reporter plasmid. pRL-luc plasmid was used as an internal control, and normalized relative luciferase activity was determined. Data are presented as mean ± s.e.m. *P* values: ^a*P* < 0.001, ^b*P* < 0.01, ^c*P* < 0.05 and ^d*P* > 0.05 vs scrambled siRNA control. Each experiment was performed three times with three tissue samples. A full color version of this figure is available at <https://doi.org/10.1530/JOE-18-0459>.

TPPP3 silencing induced mitochondria-dependent apoptosis in hESCs during *in vitro* decidualization

In our previous report, we showed that TPPP3 knockdown in mouse endometrial epithelial cells and Ishikawa cells induces apoptosis (Shukla *et al.* 2018). To explore whether TPPP3 controls the apoptosis in ESCs during decidualization, we analyzed Annexin-V/PI-stained cells by flow cytometry and TUNEL assay (Fig. 5A). In the Annexin-V/PI assay, TPPP3 knockdown increased the percentage of apoptotic cells, which was approximately 25% higher as compared to that in scrambled siRNA control group (Fig. 5B). In addition, the ~150% drop in mitochondrial membrane potential ($\Delta\psi_m$) was observed in the TPPP3-knockdown hESCs (*P* > 0.001) (Fig. 5C).

The expressions of pro-apoptotic marker Bax and anti-apoptotic marker BCL-2 were analyzed in hESCs during *in vitro* decidualization by immunoblotting. Results showed that TPPP3 knockdown noticeably reduced BCL-2 expression (by ~0.5 times) (*P* < 0.001), whereas upregulation was observed in Bax (~2.2-fold) (*P* < 0.001). The expressions of cleaved caspase-9 and cleaved caspase-3 protein were increased by ~2.3-fold as detected in TPPP3 siRNA-transfected hESCs (*P* < 0.001) (Fig. 5D).

Discussion

During decidualization, ESCs undergo proliferation and differentiation (Tang *et al.* 1994, Salamonsen *et al.* 2003, Maruyama & Yoshimura 2008). Our previous

Figure 4

TPPP3 inhibition reduces COX-2 expression. (A) Protein expression of COX-2 was examined by Western blotting (left panel). Densitometric quantitation of protein expression levels is shown as fold changes (right panel). Data are presented as mean $\pm$ S.E.M. P values: ^a $P < 0.001$, ^b $P < 0.01$, ^c $P < 0.05$ and ^d $P > 0.05$ vs scrambled siRNA control. (B). Representative micrographs demonstrating the distribution of COX-2 are shown. NF- κ B inhibitor QNZ inhibited COX-2 expression. Cell images were grasped using a Nikon fluorescence microscope ($\times 40$). Each experiment was performed three times with three tissue samples.

study demonstrated the decreased expression of TPPP3 in the endometrium of infertile women during the mid-secretory phase (Manohar *et al.* 2014a). More recently, we showed that TPPP3 plays an important role in embryo implantation in mice (Shukla *et al.* 2018). Certain other investigations also give indirect evidence of involvement of TPPP3 in reproductive functions in various species (Klein *et al.* 2010, Hayes *et al.* 2012). We hypothesized that the failure of decidualization may be caused by the decreased endometrial expression of TPPP3. In this study, we found that the inhibition of TPPP3 leads to the suppression of β -catenin/NF- κ B/COX-2 axis, induces apoptosis and impairs decidualization. To our knowledge, this is the first study to reveal the functional role and molecular mechanism of TPPP3 in decidualization.

We demonstrated herein that TPPP3 is ubiquitously expressed in mice uterus during artificially induced decidualization. Further, we studied the functional activity of TPPP3 and determined whether it is involved in these cellular events. In our experiments, TPPP3 knockdown in hESCs resulted in decreased prolactin and IGFBP-1 protein level, compared to control group during decidualization. Both decidual prolactin and IGFBP-1 act as endocrine and autocrine/paracrine factors and are believed to play

an important role at the blastocyst–maternal interface in the regulation of implantation and pregnancy (Irwin *et al.* 2001, Jabbour & Critchley 2001, Stefanoska *et al.* 2013, Gellersen & Brosens 2014).

The expression of Wnt/ β -catenin signaling target genes regulates proliferation, differentiation, stemness and immune responses, revealing a broad control of organismal and cellular functions (Ma & Hottiger 2016). β -catenin is predominantly expressed in the stromal compartment of human endometrium during the mid-secretory phase (Li *et al.* 2013b). In our previous report, we demonstrated that TPPP3 regulates β -catenin expression in mouse uterus during the peri-implantation phase (Shukla *et al.* 2018). Also, the conditional deletion of β -catenin from the stromal compartment results in failed decidualization in mice (Zhang *et al.* 2012). Our IF results suggested that TPPP3 knockdown showed reduced β -catenin expression in hESCs. The β -catenin pathway components are known to modulate inflammatory and immune responses via the interaction with NF- κ B (Nejak-Bowen *et al.* 2013). The earlier investigation has revealed efficient regulation and complex roles for β -catenin and NF- κ B signaling in the pathogenesis of certain cancers and other diseases (Du & Geller 2010). NF- κ B regulates

Figure 5

TPPP3 knockdown during *in vitro* decidualization in hESCs induce apoptosis and promote mitochondrial membrane potential ($\Delta\Psi_m$) loss. (A) TUNEL assay performed in scrambled siRNA or TPPP3 siRNA-transfected cells during *in vitro* decidualization. Cells were fixed and permeabilized, and the procedure was followed as described in the 'Materials and Methods' section. Representative figures stained for TUNEL showing a large number of TUNEL-positive cells in TPPP3 siRNA-transfected cells as compared with scrambled siRNA-transfected cells. Cell images were grasped using a Nikon fluorescence microscope at $\times 10$. (B) Flow cytometric analysis of apoptosis in scrambled siRNA or TPPP3 siRNA-transfected cells stained with Annexin-V/PI (AV⁺/PI⁻-intact cells; AV⁺/PI⁺-nonviable/necrotic cells; AV⁺/PI⁻ and AV⁺/PI⁺-apoptotic cells). Representative images of flow cytometry of transfected cells are shown in the upper panel and the percentage of apoptosis with mean $\pm$ S.E.M. is shown in the lower panel. (C) $\Delta\Psi_m$ was assessed by JC-1 staining using flow cytometry analysis. Representative images of flow cytometry of scrambled siRNA or TPPP3 siRNA-transfected cells are shown in the upper panel and the percentage loss of $\Delta\Psi_m$ with mean $\pm$ S.E.M. (D) Effect of TPPP3 knockdown on apoptotic markers. Representative blots are shown (left panels) and densitometric quantitation of protein expression levels are shown as fold change (right panel). The experiments were performed three times. *P* values: ^a*P* < 0.001, ^b*P* < 0.01, ^c*P* < 0.05 and ^d*P* > 0.05 vs scrambled siRNA control. Each experiment was performed three times with three tissue samples.

the pro-inflammatory molecules and may be involved in the inflammation associated with female reproductive events, for example, menstruation, implantation and decidualization (King *et al.* 2001). In a normal endometrium, the nuclear phosphorylated p65 (p65), the active form of NF- κ B subunit, was found to be notably increased in the secretory phase of menstrual cycle (Saegusa *et al.* 2007). We found the NF- κ B p65 expression

to be higher in decidualized cells than in stromal cells in an *in vitro* experiment. In addition, the functional blockage in TPPP3 expression evidently preceded the decrease in NF- κ B p65 expression and its nuclear translocation in hESCs even in the presence of cAMP and MPA.

NF- κ B inhibition suppressed the COX-2 expression in adenomyosis and is involved in the regulation of genes encoding pro-inflammatory cytokines/chemokines and

their receptors (Cao *et al.* 2006, Li *et al.* 2013a). COX-2, an NF- κ B target gene and its promoter, has been shown to contain several binding sequences for NF- κ B transcription factor (Inoue & Tanabe 1998, Tanabe & Tohnai 2002). Previous reports show that NF- κ B stimulates COX-2 expression in ESCs and a variety of cells (Nakao *et al.* 2002, Tsai *et al.* 2002, Sugino *et al.* 2004). Though no evidence is available for the link between TPPP3 and COX-2 so far, our results demonstrated that TPPP3 knockdown suppressed the COX-2 expression in these cells. Moreover, TPPP3 knockdown inhibited NF- κ B-luc transcriptional activation in hESCs, which might be responsible for suppressed COX-2 expression. These results confirmed that NF- κ B pathway is closely involved in COX-2 expression in ESCs during decidualization.

Since NF- κ B plays a central role in the regulation of apoptotic pathways (Ryan *et al.* 2000, Karin & Lin 2002, Shukla *et al.* 2015), we assessed the effect of TPPP3 knockdown on the expression of the apoptotic markers in hESCs. Decidualization of the endometrium and further pregnancy can be diminished by inhibiting the apoptosis of decidual cells via the mitochondrial-dependent apoptosis pathway (Liao *et al.* 2015). A dysbalance of apoptotic events in the secretory endometrium seems to be implicated in implantation disorders and consecutive pregnancy complications (Fluhr *et al.* 2013). In our previous report, we showed that TPPP3 suppression in mouse endometrial epithelial and Ishikawa cells induced apoptosis (Shukla *et al.* 2018). Here, we hypothesize that the dysregulated apoptosis may lead to interference with normal function of hESCs, resulting in failed stromal-to-decidual cell transition. We showed that TPPP3 knockdown induced mitochondria-dependent apoptosis in hESCs, which was mediated via intrinsic pathway. However, the present findings set the stage for several key future studies to elucidate the functions of TPPP3 in apoptosis in reproductive events.

In conclusion, the study demonstrated that TPPP3 plays a significant role in decidualization. Impairment of TPPP3 expression leads to the suppression of β -catenin/NF- κ B/COX-2 signaling, induces apoptosis and impairs decidualization in the endometrium. Our study has provided a new insight into the molecular mechanism of TPPP3, showing that it might act as a regulator controlling multiple facets that are critical for decidualization. From a therapeutic perspective, therapies targeted for TPPP3 could be applicable for the treatment of implantation failure caused by impaired decidualization.

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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Author contribution statement

A D conceptualized the study. A D and V S designed and executed the experiments. V S and J B K performed the experiments and analyzed the data. P S and J B K collected and processed the endometrial biopsy samples. M M processed and analyzed the biopsy samples for IHC. A D and V S drafted the manuscript. All authors have approved the final version of the manuscript.

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