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Nitration of protein phosphatase 2A increases via Epac1/PLCε/CaMKII/HDAC5/iNOS cascade in human endometrial stromal cell decidualization

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Abstract

Decidualization of the endometrial stroma is an essential differentiation process for embryo implantation and maintenance of pregnancy. We previously reported that protein phosphatase 2A (PP2A) acts as a key mediator during cAMP-induced decidualization of human endometrial stromal cells (hESCs). However, the mechanism underlying its activation has remained obscure in hESCs. In the present study, we aimed to reveal the mechanism that induces the nitration of PP2A catalytic subunit (PP2Ac) during cAMP-induced decidualization of hESCs. First, cAMP-induced PP2Ac nitration was significantly repressed using L-NAME, an inhibitor of nitric oxide synthase (NOS). Among several NOS isoforms, only inducible NOS (iNOS) was highly expressed in hESCs, indicating that iNOS directly induces the nitration of PP2Ac. Second, cAMP-induced iNOS expression and PP2Ac nitration were decreased by treatment with TSA, an inhibitor of histone deacetylase 5 (HDAC5). cAMP-induced phosphorylation of CaMKII and HDAC5 was suppressed by treatment with U73122 (an inhibitor of phospholipase C) or transfection of PLCε siRNA. Finally, small G protein Rap1 and its guanine nucleotide exchange factor Epac1 were

Abbreviations: AI, artificial insemination; ARTs, assisted reproductive technologies; CaMKII, Ca2+/calmodulin-dependent protein kinase II; cAMP, cyclic AMP; DAG, diacylglycerol; DEG, differentially expressed gene; Epac, exchange protein directly activated by cAMP, guanine nucleotide exchange factor; GEF, guanine nucleotide exchange factor; GEO, gene expression omnibus; HDAC5, histone deacetylase 5; hESCs, human endometrial stromal cells; ICSI, intracytoplasmic sperm injection; IGFBP1, insulin-like growth factor-binding protein 1; iNOS, inducible nitric oxide synthase; IP_3, inositol 1,4,5-trisphosphate; IVF, in vitro fertilization; LCMT1, leucine carboxyl methyltransferase 1; LCMT2, leucine carboxyl methyltransferase 2; L-NAME, N omega-Nitro-L-arginine methyl ester hydrochloride; MAT2A, methionine adenosyltransferase 2A; MAT2B, methionine adenosyltransferase 2B; NESCs, normal endometrial stromal cells; NO, nitric oxide; PA, phosphatidic acid; PI (4,5)P2, phosphatidylinositol 4, 5-bisphosphate; PLCε, phospholipase Cε; PLD1, phospholipase D1; PME1, protein phosphatase methylesterase 1; PP2A, protein phosphatase 2A; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; Ser, serine; Thr, threonine; TRP, transient receptor potential; TRPC, TRP canonical; TSA, trichostatin A.

found to be involved in cAMP-induced PP2A activation. Taken together, our results suggest that PP2Ac nitration during cAMP-induced decidualization of hESCs is induced through the Epac1-Rap1-PLCε-CaMKII-HDAC5-iNOS signaling pathway.

KEYWORDS

decidualization, human endometrial stromal cells, nitration, protein phosphatase 2A

1 | INTRODUCTION

Decidualization is a differentiation process for successful embryo implantation and maintenance of pregnancy; it occurs in endometrial stromal cells. It is actively induced from the middle to late secretory stage of the menstrual cycle and is accompanied by biochemical and morphological changes. During decidualization, marker genes such as *insulin-like growth factor-binding protein 1* (*IGFBP1*) and *prolactin* are highly expressed, and the morphology of endometrial stromal cells becomes larger and rounder. Decidualization also requires elevated progesterone and intracellular cAMP levels.[1] Previously, we demonstrated that phosphatidic acid (PA) promotes the decidualization of human endometrial stromal cells (hESCs) through increased phosphatase activity of PP2Ac which has an important role in decidualization by controlling the dephosphorylation of Akt and FoxO1.[1] However, the detailed mechanism of PP2Ac activation during the decidualization of hESCs is not fully understood. In the present study, we focused on identifying how PP2Ac is activated during decidualization in hESCs.

Protein phosphatase 2A (PP2A) is a conserved serine/threonine phosphatase with broad substrate specificity; it is involved in diverse cellular functions such as anticancer activity, invasion, cell motility, and cellular homeostasis.[2-4] PP2A is activated by the post-translational modification of its catalytic subunit (PP2Ac).[2] In particular, PP2Ac is regulated by tyrosine nitration.[5,6] Protein nitration largely relies on the free radical nitric oxide (NO), a gaseous free radical molecule with a short half-life that plays key roles in various biological processes and the physiology of the reproductive system.[7,8] Peroxynitrite ($ONOO^-$) is the reaction product of NO and superoxide radicals ($O_2\bullet^-$), and nitrite (NO_2^-) is a primary oxidation product of NO.[9,10] The reaction of $ONOO^-$ with CO_2 results in the formation of nitrogen dioxide ($\bullet NO_2$) and carbonate ($\bullet CO_3^-$) radicals via hemolytic cleavage. Peroxidases, heme proteins, and transition metals are also oxidized by H_2O_2, which catalyzes the oxidation of NO_2^- to $\bullet NO_2$ and $\bullet OH$. Moreover, NO can react directly with tyrosine radical (Tyr•), and $\bullet OH$ and $\bullet CO_3^-$ are the primary reactive species that mediate the oxidation of Tyr to Tyr•. The second step in the mechanism of Tyr nitration is the formation of 3-nitrotyrosine (NO_2Tyr, 3-NT) from the reaction between Tyr• and $\bullet NO_2$.[10,11] Under several oxidative conditions, NO_2Tyr levels are increased by the NO-dependent Tyr nitration of proteins; it often localizes to specific tissue regions and cell types.[10] Nitric oxide (NO) is derived from L-arginine by nitric oxide synthases (NOS), catalyzing the oxidation of L-arginine to L-citrullin and NO. There are three different isoenzymes of NOS: inducible NOS (iNOS or type II), endothelial NOS (eNOS or type III), and neuronal NOS (nNOS or type I).[8,10,12,13] According to previous studies, iNOS-dependent Tyr nitration of PP2A increases its activity in renal fibrosis and the innate immune system.[5,6,14] However, the role of PP2A nitration in the decidualization of hESCs is not known.

NO is a potent mediator of smooth muscle relaxation, adhesion, cell growth, and apoptosis.[12,15] In addition, NO induces menstruation through the induction of vasodilation and inhibition of platelet aggregation. NO is also produced by an estrogen hormone during pregnancy. iNOS localizes to the antral follicles in the ovary, but eNOS is present in the blood vessels of the ovary. After ovulation, iNOS is expressed in the external layers of the corpus luteum.[8] Although many studies have confirmed the expression of iNOS in the decidualized human endometrium, the detailed mechanism of inducing iNOS expression and NO production during the decidualization of hESCs is unclear.

Because histone proteins are densely packed by DNA, transcription regulation is strongly influenced by post-transcriptional histone modifications such as acetylation, methylation, and phosphorylation. Histone deacetylases (HDACs) are important modulators of histone acetylation for controlling the expression of target genes. For this reason, HDACs have many functions in several cell types, including differentiation, growth arrest, and cytotoxicity.[16] Production of NO and expression of iNOS, a representative target of HDACs, are regulated by the gain-/loss-of-function of HDACs.[17] Moreover, HDACs are exported from the nucleus via the Epac-PLCε-CaMKII pathway in cAMP-dependent signaling.[18] We, therefore, propose that the Epac1-Rap1-PLCε signaling cascade promotes CaMKII phosphorylation, leading to the upregulation of iNOS expression through the inactivation of HDAC5. Hereafter, we reveal the novel mechanism for the tyrosine nitration of PP2Ac via the Epac1-Rap1-PLCε-CaMKII-HDAC5-iNOS pathway during cAMP-induced decidualization of hESCs.

2 | MATERIALS AND METHODS

2.1 | Materials

For cell culture experiments, fetal bovine serum (FBS), penicillin-streptomycin, and Dulbecco's modified Eagle medium, low glucose (DMEM) were purchased from WelGENE (Gyeongsan-si, Gyeongsangbuk-do, Korea). 8-Br-cAMP was purchased from Sigma-Aldrich (St. Louis, MO, USA). Okadaic acid was purchased from Enzo (Farmingdale, NY, USA) and L-NAME was purchased from Abcam (Cambridge, MA, USA). The HDAC5 inhibitor TSA was purchased from Sigma-Aldrich (St. Louis, MI, USA). U73122 and BAPTA-AM were purchased from Calbiochem (La Jolla, CA, USA). Anti-iNOS was purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, California, USA); anti-PP2Ac, anti-Epac1, anti-phospho-CaMKII (Thr^{286}), and anti-CaMKII antibodies were purchased from Cell Signaling Technology, Inc (Danvers, MA, USA), and anti-phospho-HDAC5 (Ser^{498}) and anti-HDAC5 antibodies were purchased from Abcam (Cambridge, MA, USA). Anti-iNOS and anti-3-NT antibodies were purchased from Merck Millipore (Darmstadt, Hesse, Germany). DAF-FM diacetate was purchased from Molecular Probes (Eugene, OR, USA) and Fura-2 was purchased from Life Technologies (Grand Island, NY, USA). All other chemicals were of analytical grade.

2.2 | Endometrial stromal cell isolation

Human endometria were obtained by hysterectomy from three premenopausal women, aged 43-47 years, who underwent surgery for nonendometrial abnormalities in the Hanyang University Hospital between October 2016 and October 2018. These experiments were conducted under protocols approved by the Hanyang University Hospital's Institutional Review Board (IRB file number 2017-01-060-008), and written informed consent was obtained from all participants. Each endometrial specimen obtained was examined histologically. hESCs were isolated and cultured as described previously.[1] Briefly, endometrial tissue was collected in DMEM low-glucose media containing 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin. Tissue specimens were minced to fragments and digested in a 37°C incubator for less than 60 minutes with 0.25% collagenase (GIBCO, Grand Island, NY, USA). To filter out the endometrial stromal cells, the cell suspension was filtered twice through a sieve with a pore size of 40-μm (BD Falcon, Bedford, MA, USA). The purified stromal cells were washed with PBS (Biosesang, Seongnam, Gyeonggi, Korea) and cultured in DMEM supplemented with 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin in a humidified atmosphere at 37°C under 5% CO_2 conditions. To induce decidualization, cells were incubated with 0.5 mM 8-Br-cAMP (Sigma-Aldrich). Phase-contrast microscopy was used to verify the morphological changes associated with differentiation.

2.3 | Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from hESCs using RNAiso Plus (TaKaRa Bio Inc, Ohtsu, Shiga, Japan). cDNA was prepared for qPCR by reverse transcribing 1 μg of total RNA using RT Master Premix (Elpisbio, Daejeon, Korea). qPCR was performed using a SensiFAST SYBR No-ROX Kit (Bioline, London, UK) on a CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The primer sense and antisense sequences used were as follows: *IGFBP1* (5′-TTGGGACGCCATCAGTACCTA-3′ and 5′-TTGGCTAAACTCTCTACGACTCT-3′), *prolactin* (5′-TGACCCTTCGAGACTTG-3′ and 5′-CTTGCTCCTT GTCTTCGGG-3′), and *GAPDH* (5′-CATGAGAAGTATG ACAACAGCCT-3′and 5′-AGTCCTTCCACGATACCAAA GT-3′). The cycling conditions were as follows: initial denaturation at 95°C for 3 minutes, followed by 40 cycles consisting of denaturation at 95°C for 15 seconds and amplification at 61°C for 30 seconds.

2.4 | Transient transfection of siRNAs

PLCε, Epac1, and Rap1 siRNAs, as well as control siRNA (Dharmacon, Lafayette, CO, USA), were transiently transfected into hESCs for the loss-of-function study. Each siRNA (200 nM) was introduced into cells using RNAiMAX (Invitrogen, Carlsbad, CA, USA) transfection reagent, according to the manufacturer's protocol.

2.5 | Immunoprecipitation

Cells were lysed in immunoprecipitation buffer (10 mM Tris/HCl (pH 7.4) containing 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, protease inhibitors, and 1 mM PMSF). The lysate was incubated with PP2Ac subunit antibody (Cell Signaling) at 4°C for 24 hours, followed by capture on immobilized ProA/G immunoprecipitation beads (Amicogen, Jinju, Korea) at 4°C for 24 hours. Immunoprecipitates were washed with lysis buffer. Samples were then added to the 2× sample buffer before boiling for 5 minutes. Tyrosine-nitrated PP2Ac was detected in western blots using specific primary antibodies and HRP-conjugated secondary antibodies.

2.6 | Western blot analysis

Cells were lysed in RIPA buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1% Triton X-100, 1 mM PMSF, and 1 mM Na_3VO_4) containing a protease inhibitor cocktail (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Samples centrifuged at 12 000× for 15 minutes at 4°C. Equal amounts (40-50 μg) of protein samples were resolved by electrophoresis on 7%-10% SDS page gels and transferred onto poly(vinylidene difluoride) membranes (GE Healthcare Life Science, Piscataway, NJ, USA). The membranes were blocked with 5% non-fat dried milk for 1 hour, and then incubated overnight with primary antibodies (1 μg/mL), and then with HRP-conjugated secondary antibodies (1:2000; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 hour. Next, specific bands were detected by developing the blots using an electrochemiluminescence (ECL) solution (Thermo Fisher Scientific, Rockford, IL, USA).

2.7 | GEO data analysis

Microarray data with accession number GSE75425 were downloaded from the Gene Expression Omnibus (GEO, www.ncbi.nlm.nih.gov/geo), and data analysis and visualization of differentially expressed genes were conducted using R 3.5.3. The differentially expressed gene (DEG) sets were analyzed by hierarchical cluster analysis, which is performed using complete linkage and Euclidean distance as a measure of similarity. The DEGs were determined based on a twofold change in their expression.

2.8 | Nitric oxide imaging (DAF-FM staining)

hESCs were incubated in culture medium for 1 hour and then washed with PBS for 5 minutes. They were then stained with the NO fluorescent dye, DAF-FM (Invitrogen, Carlsbad, CA, USA) at 37°C for 30 minutes. After the experiments, hESCs were fixed in 4% paraformaldehyde in PBS for 2 minutes at room temperature. hESCs were mounted with VECTASHIELD (Vector Laboratories, Burlingame, CA, USA) containing 4, 6-diamidino-2-phenylindole (DAPI). DAF-FM was detected using a TCS SP5 confocal imaging system (Leica Microsystems, Wetzlar, Germany) at a magnification of 200×.

2.9 | Calcium imaging of cultured hESCs

We established calcium imaging experiments based on previous studies.[19] Prepared hESCs were loaded with 5 μM Fura-2 AM and 0.01% Pluronic F-127 (wt/vol; Life Technologies, Grand Island, NY, USA) for 40 minutes at 37°C. The extracellular solution (pH 7.4) contained 140 mM NaCl, 10 mM HEPES/NaOH, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM D-(1)-glucose, and 5 mM KCl. Cells were placed on an inverted microscope (Olympus IX70, Center Valley, PA, USA) and perfused continuously with the extracellular solution at a rate of 3 mL/min. hESCs were illuminated with a cooled pE-100 lamp (CoolLED Ltd., UK), and images were collected at 340 nm/380 nm wavelengths every 1 seconds using MetaFluor software (Universal Imaging Corp., Downingtown, PA, USA). Intracellular calcium concentrations were detected using microfluorometry with a camera (C11440 Hamamatsu, Shizuoka, Japan) coupled with the microscope.

2.10 | Rap1 activation assay

Rap1 activity in cells was measured using the Rap1 GTP pull-down and detection kit (Merck Millipore, Darmstadt, Hesse, Germany) according to the manufacturer's instructions. Briefly, hESCs were lysed and incubated with RalGDS-RBD agarose slurry at 4°C for 45 minutes. Pellets were washed with Rap1 Activation Lysis Buffer thrice and subjected to western blotting using anti-Rap1 antibodies.

2.11 | Statistical analysis

Statistical comparisons were made using one-way ANOVA with a post hoc Tukey's analysis using GraphPad PRISM 6 (GraphPad Software Incorporated, La Jolla, CA, USA). Values of $P < .05$ were considered to be statistically significant. Descriptive statistics were summarized as the mean ± SE (standard error) of at least three independent experiments.

3 | RESULTS

3.1 | iNOS induces PP2Ac tyrosine nitration during the decidualization of hESCs

PP2Ac protein is activated during cAMP-induced decidualization of hESCs.[1] To induce in vitro decidualization, hESCs were incubated in a 0.5 mM 8-Br-cAMP culture medium for 3 days. Consequently, the morphology of hESCs changed to a decidual-like shape (rounded and enlarged, Figure 1A), and the gene expression of *IGFBP1* and *prolactin* significantly increased (Figure 1B). These morphological and molecular changes were inhibited by pretreatment with the okadaic acid, known as PP2A inhibitor,[2,20] for 30 minutes (Figure 1A,B). PP2A is generally modulated by post-translational modifications including

nitration or methylation of its catalytic subunit.[5,10,14] We examined the change in whole gene expression patterns in hESCs during decidualization (GEO accession number GSE75425). As a result, mRNA expression of *iNOS*, a nitration mediator, is increased abundance by the decidualization of hESCs. In contrast, expression of methylation-related genes (*PME1, LCMT1, MAT2B, LCMT2,* and *MAT2A*) was attenuated or unconverted following the decidualization of hESCs (Figure 1C). Also, we validated the mRNA expression level of GEO database genes using qPCR, and consequently, *iNOS* expression was significantly increased by treatment with cAMP (Figure 1D). These data indicated that PP2A activation is increased by nitration

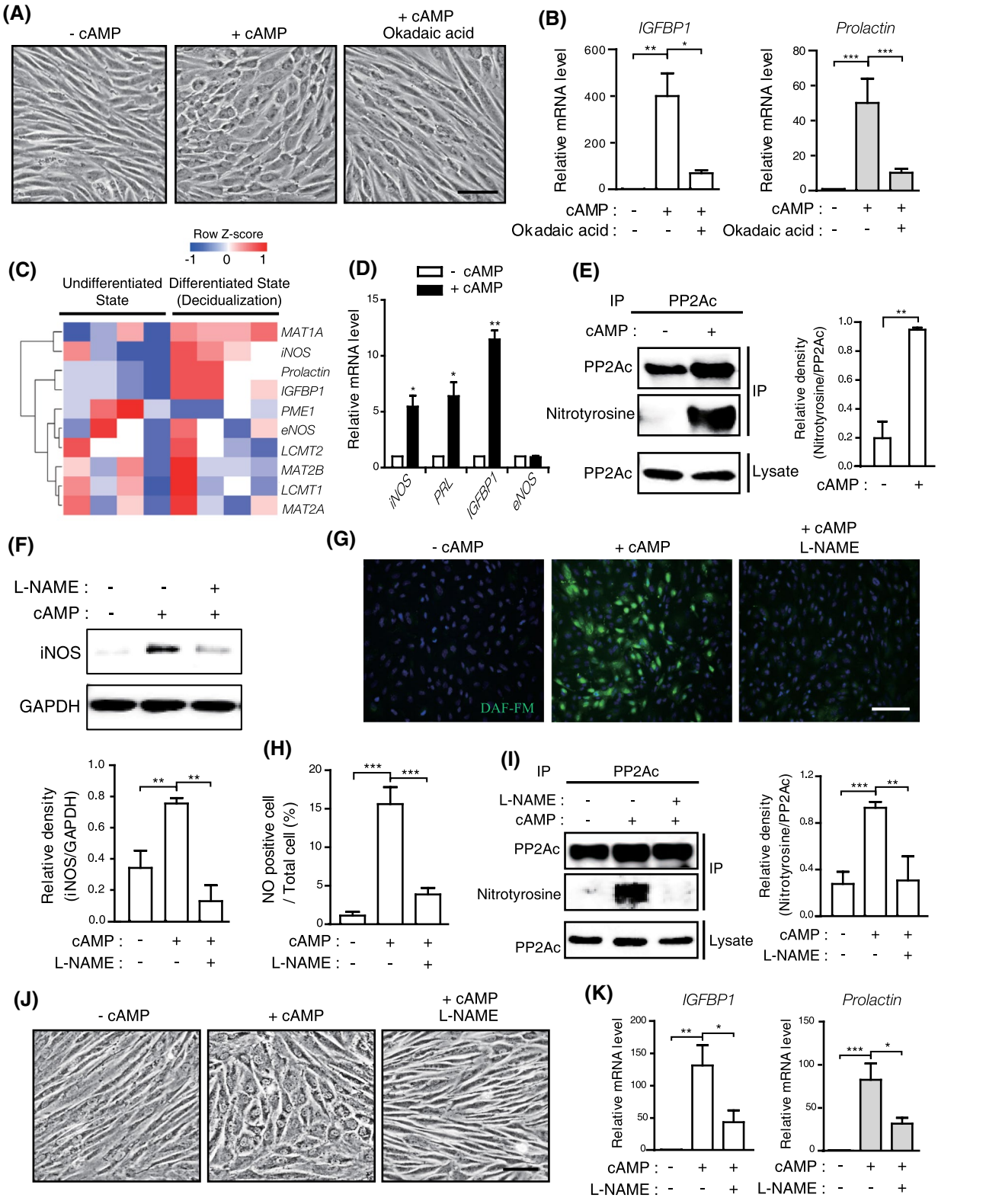

FIGURE 1 Tyrosine nitration level of PP2Ac is increased by iNOS during cAMP-induced decidualization in hESCs. A, Decidualization of hESCs was triggered by treatment with 0.5 mM 8-Br-cAMP for 3 days. hESCs were also pretreated with 30 nM okadaic acid (a PP2A inhibitor) for 30 minutes and differentiated in the presence of 0.5 mM 8-Br-cAMP for 3 days. Cells were photographed with a microscope (original magnification 200×). Scale bar, 40 μm. B, Total RNA was extracted from hESCs using TRIzol reagent, and relative mRNA expression levels of *IGFBP1* and *prolactin* were determined by qPCR. Values of mRNA were normalized to *GAPDH*. C, Heatmap of mRNA expression levels during decidualization in hESCs (GEO accession number GSE75425). The relative expression value for each gene is depicted by the Z-score. The red color indicates high expression and blue color represents the low expression of genes. D, mRNA expression level of *iNOS*, *IGFBP1*, *prolactin,* and *eNOS* were determined by qPCR. E, The hESCs were cultured in the presence of 0.5 mM 8-Br-cAMP for 24 hours Immunoprecipitation performed from hESCs with the PP2Ac antibody was analyzed by immunoblotting with anti-PP2Ac and anti-nitrotyrosine (NT) antibodies. Total cell lysates were also analyzed by immunoblotting with anti-PP2Ac. F, hESCs were pretreated with 7 mM L-NAME (a NOS inhibitor) for 30 minutes and then differentiated in the presence of 0.5 mM 8-Br-cAMP for 1 hour. The total cell lysates were analyzed by immunoblotting with anti-iNOS antibodies. G, hESCs were pre-incubated for 30 minutes with 7 mM L-NAME, followed by 50 minutes of incubation with 0.5 mM 8-Br-cAMP. Intracellular NO levels were then evaluated by staining with a NO-sensitive dye (DAF-FM). Intracellular NO levels were photographed using a microscope (original magnification 200×). Scale bar, 100 μm. H, NO-positive cells and total cells were counted within each field (12 mm cover glass) under 100 × magnification. Percentage ratio represents the ratio of the number of control NO-positive cells to that of total cells. (I) Cells were pretreated with 7 mM L-NAME for 30 minutes and then treated with 8-Br-cAMP (0.5 mM) for 24 hours Cell lysates and PP2Ac immunoprecipitates were analyzed by immunoblotting with anti-PP2Ac and anti-3NT antibodies. J, Cells were photographed with a microscope (original magnification 200×). Scale bar, 40 μm. K, mRNA expression levels of *IGFBP1* and *prolactin* were measured by qPCR. All data are presented as the mean ± SE (standard error) of at least five independent experiments. Statistical significances were assessed by one-way ANOVA with a post hoc Tukey's test at $\alpha = 0.05$. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$

during the decidualization of hESCs.[12,21,22] To confirm the tyrosine nitration of PP2A, hESCs were differentiated into decidualization by treatment with 0.5 mM cAMP for 24 hours, and cell lysates were subjected to immunoprecipitation using nitrotyrosine antibodies (Figure 1E). We demonstrated that PP2Ac is nitrated at tyrosine residues during the decidualization of hESCs. We also found that iNOS expression was increased by cAMP induction and reduced by L-NAME, known as NOS inhibitor[23] (Figure 1F). Among the various types of NOS, the expression of *eNOS* was not changed during the decidualization of hESCs (Figure 1D). We confirmed, by a NO-sensitive dye (DAF-FM), that intracellular NO levels increased considerably upon treatment with cAMP in Figure 1G. A large percentage of NO-positive cells showed decreased iNOS inhibition upon pretreatment with L-NAME (Figure 1H). As expected, PP2Ac nitration was completely abolished by iNOS inhibition during cAMP-induced decidualization (Figure 1I). Likewise, cAMP-induced decidual-like phenotype changes were completely blocked by L-NAME (Figure 1J), and gene expression of *IGFBP1* and *prolactin* was also significantly decreased by L-NAME (Figure 1K). Taken together, these findings demonstrate that iNOS directly increases the phosphatase activity of PP2Ac by promoting the tyrosine nitration of its catalytic subunit during the decidualization of hESCs.

3.2 | HDAC5 phosphorylation triggers iNOS expression during cAMP-induced decidualization

Compared with other post-translational modifications, acetylation of core histones is the most prominent effect of histone modification.[16,24] For this reason, HDACs are a key regulator of gene expression. HDACs are classified by two classes (classes I, II).[16] Trichostatin A (TSA) is the class II HDACs inhibitor (especially HDAC5[25]), and valproic acid (VPA) is the class I HDACs (HDAC1, 2 and 3) inhibitor. According to our preliminary result, treatment of TSA increased the level of *IGFBP1*, while the treatment of VPA did not regulate *IGFBP1* expression (data not shown). Therefore, we could conclude that HDAC5 has an important role in the decidualization of hESCs.[26,27] When HDAC5 exists in its dephosphorylated form, it is localized to the nucleus, shuttling back to the cytoplasm upon phosphorylation. Expression of HDAC5 target genes is, therefore, turned on by the phosphorylation of HDAC.[16] We found that Ser^{498} phosphorylation of HDAC5 was significantly increased by treatment with 0.5 mM cAMP, as well as by treatment with 200 nM TSA without cAMP, indicating that TSA mimicked the effects of cAMP in Figure 2A. The morphology of hESCs also changed into a decidual-like shape with 0.5 mM cAMP, and these results were reproduced with 200 nM TSA treatment, as shown in Figure 2B. Moreover, decidualization marker genes (*IGFBP1* and *prolactin*) were significantly expanded by treatment with TSA only, without cAMP (Figure 2C). We predicted that iNOS-dependent PP2Ac nitration is induced by HDAC5 phosphorylation during the decidualization of hESCs, because iNOS is the representative target gene of HADC5.[17] To confirm this prediction, we investigated the protein expression of iNOS by western blotting. When cells were decidualized by treatment with cAMP or TSA, protein expression of iNOS was increased (Figure 2D), and intracellular NO production and proportion of NO-positive hESCs were significantly increased by cAMP or TSA treatment (Figure 2E,F). Furthermore, PP2Ac tyrosine nitration was drastically increased by treatment with cAMP or TSA (Figure 2G). Collectively, these results indicate that cAMP stimulation increases the phosphorylation of HDAC5,

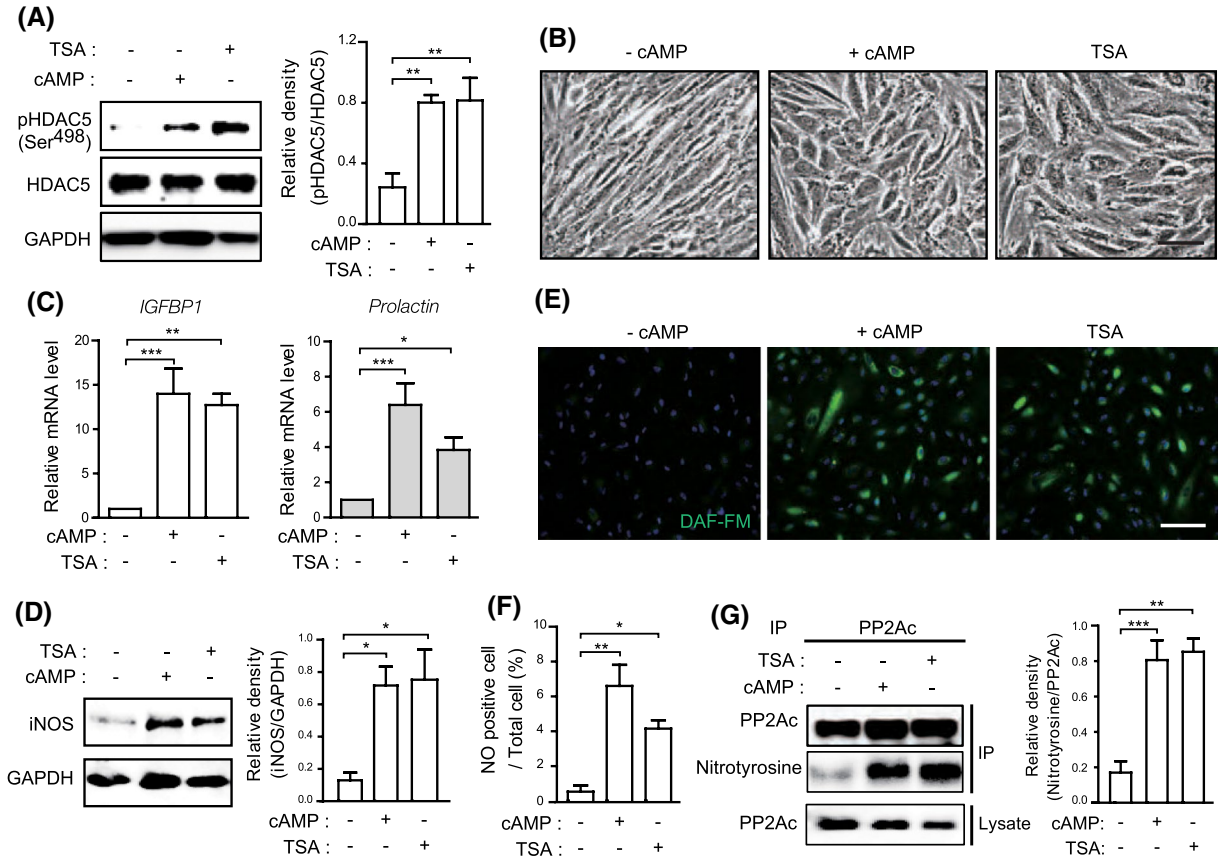

FIGURE 2 iNOS-mediated PP2Ac nitration is stimulated by the phosphorylation of HDAC5 during decidualization in hESCs. A, hESCs were treated with 0.5 mM 8-Br-cAMP or 200 nM TSA for 1 hour. Cell lysates were analyzed by immunoblotting with anti-phospho HDAC5 (S498) antibodies to measure HDAC5 phosphorylation. B, hESCs were incubated for 3 days with 0.5 mM 8-Br-cAMP or 200 nM TSA to induce decidualization, and then, the cells were photographed (original magnification 200×). Scale bar, 40 μm. C, mRNA levels of *IGFBP1* and *prolactin* were determined by qPCR. D, hESCs were differentiated with 8-Br-cAMP or TSA for 1 hour, and then, cell lysates were analyzed by immunoblotting with anti-iNOS antibodies. GAPDH was used as the loading control. E, Intracellular NO release in response to 8-Br-cAMP or TSA for 50 minutes was determined by DAF-FM fluorescence staining (original magnification 200×). Scale bar, 100 μm. F, NO-positive cells and total cells were counted within each field (12 mm cover glass) under 100× magnification. Percentage ratio represents the ratio of the number of control NO-positive cells to that of total cells. G, Cells were treated with 8-Br-cAMP or TSA for 24 hours, and then, cell lysates and PP2Ac immunoprecipitates were analyzed by immunoblotting. All data are presented as the mean ± SE (standard error) of at least five independent experiments. Statistical significances were assessed by one-way ANOVA with a post hoc Tukey's test at $\alpha = 0.05$. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$

leading to the increased gene expression of iNOS, which induces PP2A tyrosine nitration on the C subunit during the decidualization of hESCs.

3.3 | CaMKII increases PP2Ac nitration through HDAC5 phosphorylation and iNOS expression during decidualization

CaMKII is the downstream target protein of the Ca^{2+} and calmodulin complex, which regulates many cellular functions, including the immune response and inflammatory process.[28] CaMKII preserves its ability to undergo Ca^{2+}- and calmodulin-dependent auto-phosphorylation at Thr^{286}.[29] CaMKII also promotes the phosphorylation and nuclear export of HDAC5 through HDAC4 regulation.[30,31] Therefore, we hypothesized that CaMKII induces PP2Ac nitration by inducing the phosphorylation of HDAC5 during the decidualization of hESCs. To identify the effects of CaMKII, we treated hESCs with the BAPTA-AM, known as the inhibitor of CaMKII.[32] Cells were pretreated with 5 μM BAPTA-AM for 30 minutes and then differentiated with cAMP for 3 days. The cAMP-induced decidual-like morphology was clearly abolished (Figure 3A), and mRNA expression of *IGFBP1* and *prolactin* was significantly diminished by BAPTA-AM (Figure 3B). To see whether CaMKII is responsible for HDAC5 phosphorylation and iNOS expression, we performed immunoblotting. A large percentage of CaMKII protein existed in a Thr^{286}-phosphorylated form after cAMP treatment, and this phosphorylation was inhibited by pretreatment with BAPTA-AM. Moreover, iNOS expression and HDAC5 phosphorylation were reduced by CaMKII

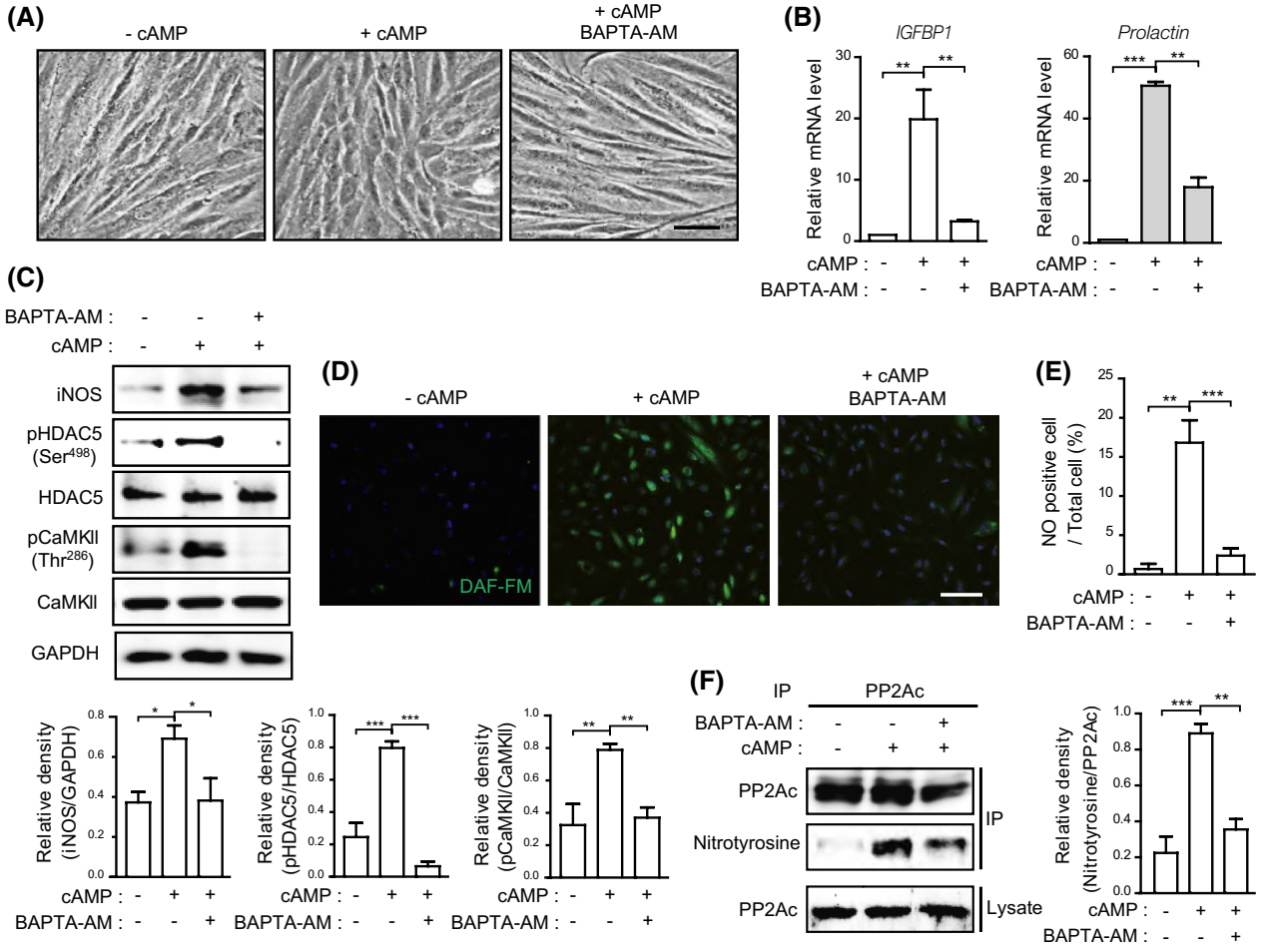

FIGURE 3 CaMKII has an impact on PP2Ac nitration through the phosphorylation of HDAC5 during decidualization. A, Decidualization of hESCs was stimulated by treatment with 0.5 mM 8-Br-cAMP for 3 days. hESCs were pretreated with 5 μM BAPTA-AM (a CaMKII inhibitor) for 30 minutes and differentiated in the presence of 0.5 mM 8-Br-cAMP for 3 days. Cells were photographed under a microscope (original magnification 200×). Scale bar, 40 μm. B, mRNA gene expression levels of *IGFBP1* and *prolactin* were determined using qPCR. Values were normalized to *GAPDH*. C and F, hESCs were pretreated with 5 μM BAPTA-AM for 30 minutes and then differentiated in the presence of 0.5 mM 8-Br-cAMP for 50 minutes. C, Using whole hESC lysates, we performed immunoblotting analyses with anti-iNOS, anti-phospho-HDAC5 (Ser^{498}), anti-HDAC5, anti-CaMKII (Thr^{286}), and anti-CaMKII antibodies. GAPDH was used as the loading control. D, NO production was measured by DAF-FM fluorescence in hESCs (original magnification 200×). Scale bar, 100 μm. E, NO-positive cells and total cells were counted within each field (12 mm cover glass) under 100× magnification. Percentage ratio represents the ratio of the number of control NO-positive cells to that of total cells. F, Cell lysates and PP2Ac immunoprecipitates were analyzed with immunoblotting. All data are presented as the mean ± SE (standard error) of at least five independent experiments. Statistical significances were assessed by one-way ANOVA with a post hoc Tukey's test at $\alpha = 0.05$. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$

inhibition (Figure 3C). Furthermore, to confirm the relation between HDAC4 as the downstream molecule of CaMKII, we checked HDAC4 phosphorylation during decidualization in hESCs. However, the phosphorylation of HDAC4 was not regulated by cAMP or cAMP/BAPTA-AM induction (data not shown), indicating that HDAC5 phosphorylation is HDAC4 independent. cAMP-induced increase in intracellular NO production and NO-positive cell numbers were significantly decreased by BAPTA-AM treatment (Figure 3D,E). As is typical, cAMP-induced tyrosine nitration of PP2Ac was substantially decreased by CaMKII inhibition (Figure 3F). These results suggest that CaMKII has an important role in decidualization through controlling PP2Ac tyrosine nitration by HDAC5 phosphorylation and iNOS expression through the HDAC4-independent pathway.

3.4 | PLC induces PP2Ac tyrosine nitration during the decidualization of hESCs

Phospholipase C (PLC) has key roles in various cellular signaling pathways, increasing intracellular Ca^{2+} concentration to promote biological activity. One of its major functions is Ca^{2+} mobilization through $Ins(1,4,5)P_3$ and DAG

production.[33] Activation of PLC is induced by many extracellular stimuli, such as hormones, neurotransmitters, antigens, and cAMP. Among these, cAMP increases intracellular Ca^{2+} through PLC/IP_3 signaling and CaMKII phosphorylation.[34] After 5 minutes of 0.5 mM cAMP treatment to induce the decidualization of hESCs, the intracellular Ca^{2+} concentration steadily increased compared to the untreated cells. However, this increase was completely abolished by pretreatment with 7 μM U73122, known as a PLC inhibitor[33-36] (Figure 4A). Next, we investigated whether PLC inhibition affected decidualization phenotypes. cAMP-induced decidual-like morphologic change in hESCs was completely ablated by PLC inhibition (Figure 4B). Likewise, gene expression of *IGFBP1* and *prolactin* was markedly decreased by pretreatment with U73122 (Figure 4C). Following these results, we wanted to explore the mechanism of PLC-mediated increase in intracellular calcium levels during the decidualization of hESCs. To clarify the role of PLC in PP2Ac nitration through the CaMKII-HDAC5-iNOS signaling pathway, we examined the phosphorylation of CaMKII and HDAC5, as well as iNOS expression. cAMP-induced CaMKII phosphorylation at Thr^{286} and HDAC5 phosphorylation at Ser^{498} were reversed by U73122. Furthermore, the protein expression of iNOS was also completely reduced by PLC inhibition (Figure 4D). Next, cells were pretreated with U73122 in the presence of cAMP, and the extracts were subjected to immunoprecipitation for the analysis of PP2Ac tyrosine nitration. The PP2Ac tyrosine nitration level increased following cAMP treatment during hESC decidualization; this was drastically suppressed by U73122 (Figure 4E). NO production and NO-positive hESCs were observed after cAMP treatment for 50 minutes and were completely suppressed by PLC inhibition (Figure 4F,G).

3.5 | Depletion of PLCε blocks cAMP-induced CaMKII-HDAC5-iNOS signaling pathway

To confirm whether PLCε was the PLC isoform responsible for regulating PP2Ac nitration by triggering the CaMKII-HDAC5-iNOS signaling pathway, inhibition of PLCε was performed using PLCε siRNA. cAMP-induced intracellular calcium level increment was suppressed by the transfection of hESCs with PLCε siRNA (Figure 5A). Furthermore, the cAMP-induced decidual-like morphology change and increase in decidualization marker gene expression were completely abolished by PLCε depletion (Figure 5B,C). CaMKII and HDAC5, downstream molecules of PLC, were dephosphorylated to a high degree following PLCε siRNA treatment, and iNOS expression was completely blocked by PLCε depletion (Figure 5D). Moreover, intracellular NO production and the number of NO-positive cells were drastically reduced by PLCε siRNA during the decidualization of hESCs (Figure 5E,F). These results indicate that PLCε, as an upstream molecule of the CaMKII-HDAC5-iNOS cascade, promotes PP2Ac tyrosine nitration during cAMP-induced decidualization of hESCs.

3.6 | Role of Epac1 in PP2Ac nitration during the decidualization of hESCs

According to several previous studies, the Epac1-Rap1 signaling pathway plays a key role in cAMP-mediated decidualization of hESCs.[37,38] Moreover, cAMP stimulates PP2A activation in an Epac-Rap1-dependent manner.[39] We hypothesized that Epac1 has a crucial function in PP2Ac tyrosine nitration during the decidualization of hESCs. To verify this, we investigated the change in intracellular calcium levels by treating hESCs with Epac1 siRNA. Calcium response was gradually increased by treatment with 0.5 mM cAMP, and this was completely repressed by the transfection of Epac1 siRNA (Figure 6A). Next, cAMP-induced decidual-like morphology and expression of decidualization marker genes were found to be substantially abolished by Epac1 depletion (Figure 6B,C). Likewise, Epac1 knockdown inhibited the phosphorylation of CaMKII and HDAC5, as well as iNOS protein expression (Figure 6D). Further, cAMP-stimulated NO production and increase in the number of NO-positive hESCs were fully inhibited by the transfection of hESCs with Epac1 siRNA (Figure 6E,F). These results indicate that Epac1 is a crucial mediator in inducing the decidualization of hESCs through PP2Ac nitration to induce the PLCε-CaMKII-HDAC5-iNOS signaling pathway.

3.7 | Rap1, the target small G protein of Epac1, is activated during decidualization in hESCs

PLCε has a Ras-binding domain (RA domain), and its activity is associated with Ras and the small Ras-like GTPase Rap1.[33,40] Because Epac1 is the guanine nucleotide exchange factor (GEF) of Rap1,[41,42] we evaluated Rap1 activity in lysates of cAMP-induced hESCs. Notably, the active form of Rap1, Rap1-GTP, was increased compared with that in undifferentiated hESCs (Figure 7A). Therefore, we confirmed that as the specific GEF, Epac1 activates the target small G protein, Rap1, during cAMP-induced decidualization of hESCs. Next, to verify whether Rap1 is involved in the decidualization, we suppressed the Rap1 gene using siRNA. The cAMP-induced intracellular calcium increase was suppressed by the transfection of Rap1 siRNA (Figure 7B). Rap1 siRNA also completely blocked cAMP-induced changes in morphology and marker gene expression during decidualization (Figure 7C,D). In addition, we assessed whether Rap1 controls the cAMP-induced

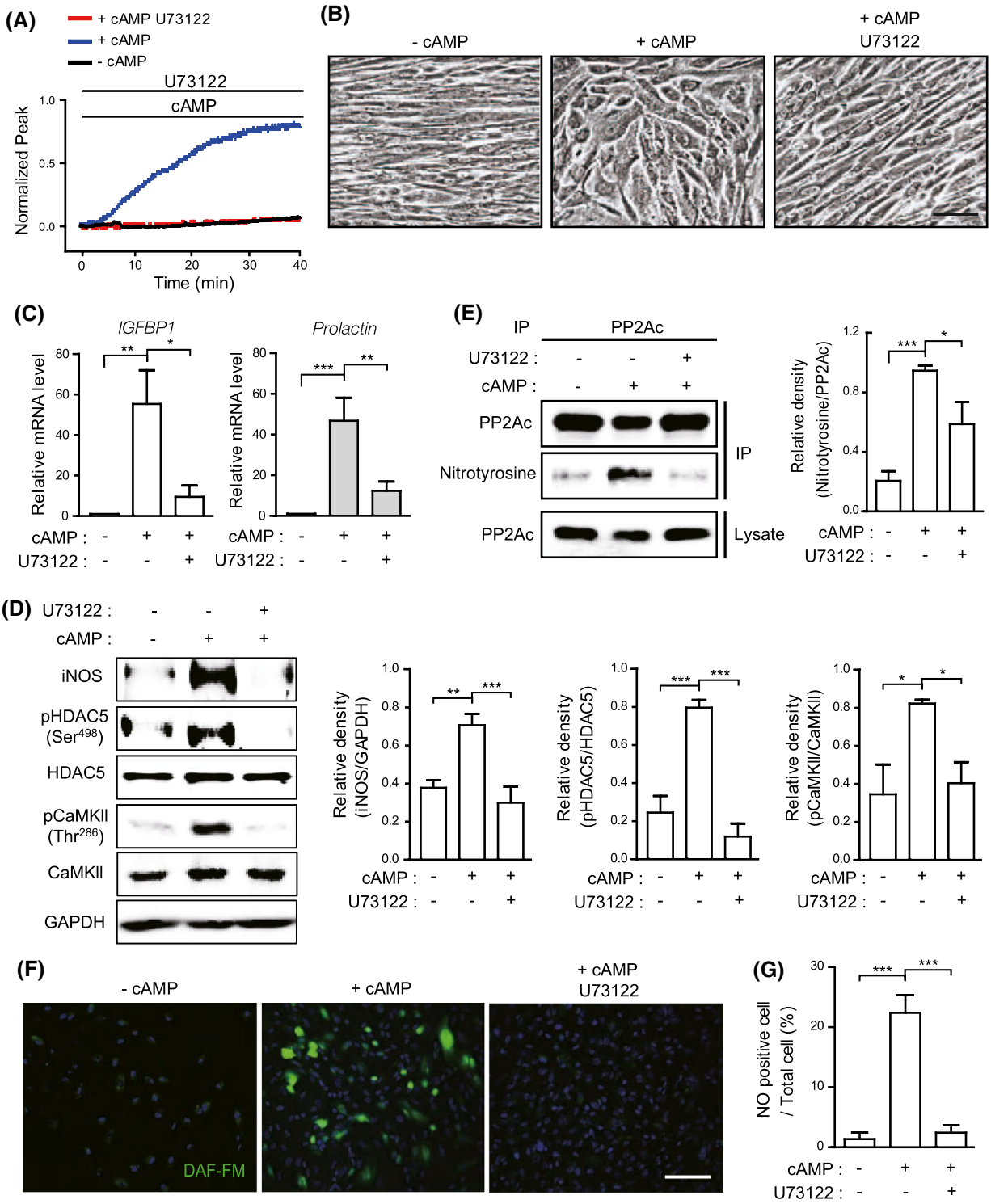

FIGURE 4 PLC increases the decidualization of hESCs through CaMKII activation. hESCs were preincubated with 7 μM U73122 for 30 minutes and differentiated in the presence of 0.5 mM 8-Br-cAMP for (A) 40 minutes, (B, C) 3 days or (D) 1 hour. A, Changes in intracellular free calcium were calculated based on the variation of the 340 nm/380 nm fluorescence ratio in Fura-2-labeled cells. B and C, Cells were photographed under a microscope (original magnification 200×, scale bar, 40 μm), and mRNA gene expression levels of *IGFBP1* and *prolactin* were determined using qPCR. D, Proteins were analyzed by western blotting with anti-iNOS, anti-phospho-HDAC5 (Ser^{498}), anti-HDAC5, anti-phospho-CaMKII (Thr^{286}), anti-CaMKII, and anti-GAPDH antibodies. E, The amount of nitrated tyrosine in PP2Ac was measured by immunoprecipitation with immunoblotting. F, NO production was measured by DAF-FM fluorescence in hESCs (scale bar, 100 μm). G, NO-positive cells and total cells were counted within each field (12 mm cover glass) under 100× magnification. Percentage ratio represents the ratio of the number of control NO-positive cells to that of total cells. All data are presented as the mean ± SE (standard error) of at least five independent experiments. Statistical significances were assessed by one-way ANOVA with a post hoc Tukey's test at $\alpha = 0.05$. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$

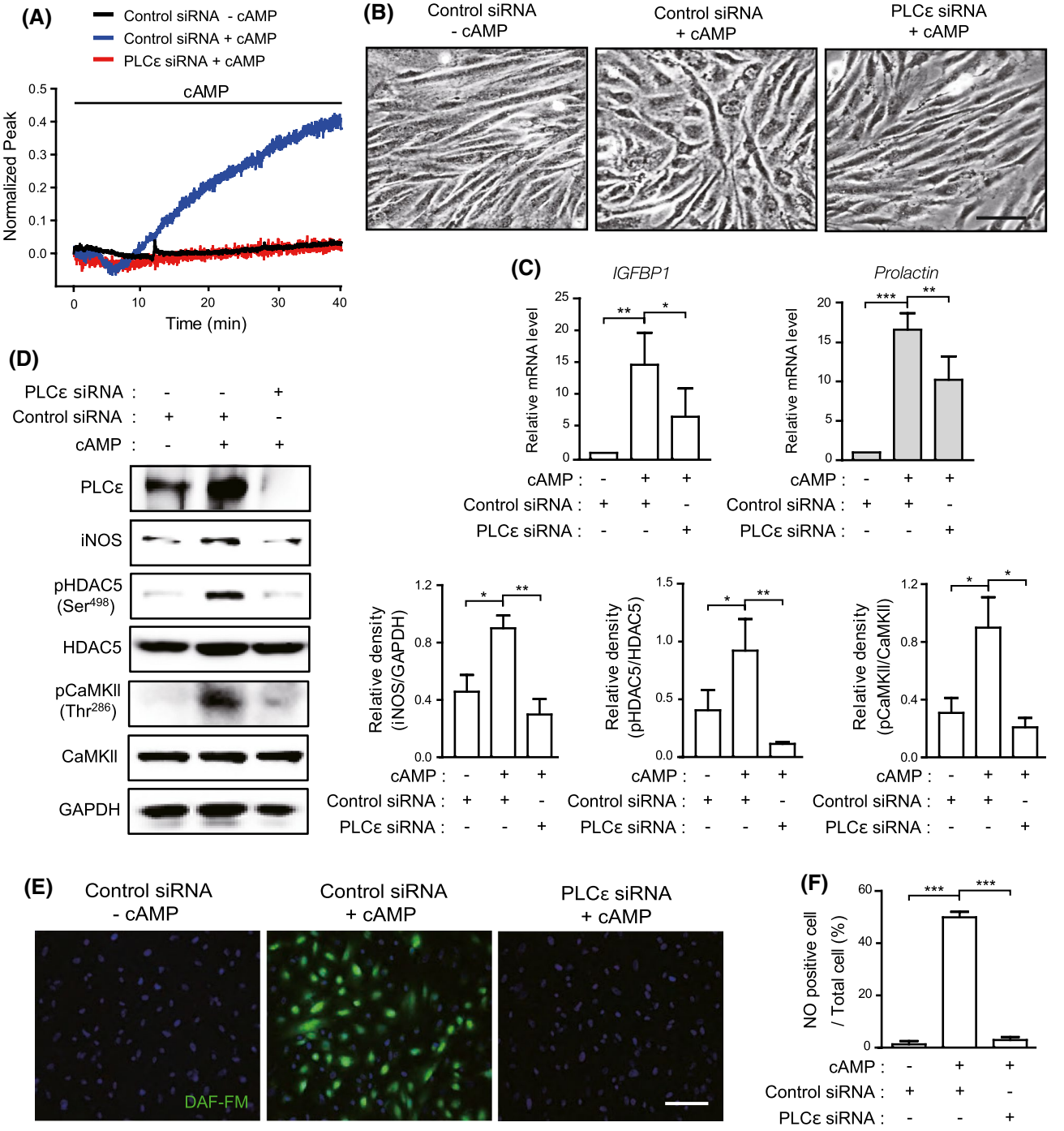

FIGURE 5 Downregulation of PLCε blocks cAMP-induced CaMKII-HDAC5-iNOS signaling pathway. A, hESCs were transfected with 200 nM control siRNA or 200 nM PLCε siRNA for 48 hours and then differentiated in the presence of 0.5 mM 8-Br-cAMP for 40 minutes. Intracellular calcium level was detected by 340 nm/380 nm fluorescence ratio in Fura-2-labeled cells. B, Cells were transfected with PLCε siRNA or control siRNA for 48 hours and then differentiated for 3 days with 8-Br-cAMP. Cells were photographed under a microscope (original magnification 200×). Scale bar, 40 μm. C, *IGFBP1* and *prolactin* mRNA expression were determined by qPCR. D, After 48 hours transfection with control siRNA or PLCε siRNA, hESCs were treated with 8-Br-cAMP for 1 hour to induce decidualization. Proteins were analyzed by western blotting with anti-iNOS, anti-phospho-HDAC5 (Ser^{498}), anti-HDAC5, anti-phospho-CaMKII (Thr^{286}), anti-CaMKII, and anti-GAPDH antibodies. E, NO production was analyzed through DAF-FM staining (original magnification 200×). Scale bar, 100 μm. F, NO-positive cells and total cells were counted within each field (12 mm cover glass) under 100 × magnification. Percentage ratio represents the ratio of the number of control NO-positive cells to that of total cells. All data are presented as the mean ± SE (standard error) of at least five independent experiments. Statistical significances were assessed by one-way ANOVA with a post hoc Tukey's test at $\alpha = 0.05$. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$

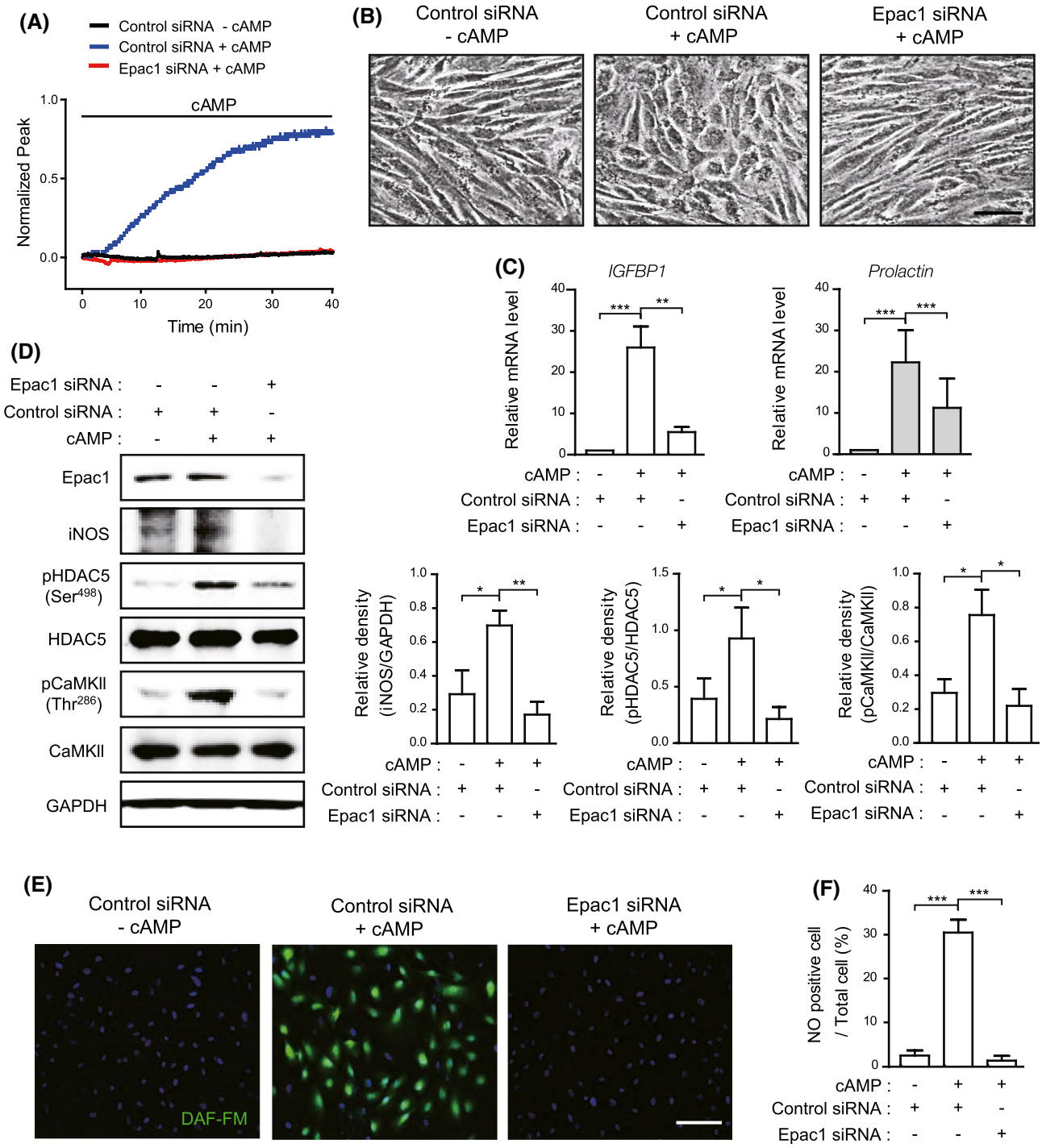

FIGURE 6 Epac1 promotes downstream signal molecules of PLCε during decidualization. A, hESCs were transfected with 200 nM control siRNA or 200 nM Epac1 siRNA for 48 hours and then differentiated in the presence of 0.5 mM 8-Br-cAMP for 40 minutes. Changes in intracellular free calcium were calculated based on the variation of the 340 nm/380 nm fluorescence ratio in Fura-2-labeled cells. B, Transfected hESCs were differentiated in the presence of 0.5 mM 8-Br-cAMP for 3 days, and then cells were photographed under a microscope (original magnification 200×). Scale bar, 40 μm. C, mRNA gene expression level of *IGFBP1* and *prolactin* were determined by qPCR. D, Proteins were analyzed by western blotting with anti-Epac1, anti-iNOS, anti-phospho-HDAC5 (Ser^{498}), anti-HDAC5, anti-phospho-CaMKII (Thr^{286}), anti-CaMKII, and anti-GAPDH antibodies. E, NO-positive cells were measured by DAF-FM staining (original magnification 200×). Scale bar, 100 μm. F, NO-positive cells and total cells were counted within each field (12 mm cover glass) under 100× magnification. Percentage ratio represents the ratio of the number of control NO-positive cells to that of total cells. All data are presented as the mean ± SE (standard error) of at least five independent experiments. Statistical significances were assessed by one-way ANOVA with a post hoc Tukey's test at $\alpha = 0.05$. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$

PP2Ac nitration signaling pathway. Rap1 knockdown in the presence of cAMP considerably repressed the phosphorylation of CaMKII and HDAC5, as well as protein expression of iNOS (Figure 7E). In addition, intracellular NO production and the number of NO-positive cells were markedly inhibited by Rap1 depletion (Figure 7F,G). Taken together, these findings indicate that Epac1 activates Rap1, and both are crucial upstream molecules of the PLCε-CaMKII-HDAC5-iNOS-PP2Ac nitration signaling cascade for the decidualization of hESCs (Figure 8A).

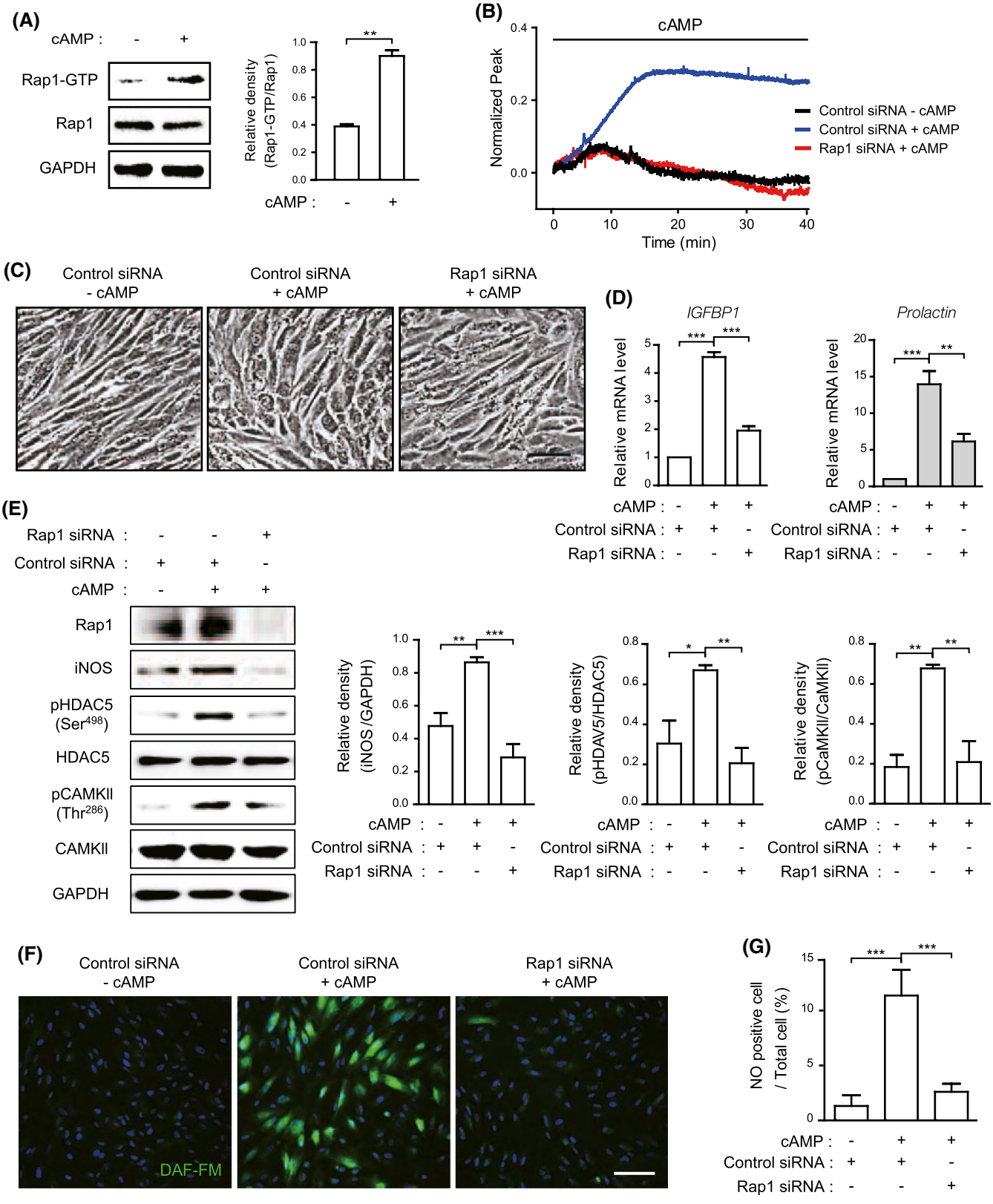

FIGURE 7 Small G protein Rap1 is activated by cAMP during decidualization in hESCs. A, hESCs were treated with 0.5 mM 8-Br-cAMP for 50 minutes, followed by cell lysis and pull-down of Rap-GTP using RalGDS-Sepharose. Cells were induced to decidualize by treatment with 0.5 mM 8-Br-cAMP for 1 hour. Quantification of Rap1-GTP from hESCs was analyzed by anti-GST pulldown assay and probing with anti-Rap1 antibodies. hESCs were transfected with 200 nM non-targeting siRNA or 200 nM Rap1 siRNA for 48 hours and then differentiated in the presence of 8-Br-cAMP for 40 minutes (B) or 3 days (C and D). B, Changes in intracellular free calcium are calculated based on the variation of the 340 nm/380 nm fluorescence ratio in Fura-2-labeled cells. C, The cells were photographed under a microscope (original magnification 200×). Scale bar, 40 μm. D, *IGFBP1* and *prolactin* mRNA expression levels were analyzed by qPCR. E, Cell lysates were analyzed by western blotting with anti-Rap1, anti-iNOS, anti-phospho-HDAC5 (Ser^{498}), anti-HDAC5, anti-phospho-CaMKII (Thr^{286}), anti-CaMKII, and anti-GAPDH antibodies. F, NO production was detected by DAF-FM staining (scale bar, 100 μm). G, NO-positive cells and total cells were counted within each field (12 mm cover glass) under 100× magnification. Percentage ratio represents the ratio of the number of control NO-positive cells to that of total cells. All data are presented as the mean ± SE (standard error) of at least five independent experiments. Statistical significances were assessed by one-way ANOVA with a post hoc Tukey's test at $\alpha = 0.05$. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$

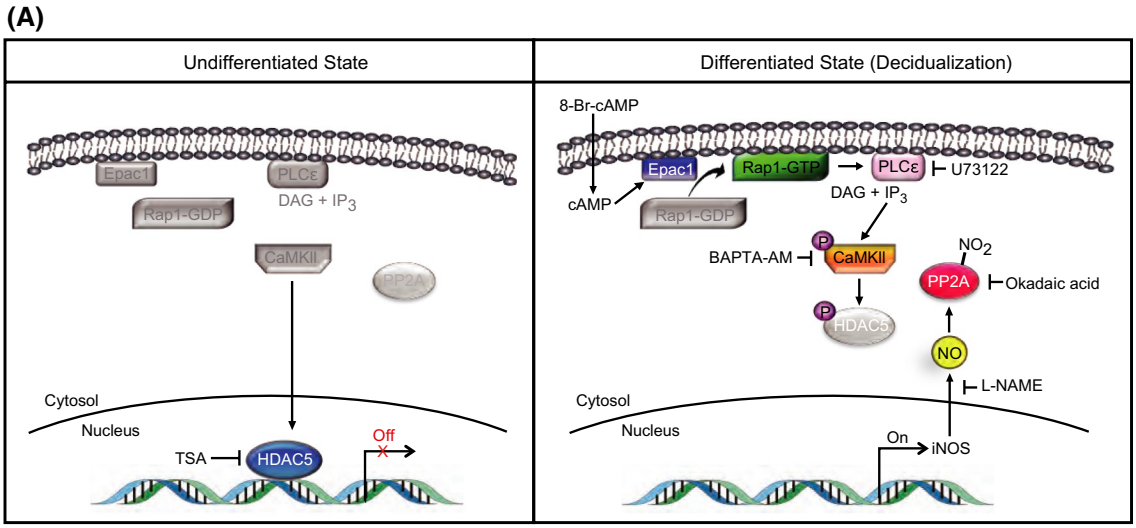

FIGURE 8 Scheme for the signaling pathway of PP2Ac nitration in the decidualization of hESCs. A, In hESCs, cAMP activates Epac1 (GEF of Rap1), and then, Epac1 stimulates the conversion of Rap1-GDP (inactive form) to Rap1-GTP (active form). Active Rap1 activates PLCε, leading to increased intracellular calcium concentration. CaMKII is activated through phosphorylation by IP_3-related intracellular calcium release. The function of HDAC5 as a transcriptional repressor is inhibited by CaMKII phosphorylation. NO is synthesized by increasing iNOS protein expression in cAMP-induced decidualization of hESCs. Consequently, PP2Ac is activated by tyrosine nitration through increased intracellular NO production. Therefore, we propose that the nitration of PP2Ac is increased by the Epac1-Rap1-PLCε-CaMKII-HDAC5-iNOS signaling pathway during cAMP-induced decidualization of hESCs

4 | DISCUSSION

Pregnancy is an intricate process that comprises discrete events such as decidualization, implantation, and placentation. Each event is essential for pregnancy, and the process is primarily coordinated by ovarian estrogen and progesterone. In particular, stromal cells surrounding the implanted blastocyst undergo differentiation into specialized cell types and become rounded and enlarged, in a process called decidualization.[43] According to many previous studies, decidualization is induced by intracellular cAMP as well as estrogen and progesterone.[1,37,38] Phospholipase D1 (PLD1) catalyzes the hydrolysis of phospholipids at the phosphodiester bond. Phosphatidylcholine is hydrolyzed to choline and PA by PLD1 activation; thus, PA is an enzymatic product of PLD1. In our previous research, we certified that both activity and expression of PLD1 increase during cAMP-induced decidualization.[44] Moreover, we reported the mechanism of PA-induced decidualization in hESCs. In this process, PP2Ac loss-of-function decreases the expression of *IGFBP1* and *prolactin* during PA-mediated decidualization. Thus, we previously suggested that PP2Ac plays a key role in decidualization.[1] By analyzing gene expression microarray data, we revealed that the expression patterns of nitration-related genes were increased by decidualization stimuli (Figure 1). Moreover, we found that tyrosine nitration of PP2Ac by iNOS plays a major part in the decidualization of hESCs (Figure 1). Through mass spectrometry assay, we evaluated several nitration residues on PP2Ac: Tyr^{127}, Tyr^{130}, Tyr^{265}, Tyr^{267}, and Tyr^{284}. Of these, Tyr^{284} and Tyr^{127} were the crucial nitration sites of PP2A that mainly control the activity of PP2Ac.[5,14] For these reasons, we considered the possibility that iNOS might be recruited to nitrate at Tyr^{284} and/or Tyr^{127} of PP2Ac during the decidualization of hESCs. According to previous

reports, not only iNOS, but also eNOS is expressed in the human uterus.[15,22] Protein expression of iNOS increased during decidualization (Figure 1); moreover, eNOS levels are not altered by decidualization stimuli (data not shown). We revealed that PP2Ac nitration is induced by iNOS during decidualization in hESCs. During decidualization, endogenous production of NO by iNOS has the crucial function of limiting platelet aggregation at the interface between maternal and fetal circulations in pregnancy. Further, NO regulates placental blood flow and suppresses contractions of the myometrium during pregnancy.[12,21,22] In this context, we suggest that PP2Ac nitration is intimately associated with implantation and maintaining pregnancy.

Many types of cells exhibit oscillations of intracellular Ca^{2+} concentration in response to diverse stimuli. Calcium, as an intracellular second messenger, binds with calmodulin to control numerous cellular processes.[28,45] We demonstrated that intracellular Ca^{2+} levels are crucial for cAMP-dependent decidualization in hESCs (Figures 3-6). The cAMP-Epac1-Rap1-PLCε-CaMKII signaling pathway sustains cAMP-induced intracellular Ca^{2+} increase. Because of this, we predicted that the production of inositol 1,4,5-trisphosphate (IP_3) by Epac1-Rap1-PLCε cascade releases Ca^{2+} from internal Ca^{2+} stores during the decidualization of hESCs.[46,47] Moreover, Ca^{2+} influx, entering through transient receptor potential (TRP) cation channels, including TRP canonical (TRPC), was increased by estrogen- and progesterone-induced decidualization in hESCs.[48] Thus, TRP proteins, members of a nonvoltage-gated Ca^{2+} entry channel superfamily, are correlated with decidualization. In this context, activated PLC hydrolyzes phosphatidylinositol 4, 5-bisphosphate (PI(4,5)P_2) to produce diacylglycerol (DAG) and IP_3. DAG and IP_3 are directly involved in TRPC channel activation.[49] Taken together, we suggest that DAG, the product of PLC, induces TRPC-mediated Ca^{2+} entry. Further, IP_3, the product of PLC, increases intracellular Ca^{2+} release via binding at the IP_3 receptor on the endoplasmic reticulum. Thus, we anticipate that the TRPC channel and IP_3 receptor increase intracellular Ca^{2+} level with the cAMP-induced Epac1-Rap1-PLCε pathway during decidualization.

CaMKII interacts with a specific docking site on HDAC4, inducing the phosphorylation of HDAC4. HDAC5 does not bind directly to CaMKII, but because HDAC4 and HDAC5 form hetero-oligomers, HDAC5 is transphosphorylated by CaMKII.[27] However, we found that HDAC5 is phosphorylated by cAMP-Epac1-Rap1-PLCε-CaMKII during the decidualization of hESCs through HDAC4-independent pathway. The cargo protein 14-3-3 binding to CaMKII-mediated phosphorylated HDAC5 is sufficient to induce the translocation of the HDAC5, which consequently activates transcription factors.[50] Also cAMP increases 14-3-3 mRNA and protein levels in hESCs.[51] Therefore, we considered the possibility that the target of HDAC5, iNOS expression, is induced by 14-3-3-mediated HDAC5 translocation from the nucleus to the cytoplasm during decidualization.

Infertility is a reproductive disorder with worldwide impacts.[52] To overcome infertility, assisted reproductive technologies (ARTs) are widely performed for couples with subfertility. A variety of ARTs exist, such as in vitro fertilization (IVF), artificial insemination (AI), and intracytoplasmic sperm injection (ICSI).[52] However, ART merely induces successful fertilization, and cannot increase the efficiency of implantation and pregnancy maintenance. Therefore, women who have conception failure cannot overcome infertility despite the application of ART. If a new drug were to be developed for enhancing the decidualization of hESCs for efficient implantation of fertilized eggs, the efficiency of ART would be significantly increased. Moreover, aberrant decidualization provokes pathologies such as endometriosis, deciduosis, and endometrial cancer.[53-55] Endometriosis is a common chronic gynecological disorder associated with pelvic pain, dyspareunia, menstrual pain, and infertility.[56] It occurs when abnormal decidualized endometriotic stromal cells flow to the outside of the endometrial cavity during menstrual periods.[1,57] Enhancing decidualization is highly pertinent to the treatment of endometriosis. Therefore, we focused on the PP2Ac nitration mechanism, which induces the decidualization of hESCs, as a potential target of endometriosis therapy. PP2A is associated with the regulation of the cell cycle, and with apoptosis.[3,58] Through efficient inhibition of its activity or loss of its functional subunits, PP2A is now widely designated as a tumor suppressor.[3] In the same context, we anticipate that the mechanism underlying PP2Ac nitration will be correlated with ovarian cancer and/or endometrial cancer. Despite the clinical importance of decidualization, the concrete mechanisms of decidualization are not fully defined in vivo due to its complexity. Instead, in vitro systems of decidualization have been investigated to overcome limitations such as the difficulty of obtaining primary human cells.[59] Therefore, in vitro studies of decidualization using human primary cells is necessary for the improvement of implantation and pregnancy function, as well as treatment of related diseases.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

S. Y. Lee, Y. Y. Lee, S. Y. Park, and J. S. Han designed the study and edited the manuscript; S. Y. Lee performed the research and wrote the draft of the manuscript; J. S. Choi supplied the hESCs for our experiments; K. S. Kim and D. S. Min commented on the experiments. All authors read and approved the final manuscript.

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