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Research Paper

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Anandamide modulates NOTCH1 signalling and inflammatory responses during human endometrial stromal cell decidualization

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In brief

Successful implantation requires coordinated decidual differentiation of endometrial stromal cells. This study shows that anandamide treatment of decidualizing cells is associated with reduced neurogenic locus notch homolog protein 1 (NOTCH1) activation and selective alterations in decidual, inflammatory, and structural programs.

Abstract

Decidualization of endometrial stromal cells is essential for human embryo implantation and pregnancy maintenance. The endocannabinoid system (ECS), particularly anandamide (AEA), has been implicated in endometrial receptivity, but the mechanisms through which AEA influences decidualization have been only partially elucidated. This study investigates the effects of AEA during decidualization of immortalized human endometrial stromal cells (HESCs), focusing on the neurogenic locus notch homolog protein 1 (NOTCH1) pathway, inflammatory mediators, cell-cycle arrest/senescence-associated markers, and cytoskeletal remodelling. Expression was analysed by quantitative real-time PCR, western blot, and immunofluorescence. Anandamide treatment during decidualization selectively reduced forkhead box O1 (*FOXO1*) and insulin-like growth factor binding protein 1 (*IGFBP1*), without affecting prolactin (*PRL*); homeobox A10 (*HOXA10*) was reduced during decidualization but was not further modified by AEA. Anandamide also attenuated NOTCH1 activation and reduced HES-related family bHLH transcription factor with YRPW motif 1 (*HEY1*) expression. Given the role of NOTCH signalling in cellular plasticity and cytoskeletal organization, NOTCH1/*HEY1* attenuation after AEA exposure was accompanied by changes in snail family transcriptional repressor 1 (*SNAI1*), vimentin organization, and N-cadherin distribution. Consistent with the inflammatory component of stromal decidualization, decidualized HESCs showed increased interleukin-1 beta (*IL1B*), IL-8/C-X-C motif chemokine ligand 8 (*CXCL8*/IL-8), and prostaglandin-endoperoxide synthase 2/cyclooxygenase-2 (*PTGS2*/COX-2), whereas AEA reduced selected inflammatory mediators. Analysis of cyclin-dependent kinase inhibitor 1A (*CDKN1A*/p21) and cyclin-dependent kinase inhibitor 2A (*CDKN2A*/p16) suggested a short-term senescence-associated response, with AEA attenuating p21 expression. These findings suggest that AEA interferes with transcriptional and structural pathways critical for decidualization, highlighting a potential role of ECS–NOTCH1 crosstalk in implantation failure and endometrial dysfunction.

Keywords anandamide, CB1 receptor, decidualization, NOTCH, endometrial receptivity, inflammation

Embryo implantation into the uterine endometrium is a highly orchestrated process essential for the establishment of pregnancy. A key event in this process is decidualization, which involves the differentiation of endometrial stromal fibroblasts into specialized secretory decidual cells. This transformation is finely tuned by a tightly regulated interplay of progesterone, increased levels of

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cyclic adenosine monophosphate (cAMP) and low levels of oestrogen (Gellersen & Brosens, 2003; Okada et al., 2018; Ramathal et al., 2010; Teklenburg et al., 2010). In humans, decidualization starts during the luteal phase of the menstrual cycle, whereas in mice, it is induced exclusively in response to the implanting embryo (Gellersen & Brosens, 2014). Although key regulatory mechanisms of decidualization have been identified, this process remains highly intricate due to the extensive crosstalk between multiple signalling pathways (Cha et al., 2012; Jia et al., 2024; Ramathal et al., 2010; Schmidt et al., 2009). Given its pivotal role in establishing endometrial receptivity, impaired decidualization has been implicated in various reproductive disorders, including implantation failure, recurrent miscarriage, pregnancy complications (e.g., preeclampsia), and infertility (Laird et al., 2006; Larsen et al., 2013; Salker et al., 2010; Ticconi et al., 2021). Despite substantial progress in elucidating the process of decidualization, the precise molecular mechanisms governing this transformation remain incompletely understood, highlighting the need for further investigation into the signalling pathways that regulate this differentiation process.

Decidualization is now recognized as a dynamic process that extends beyond the induction of classical markers such as forkhead box O1 (FOXO1), insulin-like growth factor binding protein 1 (IGFBP1), and prolactin (PRL). During this transformation, stromal cells progressively acquire distinct functional states that integrate receptivity-associated transcriptional programs, inflammatory and prostaglandin-related signals, cell-cycle regulation, and cytoskeletal remodelling. This complexity has been further highlighted by single-cell profiling studies, which have revealed specialized decidual stromal and immune cell populations at the maternal–fetal interface, contributing to implantation and pregnancy maintenance (Guo et al., 2021; Vento-Tormo et al., 2018). In this context, controlled inflammatory activation and transient senescence-like features are increasingly considered physiological components of decidual remodelling, provided that they remain temporally regulated (Brighton et al., 2017; Tomari et al., 2020). Among the transcriptional regulators of endometrial receptivity and stromal differentiation, homeobox A10 (HOXA10) contributes to decidual competence and to the temporal control of decidual inflammation, and its dysregulation has been associated with reproductive failure (Sharma et al., 2026).

Within this complex regulatory context, the endocannabinoid system (ECS) has emerged as a critical regulatory network involved in endometrial receptivity, implantation, and decidualization (Almada et al., 2015; Fonseca et al., 2013; Maccarrone, 2009a). This system comprises endogenous ligands, such as anandamide (AEA), its receptors cannabinoid receptor type 1 (CB1), cannabinoid receptor type 2 (CB2), transient receptor potential vanilloid 1 (TRPV1), and enzymes responsible for endocannabinoid synthesis and degradation (Cecconi et al., 2020; Fonseca et al., 2013; Maccarrone, 2009a). Endocannabinoids are lipid-derived signalling molecules that bind to their receptors and modulate multiple physiological functions essential for endometrial receptivity and early pregnancy maintenance (Ezechukwu et al., 2020; Maccarrone, 2009a; Maia et al., 2020; Rava & Trezza, 2023). In the endometrium, the ECS is dynamically regulated throughout the menstrual cycle and early pregnancy, ensuring a tightly controlled balance of AEA levels. Elevated levels of AEA have been shown to impair decidualization by inhibiting the proliferation and differentiation of human endometrial stromal cells (HESCs), thereby compromising endometrial receptivity (Almada et al., 2015, 2016). This effect appears to be primarily mediated by CB1 activation (Almada et al., 2016; Fonseca et al., 2013; Maccarrone, 2009a). However, in addition to CB1 and CB2, TRPV1 has emerged as an additional receptor for AEA, influencing reproductive processes (Fernandes et al., 2012; Fonseca et al., 2009; Liu et al., 2012; Nie et al., 2010; Ross, 2003). Regulation of AEA levels in the endometrium is controlled by fatty acid amide hydrolase (FAAH), the primary enzyme responsible for AEA degradation (Gasperi et al., 2005; Paria et al., 1999a). Increased FAAH expression during decidualization ensures that AEA concentrations are reduced, thereby maintaining a favourable environment for implantation (Almada et al., 2016; Fonseca et al., 2013). However, the molecular mechanism by which altered ECS activation negatively affects decidualization remains to be fully elucidated.

Another fundamental pathway implicated in decidualization is the Notch signalling pathway, which regulates cellular differentiation, proliferation, and apoptosis through direct cell-to-cell interactions. In humans, the NOTCH signalling pathway involves four receptors (NOTCH1–4), that are activated by delta-like canonical Notch ligands 1, 3, and 4 (DLL1, DLL3, DLL4) and jagged canonical Notch ligands 1 and 2 (JAG1, JAG2) ligands (Moldovan et al., 2021). Upon ligand binding, a disintegrin and metalloproteinase domain-containing proteins 10 and 17 (ADAM10 and ADAM17) and the γ-secretase complex sequentially cleave the receptor, releasing the NOTCH intracellular domain (NICD), which translocates to the nucleus and regulates the transcription of key target genes, including the HES family bHLH transcription factor 1 (*HES1*) and HES-related family bHLH transcription factor with YRPW motif 1 (*HEY1*) genes (Kopan, 2012; Moldovan et al., 2021). The NOTCH signalling pathway plays an important role in female reproductive physiology, particularly in the regulation of decidualization, implantation, and uterine repair (Afshar et al., 2012; Maccarrone, 2009a; Su et al., 2015). Several studies have demonstrated that NOTCH1 is a key regulator of decidualization in both humans and mice (Chu et al., 2011; Su & Fazleabas, 2015). Knockdown of *NOTCH* in human fibroblasts (HuF) impairs the decidualization response when these cells are treated with 17β-estradiol (E2), medroxyprogesterone acetate (MPA), and cAMP (Su & Fazleabas, 2015). Furthermore, conditional *Notch1*-knockout in the mouse uterus results in reduced decidualization, as evidenced by reduced uterine wet weight and downregulation of key decidualization markers, such as bone morphogenetic protein 2 (*Bmp2*), and Wnt family member 4 (*Wnt4*) (Afshar et al., 2012). Conversely, constitutive activation of NOTCH1 via NICD overexpression results in impaired decidualization and implantation, suggesting that tight regulation of NOTCH1 activity is required for normal endometrial function (Su et al., 2016). Importantly, studies have shown that dysregulation of NOTCH signalling has also been observed in pathological conditions such as endometriosis, where its altered expression correlates with implantation failure (Su & Fazleabas, 2015).

Emerging evidence has shown a potential interplay between the ECS and the NOTCH signalling pathways in neuronal and cancer cell models (Frampton et al., 2010; Tanveer et al., 2012). However, whether a similar interaction occurs in endometrial stromal cells and contributes to the regulation of decidualization remains unknown. In this respect, this study aimed to

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investigate whether AEA modulates NOTCH signalling during decidualization and whether this modulation is associated with changes in selected decidual regulatory components, including FOXO1/IGFBP1, HOXA10, inflammatory mediators, and cell-cycle arrest/senescence-associated markers, together with cytoskeletal remodelling. This approach may provide new insights into the molecular mechanisms underlying implantation and early pregnancy maintenance and potentially identify new therapeutic targets for reproductive disorders associated with impaired decidualization.

Materials and methods

Human endometrial stromal cell culture

Immortalized primary human endometrial stromal cell line (HESC; ATCC® CRL-4003) was maintained in culture according to the manufacturer's instructions. Specifically, cells were cultured in phenol red-free Dulbecco's modified Eagle's medium (DMEM)/F-12 medium (Cat.No. D6434 Sigma-Aldrich Merck) with 3.1 g/L glucose and 1 mM sodium pyruvate (Merck, Darmstadt, Germany), 1.5 g/L sodium bicarbonate (Merck, Darmstadt, Germany) supplemented with 1% ITS + Premix (Corning 354352), 500 ng/mL puromycin (Millipore Sigma P8833), 10% charcoal-stripped fetal bovine serum (FBS; Sigma-Aldrich F6765), 1% penicillin/streptomycin, and 100 units/mL l-glutamine (Lonza, Milan, Italy). Cultures were maintained in a humidified incubator at 37 °C in the presence of 5% CO_2 and cells were used for no more than five passages from purchase.

Collection of endometrial biopsies and isolation of primary endometrial stromal cells

Endometrial biopsies were obtained from patients undergoing diagnostic hysteroscopy using the Rampipella® endometrial biopsy device (RI.MOS. s.r.l., Mirandola, Italy). Normal saline was used for uterine distension in all procedures. Informed consent was obtained from all participants, and the study was approved by the Ethics Committee of Policlinico Tor Vergata (Prot. PTV n. 19082). All subjects donated the endometrial tissue for research purposes.

The study included two independent groups of fertile women with regular menstrual cycles and no evidence of endometrial pathology. Menstrual cycle phase was determined using menstrual history, urinary LH testing, and ultrasound evaluation. Proliferative-phase samples were collected during the late proliferative phase (approximately cycle days 11–13), based on menstrual dating and ultrasound assessment. Midsecretory-phase samples were obtained approximately 8 days after detection of the urinary LH surge, and ovulation was confirmed by concomitant ultrasound examination. Cycle-phase assignment was further verified by recording the timing of the subsequent menstrual period. The first study group consisted of three women sampled during the midsecretory phase; endometrial biopsies were used for endometrial stromal cells isolation and culture (a detailed protocol is described below). The second group consisted of six women, including three sampled during the proliferative phase and three during the midsecretory phase. Biopsies were immediately frozen in liquid nitrogen for subsequent RNA extraction. Both groups of samples were used as confirmatory controls to validate selected findings obtained in immortalized HESCs. Inclusion criteria were: regular menstrual cycles of normal length (28 ± 4 days), history of at least one uncomplicated full-term pregnancy, no previous miscarriages, normal diagnostic hysteroscopy, and absence of endometrial abnormalities at histological examination. Participants were recruited among patients referred for abnormal uterine bleeding. Only women with completely normal findings following diagnostic evaluation, performed in accordance with Federation of Gynecology and Obstetrics (FIGO) recommendations (Munro et al., 2018), were included and classified as having abnormal uterine bleeding not otherwise specified. Exclusion criteria were: abnormal hysteroscopic findings, histological evidence of endometrial abnormalities, irregular menstrual cycles, or incomplete clinical/histological information. Clinical characteristics of the participants are reported in Tables S1 and S2. For the isolation of stromal cells, fragments from secretory phase biopsies were enzymatically digested in calcium- and magnesium-free HBSS (Merck, Darmstadt, Germany) containing 0.25% collagenase (from *Clostridium histolyticum*, cat. C2674, Merck, Darmstadt, Germany), 0.02% deoxyribonuclease I (Type IV; from bovine pancreas, Merck), and 0.1% hyaluronidase (Merck) for 20 min at 37 °C with gentle agitation in a humidified atmosphere of 5% CO_2. The resulting cell suspension was allowed to settle for 2 min, then sequentially filtered through 100 µm and 20 µm mesh filters (pluriSelect, Leipzig, Germany). The flow-through was collected and centrifuged at 1500 × *g* for 5 min, washed twice in phosphate-buffered saline (PBS; Corning, New York, USA), and treated with Red Blood Cell lysis buffer (Roche, Basel, Switzerland) for 8 min to remove erythrocytes. Cells were washed again in PBS and resuspended in phenol red-free RPMI-1640 (Merck) supplemented with 10% charcoal-stripped FBS (Merck, Darmstadt, Germany), 2 mM l-glutamine, 50 U/mL penicillin, 50 µg/mL streptomycin (Lonza, Milan, Italy), and 0.1 mM sodium pyruvate (Merck, Darmstadt, Germany). Cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2. Once confluent, cells were harvested for molecular analysis or cryopreserved for subsequent use). For all experiments, cells were used at passage between 2 and 4.

Endometrial stromal cell culture treatments

Both primary and immortalized HESCs were seeded at a cellular density of 5×10^4 cells/well in 6-well culture plate. Once cells reached approximately 80% of confluence, they were serum-starved overnight before inducing their differentiation into decidual cells (dHESC). Serum starvation was performed prior to in-vitro decidualization to minimize basal signalling activity and synchronize cellular responses to subsequent treatments (Lacconi et al., 2023). To this end, HESCs were cultured in differentiation medium containing 1 µM medroxyprogesterone acetate (MPA, Merck, Darmstadt, Germany) and 0.5 mM 8-Bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP, Merck, Darmstadt, Germany) in phenol red-free DMEM/F-12 supplemented with 2% charcoal-stripped FBS, as previously described (Gellersen &

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Brosens, 2014). This protocol was selected based on prior studies that identified the combination of cAMP and MPA as the most physiologically relevant system to mimic in-vivo decidualization (Doi-Tanaka et al., 2024; Gellersen & Brosens, 2014; Lacconi et al., 2023). Cells were maintained under these conditions for 4 and 6 days with medium renewal every 48 hr. The HESCs cultured in the absence of differentiation stimuli for 4 days and 6 days, as control groups, were included. Ethanol, used to dissolve MPA, was added to control media. Details of the treatment protocols and experimental conditions are shown in Figure S1A and B. Decidualization was confirmed by quantitative real-time PCR (qRT-PCR) analysis of key decidual markers, including *FOXO1*, *IGFBP1*, and *PRL* (Figure S1C). To investigate the effects of endocannabinoids on decidualization process, HESCs were treated with differentiation medium supplemented with 10 µM N-arachidonoylethanolamine (anandamide, AEA, Cayman Chemical, Item No. 90050). The concentration of AEA was selected based on preliminary dose–response experiments conducted at both 24 and 72 hr of treatment (Figure S2A), as well as on published studies (Almada et al., 2017). Details of treatment protocols are reported in Figure S3A. Images were acquired using an inverted microscope Axiovert 200M with Zeiss AxioCam HRc (Carl Zeiss).

Quantitative real-time PCR analysis

Gene expression levels were assessed using qRT-PCR. Total RNA was extracted from both undifferentiated and differentiated cells using TRIzol Reagent (TriPure Isolation Reagent, Roche Diagnostics GmbH, Mannheim, Germany; Cat. No. 11667165001), according to the manufacturer's protocol. RNA concentration and purity were determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). For cDNA synthesis, 1 µg of total RNA was reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany; Cat. No. 205313) following the manufacturer's protocol. Real-time PCR was performed using the SYBR Fast qPCR kit (Bio-Rad; Cat. No. 1725124) on a LightCycler 96 Real-Time PCR System (Roche Diagnostics GmbH, Mannheim, Germany), following the manufacturer's protocol. Primer sequences are listed in Table 1. Primer efficiency was calculated from standard curves, and amplification specificity was verified by melting curve analysis. Relative gene expression was calculated using the $2^{-\Delta Ct}$ method, with normalization to the housekeeping gene ribosomal protein L17 (*RPL17*). For graphical representation, values were scaled by a factor of 10,000 to improve visualization. All qRT-PCR experiments were performed in technical triplicates for each biological replicate, and technical replicates were averaged prior to analysis. For immortalized HESCs, each independent experiment represents one biological replicate derived from independently seeded and treated cultures. For primary endometrial stromal cells, each biological replicate corresponds to cells derived from an independent patient biopsy.

Western blot analysis

The HESC and dHESC cell lysate were prepared with RIPA buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.5% NP-40, 5 mM ethylenediaminetetraacetic acid [EDTA], 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate), supplemented with an EDTA-free protease inhibitor cocktail (Roche, Penzberg, Germany). Lysates were incubated on ice for 30 min, followed by centrifugation at 13,000 rpm for 10 min at 4 °C. Protein samples (30 µg) were separated by electrophoresis in precast Bis-Tris gels (NuPAGE 4–12%, Invitrogen, Thermo Fisher Scientific) and transferred to polyvinylidene difluoride membranes (Amersham Hybond® P, Merck,

Table 1 Oligonucleotide primer sets used for the study.

Gene	RefSeq Acc. No.	Forward primer	Reverse primer
***CDKN1A*/p21**	NM_000389.5	5′-TGTCCGTCAGAACCCATGC-3′	5′-AAAGTCGAAGTTCCATCGCTC-3′
***CDKN2A*/p16**	NM_000077.5	5′-GAGAACAAGTTCGTGGACGAAGA-3′	5′-CTTCACTGCCTGACTCTTGGTG-3′
CNR1	NM_001424098.1	5′-GTATGCTCTGCCTGCTGAA-3′	5′-CTGTGAACACTGGCTGCATT-3′
CNR2	NM_001841.3	5′-GGTCTTGGCACTCTCTTCTT-3′	5′-GTCCCAACACTCATCAGCAA-3′
FAAH	NM_001441.3	5′-CTGGAGACCAAACAGAGCCTTG-3′	5′-CACTGAAGAGCCCACCTGTTGA-3′
FOXO1	NM_002015.4	5′-TCATACTTCATCTCATTCTCCCTTCT-3′	5′-TTCTATTCCATTTGCTACCCATCT-3′
HEY1	NM_001040708.2	5′-CATCGAGGTGGAGAAGGAGAGT-3′	5′-GACATGGAACCTAGAGCCGAACT-3′
HOXA10	NM_018951.4	5′-TCCAGTGTCTGGTGCTTCGTGT-3′	5′-CTTCCGAGAGCAGCAAAGCCTC-3′
IGFBP1	NM_000596.4	5′-CCTGCCAAACTGCAACAAGA-3′	5′-TCCCATTCCAAGGGTAGACG-3′
***CXCL8*/IL-8**	NM_000584.4	5′-GAGAGTGATTGAGAGTGGACCAC-3′	5′-CACAACCCTCTGCACCCAGTTT-3′
***IL1B*/IL-1β**	NM_000576.3	5′-CCACAGACCTTCCAGGAGAATG-3′	5′-GTGCAGTTCAGTGATCGTACAGG-3′
PRL	NM_000948.6	5′-GAGGAGCAAACCAAACGGCTTC-3′	5′-AAGGCGAGACTCTTCATCAGCC-3′
***PTGS2*/COX-2**	NM_000963.4	5′-CGGTGAAACTCTGGCTAGACAG-3′	5′-GCAAACCGTAGATGCTCAGGGA-3′
RPL17	NM_001199345.2	5′-ACGAAAAGCCACGAAGTATCTG-3′	5′-GACCTTGTGTCCAGCCCCAT-3′
***SNAI2*/SLUG**	NM_003068.5	5′-CAAGGCGTTTTCCAGACCCTG-3′	5′-TTGACCTGTCTGCAAATGCTCT-3′
SNAI1	NM_005985.4	5′-CACTATGCCGCGCTCTTTC-3′	5′-GCTGGAAGGTAAACTCTGCATTAGA-3′
TRPV1	NM_080704	5′-GTGGACAGCTACAGTGAGATGC-3′	5′-GGAAGCCACATACTCCTTGAGG-3′
ZEB1	NM_001128128.3	5′-ACTCAACTACGGTCAGCCCT-3′	5′-TGGGCGGTGTAGAATCAGAG-3′

CDKN1A/p21 = cyclin-dependent kinase inhibitor 1A; *CDKN2A*/p16 = cyclin-dependent kinase inhibitor 2A; *CNR1* = cannabinoid receptor 1; *CNR2* = cannabinoid receptor 2; *FAAH* = fatty acid amide hydrolase; *FOXO1* = forkhead box O1; *HEY1* = reduced HES-related family bHLH transcription factor with YRPW motif 1; *HOXA10* = homeobox A10; *IGFBP1* = insulin-like growth factor binding protein 1; *CXCL8*/IL-8 = C-X-C motif chemokine ligand 8/interleukin-8; *IL1B*/IL-1β = interleukin-1 beta; *PRL* = prolactin; *PTGS2*/COX-2 = prostaglandin-endoperoxide synthase 2/cyclooxygenase-2; *RPL17* = ribosomal protein L17; *SNAI2*/SLUG = snail family transcriptional repressor 2; *SNAI1* = snail family transcriptional repressor 1; *TRPV1* = transient receptor potential vanilloid 1; *ZEB1* = zinc finger E-box-binding homeobox 1.

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Darmstadt, Germany). Membranes were then blocked for 1 hr at room temperature with 5% (w/v) nonfat dry milk in PBS containing 0.05% (v/v) Tween-20 (PBS-T), and incubated overnight at 4 °C with primary antibodies. The following primary antibodies were used: rabbit anti-CB1 (1:1,000, Cayman Chemical, Item No. 101500), rabbit anti-NOTCH1 (1:1,000, D6F11 Cell Signaling Tecnology) and mouse anti-GAPDH (1:2,000, clone 6C5, Santa Cruz, CA, USA, #sc-32233). Following washing, membranes were incubated for 1 hr at room temperature with horseradish peroxidase–conjugated secondary antibodies: antirabbit (1:10,000, Amersham Biosciences, NA934V), and antimouse (1:5,000, Amersham Biosciences, NA9310V), diluted in 5% nonfat dry milk in PBS-T. Immunoreactive bands were visualized using Amersham ECL Prime (Amersham Biosciences), according to the manufacturer's protocol. Chemiluminescent signals were detected using the ImageQuant LAS 4000 mini system (GE Healthcare). Densitometric analysis of the bands was performed using ImageJ software. For normalization of protein expression, membranes were stripped using the Re-Blot Plus Mild Solution (10X) (Merck KGaA, Darmstadt, Germania) and reprobed with mouse anti-GAPDH. Results were normalized to housekeeping proteins. Western blot experiments were performed on at least three independent biological replicates from independent cell cultures.

Immunofluorescence analysis

The HESCs and dHESCs were cultured on glass coverslips, washed three times with PBS and fixed with cold methanol for 5 min at −20 °C. After fixation, cells were air dried under a chemical hood and then rehydrated with PBS. Nonspecific binding was blocked by incubation with 10% donkey serum in PBS for 1 hr at room temperature. Cells were then incubated overnight at 4 °C with the following primary antibodies diluted in BSA 0,1% in PBS: rabbit anti-Vimentin (VIM) (1:200, ab45939, Abcam), mouse anti-Smooth Muscle Actin (α-SMA) (1:250, clone 1A4, A2547, Sigma), rabbit anti-N-Cadherin (N-Cad) (1:100, 13116, Cell Signalling Technology), rabbit anti-Cytokeratin (CK) (1:600, Z0622, Dako), and mouse anti-E-Cadherin (E-Cad) (1:100, 4A2, Cell Signalling Technology). Specificity of CK and E-Cad antibodies was validated using positive controls, as shown in Figure S5A and B. The following day, cells were washed with PBS and incubated for 1 hr at room temperature with the appropriate secondary antibodies: Alexa-Fluor-568-conjugated donkey antirabbit and Alexa-Fluor-488-conjugated donkey antimouse secondary antibody both diluted in BSA 0.1% in PBS. Nuclei were counterstained using Hoechst 33242 (0.5 µg/mL, Invitrogen, Carlsbad, CA, USA) diluted in PBS. After extensive washing in PBS, coverslips were mounted using ProLong Gold Antifade Mountant (P36934, Invitrogen, ThermoFisher Scientific, Waltham, MA, USA). Images were acquired using a Nikon eclipse Ti-S microscope equipped with NIS-Elements BR 4 software and the LAS AF software (2.6.0.7266, Leica Microsystems). Immunofluorescence experiments were performed on at least three independent biological replicates derived from independent cell cultures.

Statistical analysis

All data are expressed as mean ± SEM from at least three independent experiments. Each experimental time point was analysed independently. When two groups were compared, statistical significance was assessed using Student's *t*-test. Comparisons involving more than two groups within the same time point were analysed by one-way analysis of variance followed by Tukey's post hoc test. A p-value <0.05 was considered statistically significant. All analyses were performed using GraphPad Prism 9 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Effect of AEA on in-vitro decidualization of HESCs

To validate the in-vitro decidualization protocol used in this study, the efficiency of stromal cell differentiation was assessed by analysing both morphological changes and gene expression of established decidualization markers (Figure 1, Figure S1). Specifically, morphological assessment revealed the characteristic transformation of fibroblast-like stromal cells into polygonal decidualized cells, characterized by an increase in cell size and a shift toward an epithelioid morphology (Figure S1B), consistent with previous descriptions of decidualization in vitro (Okada et al., 2018; Pan-Castillo et al., 2018). In parallel, qRT-PCR analysis confirmed a significant upregulation of key decidualization markers, including *FOXO1*, *PRL*, and *IGFBP1* (Figure 1B), confirming the induction of decidualization at the molecular level. To investigate the involvement of the ECS in HESCs and their decidualized counterparts (dHESCs), the expression of its molecular components, including cannabinoid receptor 1 (*CNR1*, encoding CB1), cannabinoid receptor 2 (*CNR2*, encoding CB2), *TRPV1*, and *FAAH*, was analysed by qRT-PCR. The results revealed the presence of *CNR1* mRNA in HESCs, and a significant upregulation was observed in dHESCs after both 4 and 6 days of decidualization (Figure 2A). The increased expression of CB1 in dHESCs was further confirmed by western blot analysis (Figure 2B). In contrast, and consistent with previous studies (Almada et al., 2016; Neradugomma et al., 2019), *CNR2* expression was undetectable in both HESCs and dHESCs (Figure S4), indicating a limited functional role for CB2 in endometrial stromal cells. *TRPV1*, on the other hand, was expressed at relatively high levels in undifferentiated HESCs, and such levels remained unchanged upon decidualization as demonstrated by qRT-PCR analysis (Figure 2C and Figure S4). These results suggest that CB1 and, potentially TRPV1, are the main effectors of ECS signalling in these cells. *FAAH* expression was also detected in both HESCs and dHESCs, but no significant modulation was observed during the decidualization process (Figure 2D). Previous studies have demonstrated that dysregulated levels of AEA negatively impact endometrial decidualization (Almada et al., 2016). In the present study, we confirmed that such effect also occurs in our in-vitro model. To this end, HESCs were cultured under decidualizing conditions and AEA was added to the culture medium as detailed in Figure S3A. Morphological analysis showed that decidualization was evident in both untreated and AEA-treated conditions, indicating that AEA does not affect the induction of the epithelioid phenotype characterizing decidual cells (Figure 1A). Despite the lack of visible morphological differences, gene expression analysis revealed that AEA modulates the expression of key decidualization markers in a time-dependent manner.

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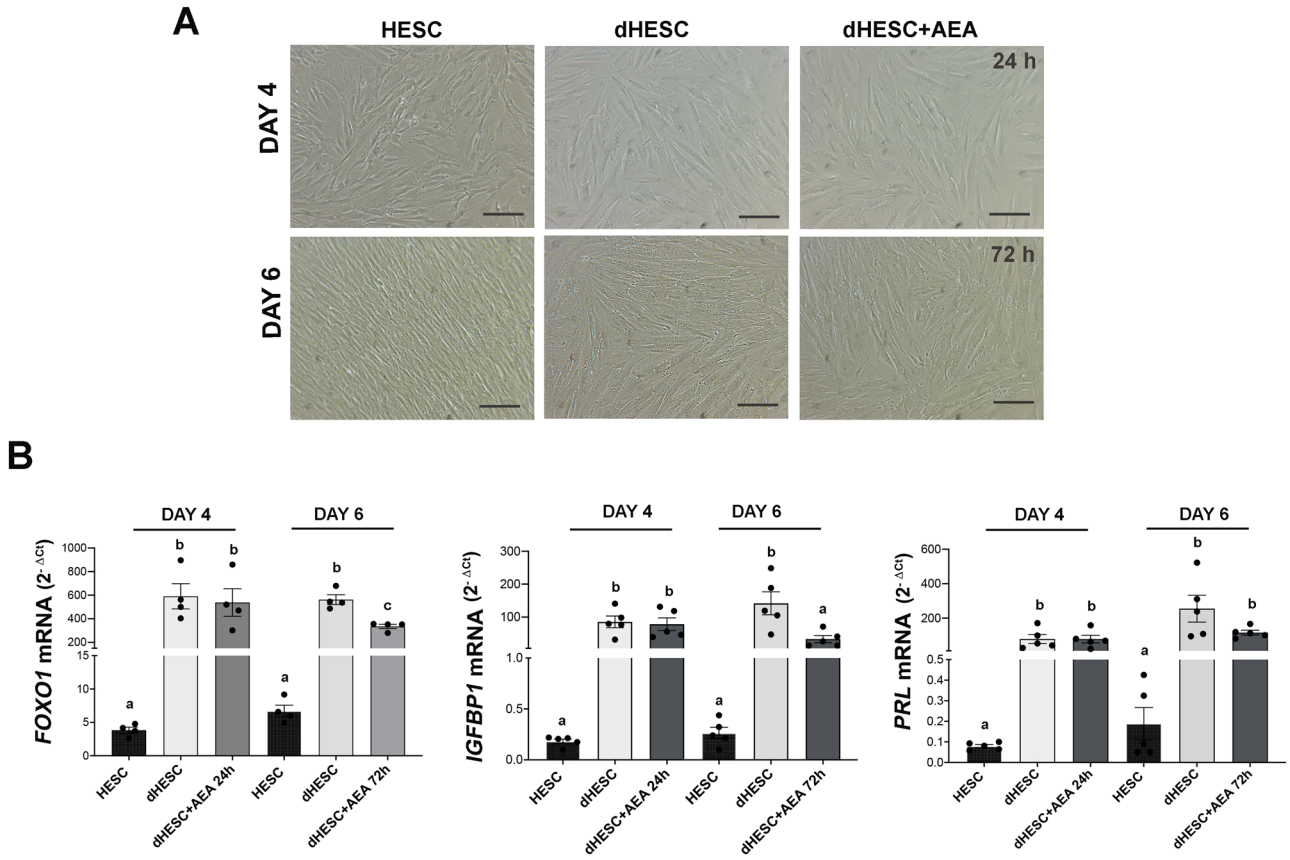


Figure 1 Effect of anandamide (AEA) on in-vitro decidualization of human endometrial stromal cells (HESCs) at 4 and 6 days of culture. The HESCs were cultured under standard decidualizing conditions for 4 and 6 days, with or without the addition of 10 µM AEA administered during the final 24 and 72 hr. (A) Phase-contrast microscopy images showing the morphological characteristics of HESCs, decidualized HESCs (dHESCs), and dHESCs treated with AEA. Images were acquired using an Axiovert 200 M inverted microscope equipped with a Zeiss AxioCam HRc (Carl Zeiss) at 5× magnification. Scale bars: 100 µm. (B) Quantitative real-time PCR analysis of forkhead box O1 (*FOXO1*), insulin-like growth factor binding protein 1 (*IGFBP1*), and prolactin (*PRL*) mRNA expression in HESCs, dHESCs, and dHESCs treated with AEA. Data are presented as mean ± SEM from at least four independent biological replicates per group; individual dots represent biological replicates. Statistical comparisons were performed separately for each time point using one-way analysis of variance followed by Tukey's post hoc test. For multiple groups comparison, different superscript letters are used to denote statistically significant differences, while the same letter indicates that differences are not statistically significant ($p < 0.05$).

Specifically, qRT-PCR analysis showed that, while no significant changes were observed after 24 hr of treatment, prolonged exposure to AEA up to 72 hr resulted in a significant reduction in the expression of *FOXO1* and its downstream target gene *IGFBP1* (Figure 1B). In contrast, *PRL* expression was unaffected by AEA treatment at both time points (Figure 1B). These findings suggest that prolonged cell exposure to AEA impairs molecular progression of decidualization, specifically by downregulating *FOXO1* and *IGFBP1*.

Anandamide regulation of CNR1, TRPV1, and FAAH in HESC and dHESC

Since AEA is an endogenous ligand for both CB1 and TRPV1, its effect on the expression of these receptors, as well as FAAH, the main AEA-degrading enzyme, was investigated in HESCs undergoing decidualization. To this aim, dHESCs were treated with AEA for 24 and 72 hr and the mRNA levels of *CNR1*, *TRPV1*, and *FAAH* were assessed by qRT-PCR. The results showed a significant downregulation of *CNR1* in AEA-treated dHESCs compared with untreated dHESCs (Figure 2A, Figures S2B and 3B). However, western blot analysis did not show a corresponding decrease in CB1 protein levels (Figure 2B, Figure S2C). Concerning *TRPV1*, no significant modulation of mRNA expression was detected upon AEA treatment in dHESCs at both time points (Figure 2C). Nevertheless, a downward trend in *TRPV1* expression was observed at 72 hr after AEA treatment in dHESCs, although this reduction was not statistically significant. Regarding *FAAH*, AEA treatment did not alter its mRNA levels (Figure 2D), indicating that *FAAH* mRNA levels remain stable under these experimental conditions.

Effect of AEA on HOXA10, inflammatory mediators, and cell-cycle arrest/senescence-associated markers during decidualization

To further investigate whether AEA affects additional regulatory components of the decidual program, the expression of *HOXA10*, cell-cycle arrest/senescence-associated markers, and selected

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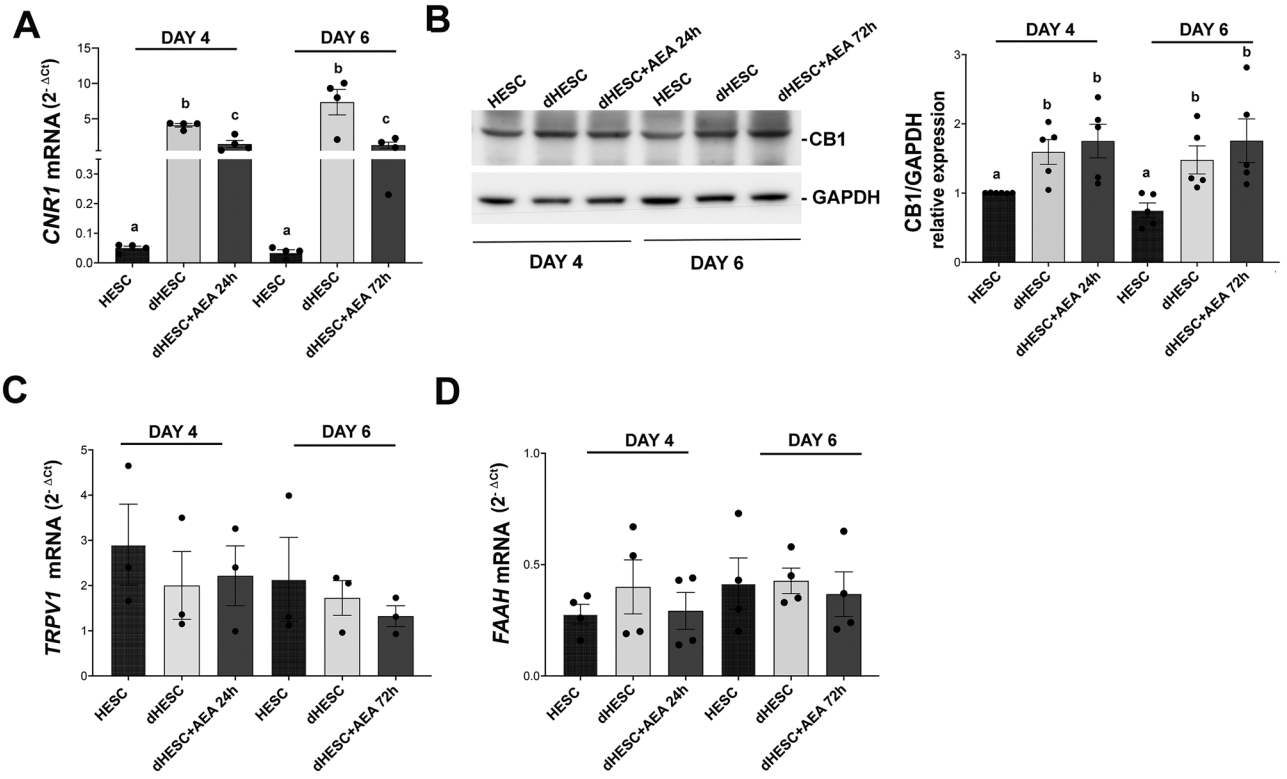


Figure 2 Effects of anandamide (AEA) treatment on cannabinoid receptor type 1 (CB1), transient receptor potential vanilloid 1 (TRPV1), and fatty acid amide hydrolase (FAAH) in human endometrial stromal cells (HESCs) and decidualized HESCs (dHESCs) at 4 and 6 days of culture. The HESCs were cultured under standard decidualizing conditions for 4 and 6 days, with or without 10 µM AEA administered during the final 24 and 72 hr, respectively. (A) Quantitative real-time PCR (qRT-PCR) analysis of including cannabinoid receptor 1 (*CNR1*) mRNA expression in HESCs and dHESCs with and without AEA. (B) Representative western blot analysis of CB1 protein expression in HESCs and dHESCs with or without AEA exposure, with corresponding densitometric quantification. (C) qRT-PCR analysis of *TRPV1* mRNA expression under the same experimental conditions. (D) qRT-PCR analysis of *FAAH* mRNA expression in HESCs and dHESCs, with or without AEA treatment. Data are presented as mean ± SEM from at least three independent biological replicates per group; individual dots represent biological replicates. Statistical comparisons were performed separately for each time point using one-way analysis of variance followed by Tukey's post hoc test. For multiple groups comparison, different superscript letters are used to denote statistically significant differences, while the same letter indicates that differences are not statistically significant ($p < 0.05$).

inflammatory mediators was analysed. *HOXA10*, a transcription factor associated with endometrial receptivity and decidual inflammation (Mishra & Modi, 2025; Sharma et al., 2026), showed a distinct temporal profile compared with the other studied decidual markers (*FOXO1*, *IGFBP1*, *PRL*). In our experimental conditions, *HOXA10* expression was significantly reduced in dHESCs compared with HESCs at both day 4 and day 6 of decidualization (Figure 3A). Anandamide treatment did not affect *HOXA10* levels compared with untreated dHESCs at either time points (Figure 3A). With respect to decidual inflammation selected inflammatory mediators were then analysed. Interleukin-1 beta (*IL1B*)/IL-1β was markedly upregulated in dHESCs compared with HESCs at both time points, whereas AEA significantly reduced its expression after both 24 and 72 hr of exposure (Figure 3B). prostaglandin-endoperoxide synthase 2/cyclooxygenase-2 (*PTGS2*/COX-2) expression was also increased in dHESCs, and AEA significantly reduced its expression after 72 hr of exposure (Figure 3C). In contrast, IL-8/C-X-C motif chemokine ligand 8 (*CXCL8*/IL-8) was induced during decidualization but showed a less consistent modulation following AEA treatment (Figure 3D). As decidual inflammation has been linked to transient activation of stress- and senescence-associated genes, the analysis was extended to cyclin-dependent kinase inhibitor 1A (*CDKN1A*/p21) and cyclin-dependent kinase inhibitor 2A (*CDKN2A*/p16). *CDKN1A*/p21 mRNA levels were increased in dHESCs compared with HESCs, and were reduced following AEA exposure, at both exposure times (Figure 3E). Conversely, *CDKN2A*/p16 mRNA levels did not show any increase during decidualization and were not significantly modulated by AEA treatment (Figure 3F). These findings suggest that AEA does not uniformly affect the decidual response but selectively modulates specific components of the decidual program.

Modulation of NOTCH1 signalling by AEA during decidualization

Given the well-established role of NOTCH1 in the decidualization process (Afshar et al., 2012; Maccarrone, 2009b; Su et al., 2015), and its regulation by endocannabinoids in other cell models (Frampton et al., 2010; Laezza et al., 2012; Tanveer et al., 2012), the potential modulation of NOTCH1 activation by AEA during decidualization was investigated. To address this, western blot analysis was performed to examine the expression of full-length NOTCH1 (NOTCH1-FL) and its cleaved transmembrane domain

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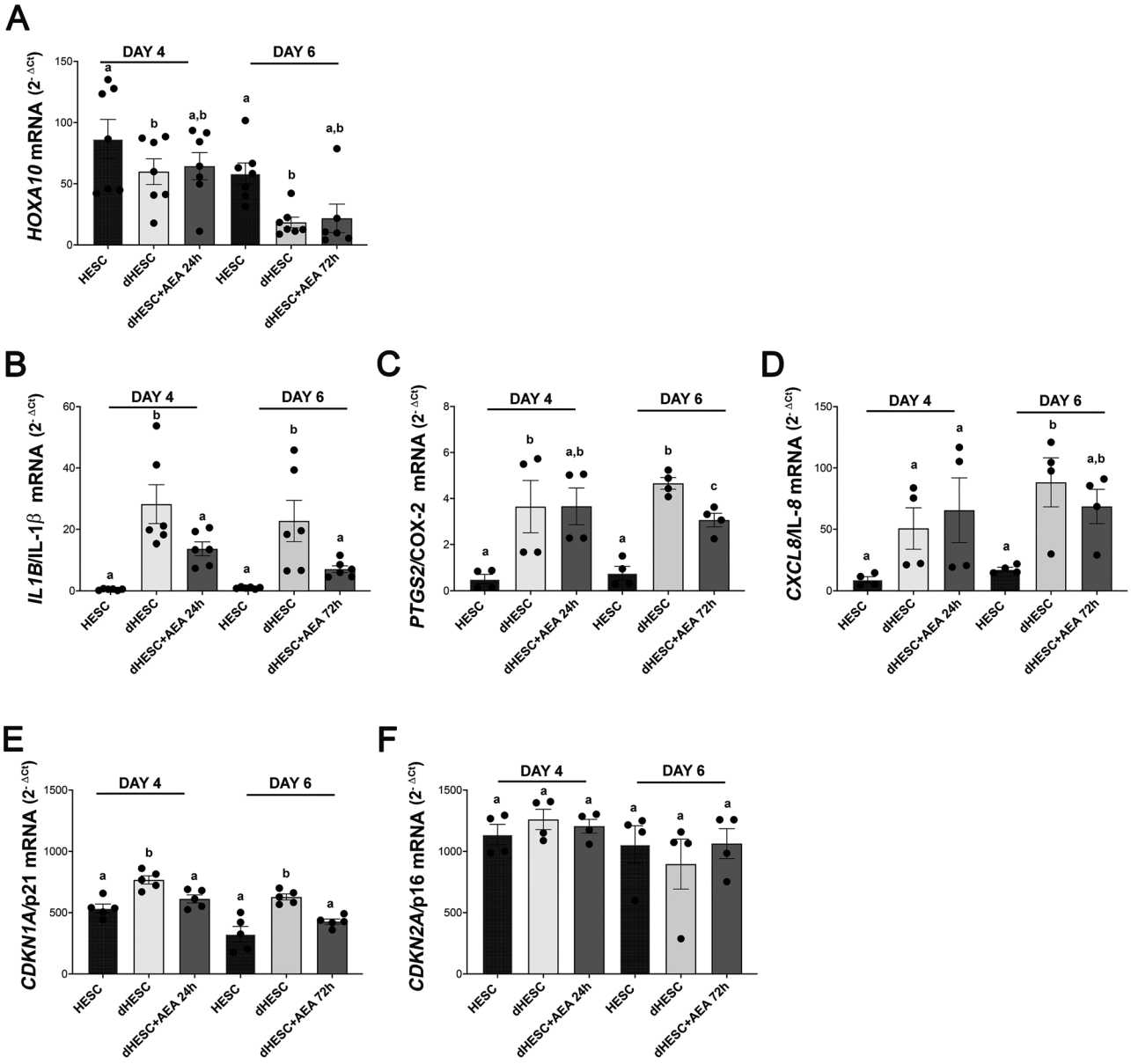


Figure 3 Anandamide (AEA) modulation of homeobox A10 (*HOXA10*), inflammatory mediators, and cell-cycle arrest-associated markers during decidualization. (A) Quantitative real-time PCR analysis of *HOXA10* mRNA expression. (B) Interleukin-1 beta (*IL1B*/IL-1β). (C) Prostaglandin-endoperoxide synthase 2/cyclooxygenase-2 (*PTGS2*/COX-2). (D) C-X-C motif chemokine ligand 8/interleukin-8 (*CXCL8*/IL-8). (E) Cyclin-dependent kinase inhibitor 1A (*CDKN1A*/p21). (F) Cyclin-dependent kinase inhibitor 2A (*CDKN2A*/p16) mRNA expression in human endometrial stromal cells (HESCs), decidualized HESCs (dHESCs), and AEA-treated dHESCs after 4 and 6 days of decidualization. Data are presented as mean ± SEM from at least four independent biological replicates per group; individual dots represent biological replicates. Statistical comparisons were performed separately for each time point using one-way analysis of variance followed by Tukey's post hoc test. For multiple-group comparisons, different letters indicate statistically significant differences, whereas groups sharing the same letter are not significantly different ($p < 0.05$).

(NOTCH1-NTM), which represent the active form of NOTCH1 receptor. In agreement with previous reports, decidualization induces a marked increase in NOTCH1 activation (Afshar et al., 2012; Su & Fazleabas, 2015), as evidenced by a significant upregulation of NOTCH1-NTM expression at day 4, which was maintained at day 6 (Figure 4A). However, AEA treatment significantly reduced NOTCH1 activation after 24 hr (day 4) and this reduction persisted after 72 hr (day 6) (Figure 4A). To further confirm the effect of AEA on NOTCH1 pathway activation, the expression of *HEY1*, a canonical downstream target gene of NOTCH1, was assessed. The qRT-PCR analysis revealed a significant upregulation of *HEY1* mRNA in dHESCs at both day 4 and day 6, consistent with active NOTCH1 signalling during decidualization (Figure 4B). However, *HEY1* expression was significantly reduced in AEA-treated dHESCs at both time points, consistent with the suppression of NOTCH1 activation. Altogether, these results highlight the modulatory role of AEA in regulating Notch signalling during the critical phase of decidualization and suggest that AEA-induced suppression of NOTCH1 activation may contribute to impaired decidualization progression.

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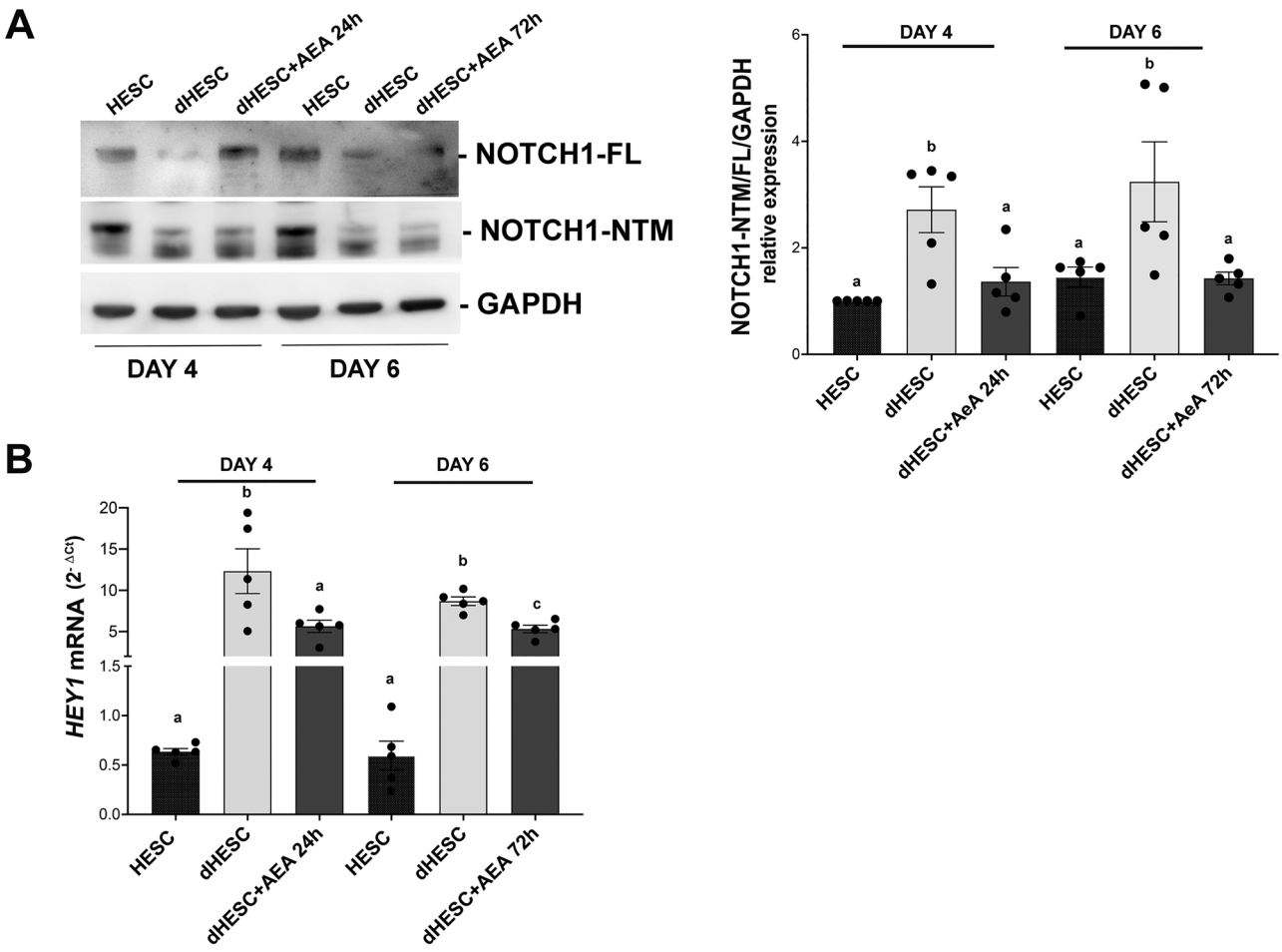


Figure 4 Modulation of neurogenic locus notch homolog protein 1 (NOTCH1) signalling by anandamide (AEA) during decidualization. Human endometrial stromal cells (HESCs) were cultured under standard decidualizing conditions for 4 and 6 days, with or without the addition of 10 µM AEA administered for the final 24 and 72 hr. (A) Representative western blot analysis of full-length NOTCH1 (NOTCH1-FL) and its cleaved transmembrane domain (NOTCH1-NTM) in HESCs and decidualized HESCs (dHESCs) with or without AEA, with corresponding densitometric quantification. The antibody used detects both NOTCH transmembrane (NTM) and NOTCH intracellular domain (NICD), which may appear as closely migrating bands. Densitometric analysis was performed on both bands, and the NTM/FL ratio normalized to GAPDH is shown. (B) Quantitative real-time PCR analysis of HES-related family bHLH transcription factor with YRPW motif 1 (*HEY1*) mRNA expression in HESCs and dHESCs with or without AEA. Data are presented as mean ± SEM from five independent biological replicates per group; individual dots represent biological replicates. Statistical comparisons were performed separately for each time point using one-way analysis of variance followed by Tukey's post hoc test. For multiple groups comparison, different superscript letters are used to denote statistically significant differences, while the same letter indicates that differences are not statistically significant ($p < 0.05$).

Anandamide regulation of cell state transition and cytoskeletal components

Decidualization involves extensive cellular remodelling and plasticity, with changes in cytoskeletal organization and transcriptional regulation. A few authors have proposed that these cellular changes may, in part, resemble features of the mesenchymal-to-epithelial transition (Pan-Castillo et al., 2018; Zhang et al., 2013). To further clarify this aspect, the expression of key regulators of cell state transition and cytoskeletal components were investigated. Differently from what was reported previously (Zhang et al., 2013), but consistent with recent reports (Doi-Tanaka et al., 2024; L. Wu et al., 2020), qRT-PCR analysis showed a significant upregulation of snail family transcriptional repressor 1 (*SNAI1*) in dHESCs compared with HESCs (Figure 5A). However, when decidualizing cells were treated for 24 hr with AEA, a significant downregulation of *SNAI1* was observed, and this effect persisted after 72 hr of AEA treatment (Figure 5A).

Consistent with these results, analysis of primary human endometrial stromal cells confirmed that *SNAI1* mRNA levels significantly increase upon decidualization and are downregulated following AEA treatment (Figure 5B).

To further clarify *SNAI1* upregulation observed during decidualization in both the cell line and primary stromal cells, the expression of *SNAI1*, snail family transcriptional repressor 2 (*SNAI2*)/SLUG, and zinc finger E-box-binding homeobox 1 (*ZEB1*) was examined in primary endometrial stromal cells isolated from endometrial biopsies collected during the proliferative and secretory phases of the menstrual cycle (Figure 5C). The proliferative phase predominantly consists of proliferating stromal cells, while the secretory phase is characterized by epithelioid differentiation and secretory activity. *SNAI1* expression was significantly higher in cells from the secretory phase compared with the proliferative

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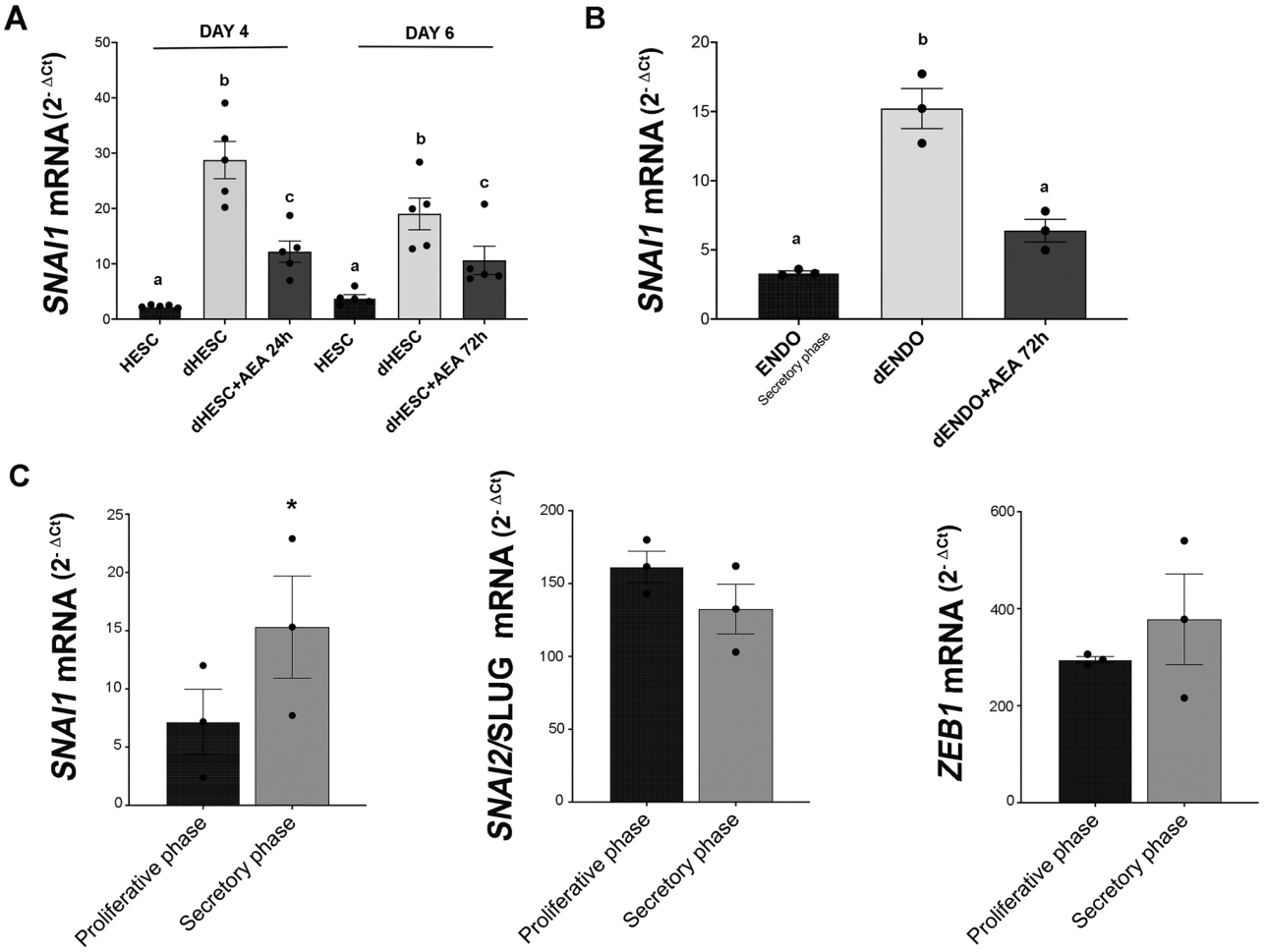


Figure 5 Snail family transcriptional repressor 1 (SNAI1) modulation by anandamide (AEA) and expression of related genes in endometrial cells and tissues. (A) Quantitative real-time PCR (qRT-PCR) analysis of *SNAI1* mRNA in immortalized human endometrial stromal cells (HESCs) and decidualized HESCs (dHESCs) cultured under standard decidualizing conditions for 4 and 6 days, with or without 10 μM AEA added during the final 24 or 72 hr of treatment. (B) qRT-PCR analysis of *SNAI1* expression in primary human endometrial stromal cells (ENDO), following in-vitro decidualization (dENDO) and after 72 hr of AEA treatment under decidualizing conditions. (C) qRT-PCR analysis of *SNAI1*, *SNAI2*/SLUG, and zinc finger E-box-binding homeobox 1 (*ZEB1*) mRNA levels in human endometrial biopsies collected during the proliferative and secretory phase of the menstrual cycle. Data are presented as mean ± SEM from at least three independent biological replicates per group (A) or from $n = 3$ independent patient biopsies per group (B, C), normalized to endogenous controls; individual dots represent biological replicates. For panel (A), statistical comparisons were performed separately for each time point using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. For panel (B), comparisons among the three groups were analysed using one-way ANOVA followed by Tukey's post hoc test. For multiple group comparisons, different superscript letters are used to denote statistically significant differences, while the same letter indicates that differences are not statistically significant ($p < 0.05$). In (C), comparisons between proliferative and secretory phases were evaluated using Student's *t*-test (*$p < 0.05$).

phase, indicating that *SNAI1* upregulation is a feature of the secretory endometrium and not limited to in-vitro decidualization. By contrast, levels of *SNAI2*/SLUG and *ZEB1*, typically associated with mesenchymal identity, did not significantly change between phases and remained consistently high.

To better correlate these transcriptional findings with cellular phenotype, immunofluorescence analysis of cytoskeletal and adhesion markers was performed in HESCs, dHESCs, and AEA-treated dHESCs (Figure 6) to qualitatively investigate their cellular distribution and organization. Vimentin was persistently expressed in dHESCs and appeared arranged in thick, parallel bundles compared with a diffuse pattern in HESCs, indicative of cytoskeletal remodelling. Upon AEA treatment, vimentin shifted from an extended cytoplasmic filamentous network to a shorter and more perinuclear distribution, suggesting that AEA may affect cytoskeletal architecture in decidualized cells. Likewise, α-SMA immunoreactivity was observed only in dHESCs and remained unaffected by AEA treatment (Figure 6).

The expression of mesenchymal and epithelial cadherins was also investigated. Immunofluorescence analysis revealed a more intense signal for N-Cad in dHESCs compared with undifferentiated HESCs and an overall redistribution toward a more spotted and discontinuous, punctate membrane pattern after AEA treatment (Figure 6). In contrast, epithelial markers such as E-Cad and CK were not detectable under any condition (Figure 6), consistent with the persistent expression of *SNAI2*/SLUG and *ZEB1*. Altogether these results suggest that decidual cells do not acquire a complete epithelial phenotype.

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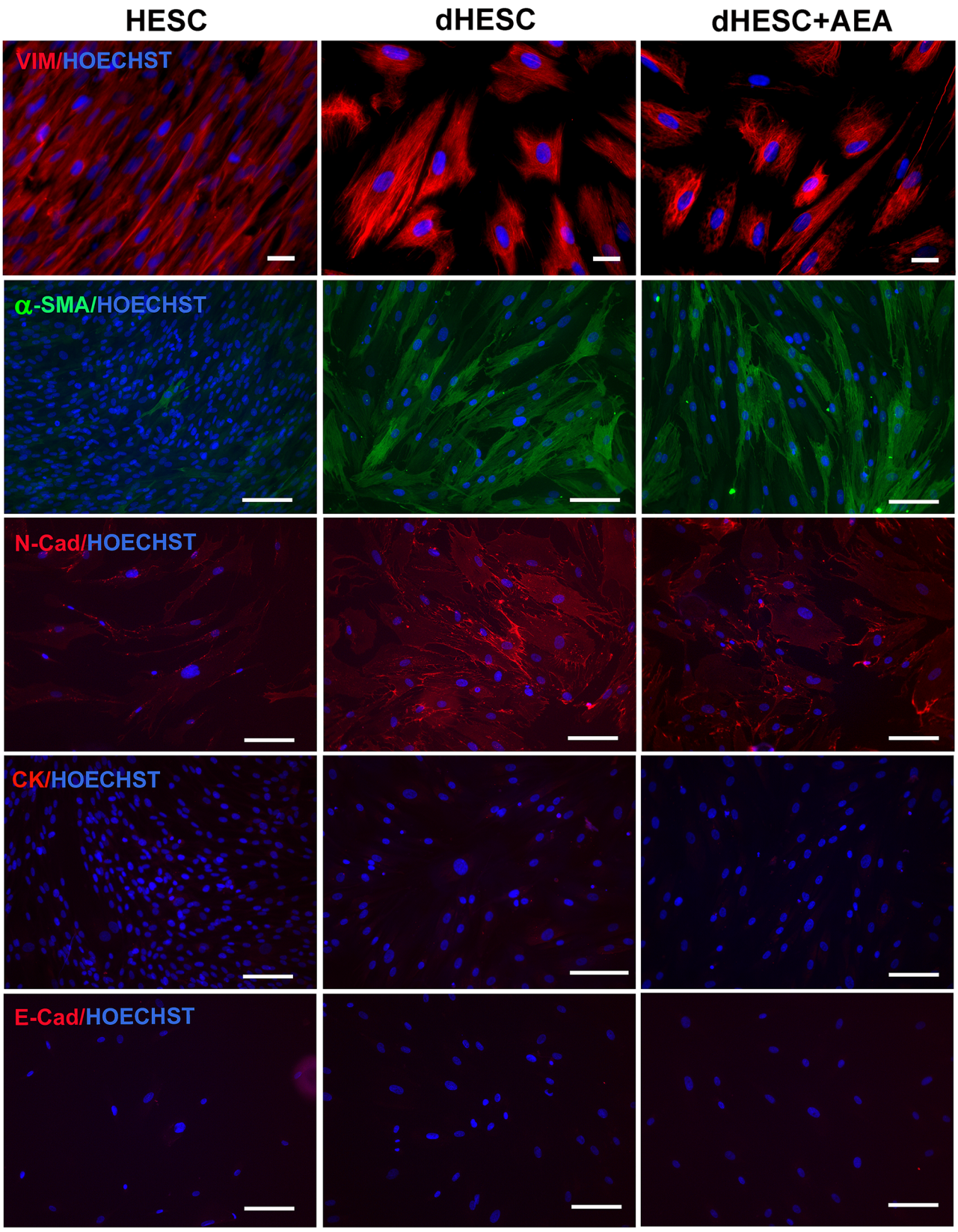


Figure 6 Anandamide (AEA)-induced modulation of cytoskeletal and adhesion-related markers in decidualizing human endometrial stromal cells (HESCs). Representative immunofluorescence images showing the expression and distribution of Vimentin (VIM), α-Smooth Muscle Actin (α-SMA), N-Cadherin (N-Cad), Cytokeratin (CK), and E-Cadherin (E-Cad) in undifferentiated human endometrial stromal cells (HESCs), decidualized HESCs (dHESCs), and dHESCs treated with 10 µM AEA for 72 hr (dHESC + AEA). Nuclei were counterstained with Hoechst 33242 (blue). Images are representative of three independent biological replicates from independently seeded and treated cultures. Scale bar: 25 µm (VIM); 50 µm (α-SMA, N-Cad , CK, and E-Cad).

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Discussion

Decidualization is increasingly recognized as a heterogeneous process involving distinct but coordinated regulatory components, including receptivity-associated transcription factors, inflammatory mediators, and cell-cycle arrest-related markers that collectively establish endometrial receptivity and maternal immune tolerance. Within this regulatory framework, the ECS is increasingly recognized as a modulator of endometrial receptivity and decidualization (Almada et al., 2016; Taylor et al., 2010). Anandamide, the primary endogenous endocannabinoid, exerts its biological effects predominantly through the CB1 receptor, although evidence suggests that other receptors may also be involved in mediating AEA responses (Di Marzo, 2008; Pertwee, 2015). Despite growing evidence for ECS involvement in reproductive physiology, the molecular mechanisms by which its components modulate decidualization remain incompletely understood (Ezechukwu et al., 2020; Maccarrone, 2009a; Paria et al., 1999b). In the present study, the potential crosstalk between ECS signalling and the NOTCH pathway, a key regulator of stromal cell differentiation and endometrial receptivity, was investigated (Afshar et al., 2012). The analysis was further extended to selected decidual regulatory components, including inflammatory and cell-cycle arrest-associated markers, together with cytoskeletal remodelling.

To this purpose, a well-established in-vitro model that mimics in-vivo decidual transformation (Doi-Tanaka et al., 2024), was used in this study. Anandamide significantly downregulated the decidual markers *FOXO1* and *IGFBP1* without affecting *PRL* expression. This selective effect suggests that AEA does not act uniformly on decidual differentiation but rather affects specific transcriptional components of the decidual program. Such distinct effect may depend on AEA-mediated regulation of pathways upstream of *FOXO1*, whose expression regulates, in turn, the expression of *IGFBP1* but not that of *PRL*, confirming that different pathways are at play to drive endometrial receptivity (Brosens et al., 1999; Gellersen & Brosens, 2014; Ujvari et al., 2017).

Several studies have demonstrated that the NOTCH1 signalling pathway is a key regulator of decidualization (Afshar et al., 2012; Y. Wu et al., 2020). Su et al. further clarified this role by showing that reduced NOTCH1 activation in human endometrial stromal cells leads to *HEY1* downregulation, which is associated with reduced *FOXO1* expression (Su et al., 2015, 2016). These findings support the existence of a NOTCH1–HEY1–FOXO1 regulatory axis, in which HEY1 acts as an intermediate transcriptional regulator promoting FOXO1 expression, one of the master regulators of decidualization. Disruption of NOTCH1 impairs decidual transformation in both in-vitro and in-vivo models (Su et al., 2015; Su & Fazleabas, 2015). In this respect, in the present study, the impact of AEA on the NOTCH pathway was assessed. To the best of our knowledge, no previous studies have investigated whether AEA modulates NOTCH signalling during endometrial stromal cell decidualization. The data show that AEA reduced NOTCH1 activation in decidualizing endometrial stromal cells, as indicated by decreased levels of the cleaved intracellular form of NOTCH1 and reduced *HEY1* expression, after 24 and 72 hr of AEA treatment. This effect may be mediated by the impact of endocannabinoid signalling on γ-secretase activity, as reported in other cell systems (Frampton et al., 2010). Although this study focused specifically on NOTCH1, due to its well-established role in decidualization (Afshar et al., 2012; Su et al., 2015; Y. Wu et al., 2020), and the consistent modulation observed upon AEA treatment, other NOTCH receptors (NOTCH2–4) may also participate in endometrial stromal cell differentiation. Therefore, further mechanistic studies, including NOTCH gain- and loss-of-function approaches, in decidualized cells exposed to AEA, are warranted to establish a direct causal relationship between ECS and NOTCH pathways.

A key regulator of endometrial receptivity, stromal decidual competence, and implantation is HOXA10 (Ashary et al., 2020; Du & Taylor, 2015). Therefore, its expression was analysed to determine whether it was affected by AEA exposure during decidualization. In the present model, *HOXA10* showed a profile distinct from that of *FOXO1*, *IGFBP1*, and *PRL*, as its expression was reduced during decidualization and remained low in AEA-treated dHESCs. Thus, the effects of AEA on the markers examined were not accompanied by detectable changes in HOXA10 expression. However, this finding does not exclude a contribution of HOXA10 at earlier stages of decidualization, and this reduction does not necessarily conflict with the established role of HOXA10 in decidual competence, as HOXA10 regulation during endometrial receptivity and decidualization is highly dynamic and stage dependent.

Recent evidence indicates that HOXA10 contributes to the temporal control of decidual inflammation and that its dysregulation is associated with impaired implantation and early pregnancy loss (Sharma et al., 2026). In line with this, the expression of selected inflammatory mediators, such as *IL1B*/IL-1β, *CXCL8*/IL-8 and *PTGS2*/COX-2, was significantly upregulated during decidualization. Anandamide did not modify *HOXA10* expression, while it reduced selected inflammatory mediators, particularly *IL1B*/IL-1β and *PTGS2*/COX-2. These data suggest that AEA modulates the inflammatory component of the decidual program without a detectable effect on *HOXA10* expression. More recently, immune-associated transcriptional regulators have also emerged as critical components of the decidual program. In particular, the autoimmune regulator AIRE, originally characterized for its role in central immune tolerance, has been identified as a local regulator of murine uterine decidualization, supporting the concept that immune regulatory pathways actively participate in stromal differentiation and establishment of a receptive decidual environment (Soumya et al., 2016). Within this framework, the ability of AEA to attenuate selected inflammatory mediators is consistent with a possible role for endocannabinoid signalling in the regulation of immune-associated pathways during decidualization, although whether AIRE is involved in AEA-mediated effects remains to be established. In addition to the inflammatory component, decidualization has been reported to involve a transient senescence-like response in a subset of endometrial stromal cells, contributing to cell-cycle arrest, tissue remodelling and inflammatory mediator production (Brighton et al., 2017; Tomari et al., 2020). In this context, the different modulation of *CDKN1A*/p21 and *CDKN2A*/p16 suggests that, in this relatively short-term decidualization model, the cell-cycle arrest component associated with stromal differentiation does not correspond to a fully established senescent phenotype. This interpretation is consistent with the notion that *CDKN1A*/p21 and *CDKN2A*/p16 belong to distinct senescence-associated pathways, with *CDKN1A*/p21 reflecting a more dynamic cell-cycle arrest/stress response and *CDKN2A*/p16 being more commonly linked to a stable senescence program

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(Kumari & Jat, 2021; Wagner & Wagner, 2022). The reduction of *CDKN1A*/p21 after AEA exposure, together with decreased *IL1B*/IL-1β and *PTGS2*/COX-2 expression, suggests that AEA attenuates selected inflammatory and cell-cycle arrest-associated features of the decidual program, without inducing a generalized modulation of senescence-associated markers.

To further define the ECS components potentially involved in AEA-mediated effects, the expression of the main AEA-responsive receptors was analysed. *CNR1* was upregulated upon decidualization, whereas *CNR2* remained undetectable, supporting a predominant role for CB1 in endometrial stromal cell function (Fonseca et al., 2013; Neradugomma et al., 2019). *TRPV1* was also expressed and remained stable during decidualization. Although its functional involvement was not directly addressed, TRPV1 may contribute to AEA responsiveness through calcium-dependent mechanisms, which have been implicated in NOTCH1 cleavage and signalling (Hounjet & Vooijs, 2021; Shuba, 2021). Anandamide reduced *CNR1* mRNA levels in dHESCs without producing a corresponding decrease in CB1 protein expression. This discrepancy may reflect post-transcriptional regulation, receptor turnover, internalization, or desensitization, as previously described for cannabinoid receptors (Haspula & Clark, 2020; Hsieh et al., 1999; Wu et al., 2008) and is consistent with previous reports showing modulation of *CNR1* transcripts without major changes in CB1 protein levels after cannabinoid exposure (Almada et al., 2016; Neradugomma et al., 2019). Overall, this expression profile supports CB1, and potentially TRPV1, as candidate mediators of AEA action, although receptor-specific functional studies are required to define their relative contribution to NOTCH1 modulation and decidual remodelling.

NOTCH signalling participates in transcriptional programs regulating cellular plasticity, mesenchymal-associated features and cytoskeletal organization, including *SNAI1* expression, cadherin dynamics and cytoskeletal marker regulation (Wang et al., 2024). Recent evidence further indicates that cytoskeletal remodelling is not merely a structural consequence of decidualization but actively contributes to the transcriptional regulation of the decidual program. In particular, silencing of the cytoskeletal regulator dedicator of cytokinesis 1 (*DOCK1/DOCK180)* abolishes decidualization by preventing the nuclear translocation of AIRE, thereby linking cytoskeletal organization with immune-associated transcriptional regulation (Mohan et al., 2018). Furthermore, *DOCK1* knockdown has recently been shown to reduce NOTCH signalling activity in acute myeloid leukaemia models (Lin et al., 2025). Although a functional relationship between DOCK1 and NOTCH signalling has not yet been demonstrated in decidualizing endometrial stromal cells, these findings raise the possibility that cytoskeletal dynamics and NOTCH signalling participate in coordinated regulatory networks contributing to the establishment of the decidual phenotype. In line with this, the attenuation of NOTCH1/*HEY1* signalling observed following AEA exposure may contribute to the structural and adhesion-related changes detected in decidualizing stromal cells. In decidualizing HESCs, decidualization was associated with marked morphological remodelling, increased *SNAI1* expression, reorganization of vimentin filaments into thick parallel bundles, persistent α-SMA expression and increased N-Cad signal. Conversely, CK and E-Cad were undetectable. This profile suggests that decidualization involves cytoskeletal and adhesion-related remodelling while maintaining a stromal/mesenchymal identity, in line with evidence describing decidualization as a dynamic process of stromal plasticity and tissue remodelling (Doi-Tanaka et al., 2024; Pan-Castillo et al., 2018; L. Wu et al., 2020; Zhang et al., 2013). Consistently, *SNAI1* increased in primary decidualized stromal cells and in secretory-phase endometrial tissue, compared with proliferative, further supporting its association with stromal remodelling during decidual preparation and implantation-related tissue adaptation (Ma et al., 2006; L. Wu et al., 2020). Anandamide partially altered this structural organization by reducing *SNAI1* expression and modifying vimentin and N-Cad organization, while α-SMA remained expressed and epithelial markers remained undetectable. These observations suggest that AEA interferes with selected transcriptional, cytoskeletal and adhesion-related features required for the full organization of the decidual phenotype. Together with the attenuation of NOTCH1/*HEY1* signalling, these findings support the view that AEA affects specific structural components of the decidual program, including cytoskeletal organization and adhesion-related features, while preserving the overall stromal identity of decidualizing cells.

Overall, the present findings support the hypothesis that AEA selectively modulates multiple components of the decidual program rather than uniformly affecting stromal decidual differentiation. Although further mechanistic studies are required to establish whether NOTCH1 directly mediates the effects of AEA on decidual regulatory programs, these data support a previously unexplored crosstalk between endocannabinoid signalling and NOTCH1 signalling in human endometrial stromal cells. This may contribute to a better understanding of how altered endocannabinoid signalling influences endometrial receptivity, implantation-related processes and fertility.

Supplementary material

Supplementary material is available at *Reproduction* online.

Author contributions

Conceptualization: L.C., G.L.S., and M.Maccarrone; Methodology: V.G., C.B., T.C., and N.B.; Formal analysis: G.L.S., V.L., T.C., and M.Massimiani; Statistical analysis: L.C. and G.L.S.; Investigation: I.C., C.S. T.C., and V.L. Resources: L.C.; Visualization: I.C., V.L., and G.L.S.; Project administration: L.C.; Supervision: L.C. and G.L.S.; Writing—original draft: L.C. and G.L.S.; Writing—review & editing: L.C., G.L.S., M.Maccarrone, V.L., and M.Massimiani.

Conflicts of interest

The authors declare no conflict of interest to disclose.

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Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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