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IL17A Suppresses *IGFBP1* in Human Endometrial Stromal Cells

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Abstract: Interleukin (IL) 17A has been implicated in preeclampsia, preterm labor, and miscarriage. IL17A production in non-lymphoid tissues is mainly carried out by unconventional γδ17T cells. Innate lymphoid cells (ILCs) 3, a subgroup of innate lymphocytes, can also be a source of IL17A in the endometrium and are required from implantation to early pregnancy, with their regulation ensuring that pregnancy continues. Herein, we examined the expression of γδ17T cells and ILC3 regulators *IL1B*, *IL23A*, and *IL17D* and IL17A receptors (*IL17RA*/*IL17RC*) in human endometrial stromal cells (EnSCs) and cell lines (KC02-44D). Accordingly, quantitative polymerase chain reaction and immunoblotting were employed. *IL1B*, *IL23A*, and *IL17D* were significantly upregulated in decidualized EnSCs and KC02-44D cells. A significant augmentation in *IL17RA*/*IL17RC* was also observed in decidualization. IL17A stimulation of KC02-44D cells during decidualization suppressed the decidualization marker *IGFBP1*. The involvement of transcription factor Forkhead box protein O1 (FOXO1) in this repression was reflected by its translocation from the nucleus into the cytoplasm. A role for IkB kinase alpha in FOXO1 phosphorylation-mediated migration was also suggested. Taken together, our findings indicate that the secretion of IL17A by γδ17T and ILC3 cells in the uterus contributes to EnSCs function and may play critical roles in regulating IGFBP1-mediated implantation and fetal growth.

Keywords: innate immune response; IL17A; IGFBP1; IL17D; stromal cells; embryo implantation

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1. Introduction

The human menstrual cycle takes approximately 28 days, during which the proliferation of the functional layer, secretory changes in epithelial cells, stromal cell decidualization, and menstruation occur periodically within the endometrium [1]. After the follicle transforms into the corpus luteum, the uterine gland secretion is activated by progesterone, which is produced by the corpus luteum. Progesterone also acts on endometrial stromal cells (EnSCs) to induce morphological and functional differentiation, known as decidualization, thus providing a microenvironment suitable for implantation of the embryo in humans. Upon decidualization, EnSCs produce and secrete cytokines, chemokines, and growth factors, such as insulin-like growth factor binding protein 1 (IGFBP1), angiogenic factors, and prolactin (PRL) [2]. Abnormal decidualization leads to placental dysplasia and underdevelopment of the spiral artery, which can cause subsequent implantation failure and miscarriage [2–4].

In early pregnancy, the embryonic and maternal cells have been found to interact [5]. The prediction of receptor–ligand molecular pairs in the placenta and decidua of women at 6–14 weeks of gestation indicated the presence of pregnancy-specific cell subsets and revealed that EnSCs and immune cells in the decidua interact with embryo-derived extravillous trophoblasts. Data suggest that the inflammatory cytokine interleukin (IL)17A may have a pathological role in preeclampsia, premature birth, and miscarriage, with previous studies having focused on IL17A produced by immune cells at the maternal–fetal interface [6–8]. IL17A is part of the IL17 family, which plays important roles in immune responses and host defense [9,10]. Interestingly, members of this family have been proposed to have unique and non-overlapping functions against cancer, autoimmune disease, and

infection. IL17A, the first member of this family to be identified, plays a critical role during infection. Persistently high levels of IL17A and homologous proteinIL17F induce inflammation. Meanwhile, IL17B has been implicated in tumorigenesis [11]. IL17C influences mucosal barrier integrity and is implicated in autoimmune diseases [12,13]. IL-17D is a newly identified cytokine, whose receptor was recently identified as CD93 [14,15]. IL-17E (IL-25) enhances allergic inflammatory responses and is involved in host defense [16–18]. A dysregulation of IL17A has been reported in unexplained recurrent pregnancy loss [19–22]. There are emerging reports that endometrial IL17A promotes trophoblast migration [23,24].

Originally, IL17A was thought to be produced mainly by Th17 cells, with a subsequent report showing that IL17A-secreting Th17 cells induced human trophocyte invasion in first-trimester trophocytes [25]. However, γδ17T cells have recently attracted attention as an alternative source of IL17A in non-lymphoid tissues, including the uterus [26–28]. Murine uterine γδT cells produce high levels of IL17A; therefore, they are called γδ17T cells [23]. Further, the reduction of Th17 cell reactivity and reversal of the Th17/Treg imbalance did not improve pregnancy outcomes in recurrent implantation failure, suggesting that Th17 cell-derived IL17A exerts minimal effects in the endometrium. Innate lymphoid cells (ILCs) 3 are another potential source of IL17A within the endometrium, as they express RORɣt [29–32]. Although ILC3 almost disappear from the decidua in late pregnancy [30,33], an increase in ILC3 numbers in the decidua causes early delivery [34], suggesting the need for ILC3 production between implantation and early pregnancy as well as its suppression during continued pregnancy. Decreased ILC production or abnormal functional interactions of ILCs with the human decidua may cause fetal death [35]. Despite a marked increase in ILC3 within the endometrium during the implantation window being associated with autoimmune thyroid disease and female infertility, the function of ILC3 has been reported to be suppressed in that period [36]. Thus, we hypothesized that γδ17T cells and ILC3 may be regulated by endometrium-derived cells, as well as other immune cells, including uterine natural killer (uNK) cells specifically found in the endometrium [33,37].

In this study, we observed for the first time the upregulation of *IL1B*, *IL23A* (which enhances the production of IL17A by γδ17T [38–40] and ILC3 [41]), and *IL17D* (which specifically regulates ILC3 function via the CD93 receptor [14,15]) in decidualized EnSCs. Based on the increase in IL17A receptor expression during decidualization, we investigated whether IL17A secreted by activated-γδ17T and ILC3 could affect decidualization through a feedback mechanism. IL17A downregulated decidualization marker *IGFBP1* in decidualized EnSCs and the KC02-44D cell line and regulatory mechanisms for *IGFBP1* downregulation by Forkhead box protein O1 (FOXO1) phosphorylation.

2. Materials and Methods

2.1. Ethical Statement

The study was explained to all eligible patients, and we obtained informed consent from all participants. The Kansai Medical University review board approved this study (ID: 2006101), and it was conducted in accordance with the Helsinki Declaration. Human uteri with a benign myoma were donated by six patients (42–50 years) with regular menstrual cycles (Table 1). Patients who received preoperative hormone therapies were not included in this study. Histologically normal endometria were obtained from all subjects.

Table 1. Patient information.

Sample No.	Materials	Methods		Age, Years	Menstrual Cycle Phase at the Time of Collection
1	Primary culture EnSCs	Treated with E2 + MPA for 12 days	RT-qPCR	50	Proliferative
2	Primary culture EnSCs	Treated with E2 + MPA for 12 days	RT-qPCR	45	Mid-secretory
3	Primary culture EnSCs	Treated with E2 + MPA for 12 days	RT-qPCR	48	Late secretory
4	Primary culture EnSCs	Treated with E2 + MPA for 12 days	RT-qPCR	50	Mid-secretory
5	Primary culture EnSCs	Treated with E2 + MPA for 12 days	RT-qPCR	44	Late secretory
6	Primary culture EnSCs	Treated with E2 + MPA for 12 days	RT-qPCR	49	Proliferative

RT-qPCR, reverse transcription–quantitative polymerase chain reaction; E2, estradiol; MPA, medroxyprogesterone acetate; EnSC, endometrial stromal cells.

2.2. Decidualization of Human EnSCs

Human EnSCs were purified from the endometrium through a previously described method [42]. For EnSCs culture, DMEM-F12 medium without phenol red was purchased from Thermo Fisher Scientific (Waltham, MA, USA) and supplemented with Glutamax (2 mmol/L, Thermo Fisher Scientific), 10% Charcoal Stripped-Fetal Bovine Serum(CS-FBS; Biowest, Nuaillé, France), streptomycin (100 µg/mL), and penicillin (100 IU/mL) (DMEM-F12/CS-FBS medium). The cells were then cultured in a 37 °C humidified environment with 5% CO_2 until near confluence, changing the medium every 3 days. EnSCs were seeded anew, and grown until confluence prior to experiments. EnSCs were then stimulated with 10^{-8} mol/L estradiol (E2) and 10^{-7} mol/L medroxyprogesterone acetate (MPA) for up to 2 weeks for induction of decidualization [43,44]. Unstimulated cells were prepared as a control group.

2.3. Human EnSC Cell Line KC02-44D and Treatment

Because patient-derived EnSCs can vary greatly in their responsiveness to stimulation, owing to the influence of many confounding factors, including differences in the timing of collection during the menstrual cycle, the expression of cytokines that may regulate IL17A-producing cells was examined using KC02-44D cells (American Type Culture Collection, Manassas, VA, USA), an established human EnSCs cell line [45]. KC02-44D were cultured in DMEM medium without phenol red containing Glutamax (2 mmol/L, Thermo Fisher Scientific), 10% Fetal Bovine Serum (EU Origin), Charcoal Stripped (CS-FBS, Biowest, France), streptomycin (100 µg/mL), and penicillin (100 IU/mL). Cells were cultured until near confluence, changing the medium every 3 days. KC02-44D were treated with 10^{-8} mol/L E2, 10^{-7} mol/L MPA, and 0.5 mM 8-Bromo-cAMP (Sigma-Aldrich Co., LLC, St. Louis, CO, USA) (E2 + MPA + cAMP treatment) for up to 6 days for the induction of decidualization [46]. Unstimulated cells were prepared as a control group. To test the effects of IL17A on decidualization, in addition to the control and E2 + MPA + cAMP treatments, 10 ng/mL IL17A (Recombinant Human IL-17A, Fujifilm Corp., Tokyo, Japan) or E2 + MPA + cAMP + 10 ng/mL IL17A stimulations were conducted for up to 6 days.

2.4. Quantitative Polymerase Chain Reaction (qPCR)

The extraction of total RNA from EnSCs or KC02-44D cells with or without treatment was conducted using the Sepasol®-RNA I Super G (Nacalai Tesque Inc., Kyoto, Japan). Reverse transcription was performed using a ReverTraAce qPCR RT master mix with gDNA remover (Toyobo, Osaka, Japan). qPCR analysis was conducted on a LightCycler96 (Roche Diagnostics K.K., Tokyo, Japan) and Thunderbird Next qPCR Mix (Toyobo). The qPCR primers are presented in Table 2. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method [47]. Hypoxanthine-phospho-ribosyl-transferase 1 (*HPRT1*) was used as a housekeeping gene.

Table 2. Primers for qPCR.

Gene Symbol	Definition	Primer Name	Sequence (5′-3′)
HPRT1	Hypoxanthine Phosphoribosyltransferase 1	895F 1034R	CTAGTTCTGTGGCCATCTGCTTAG GGGAACTGATAGTCTATAGGCTCATAGTG
PRL	Prolactin	374F 623R	ATTCGATAAACGGTATACCCATGGC TTGCTCCTCAATCTCTACAGCTTTG
IGFBP1	Insulin-like Growth Factor Binding Protein 1	636F 791R	CTATGATGGCTCGAAGGCTC TTCTTGTTGCAGTTTGGCAG
HAND2	Heart and Neural Crest Derivatives expressed 2	1479F 1552R	AGAGGAAGAAGGAGCTGAACGA CGTCCGGCCTTTGGTTTT
IL15	Interleukin 15	165F 351R	GTTCACCCCAGTTGCAAAGT CCTCCAGTTCCTCACATTC

Table 2. *Cont.*

Gene Symbol	Definition	Primer Name	Sequence (5′-3′)
IL1B	Interleukin 1 beta	162F 305R	AGCTGATGGCCCTAAACAGATG TTGTCCATGGCCACAACAAC
IL23A	Interleukin23, alpha subunit p19	71F 196R	ATCAGGCTCAAAGCAAGTGG AGCAACAGCAGCATTACAGC
IL17A	Interleukin 17A	1685F 1777R	TCTCTTCCTCAAGCAACACTCC AAAGTTCGTTCTGCCCCATC
IL17B	Interleukin 17B	594F 667R	GCACCTGCATCTTCTGAATCAC ACAAAGGTGCAAGGAGGATG
IL17C	Interleukin 17C	883F 1012R	TGCAGAAAAGGTGTCACACG AAACAGGGGTACTTCCAAGGAG
IL17D	Interleukin 17D	1791F 1925R	TGGAACGTGACATCTTTGCC AAGCCTCCAGATTGATCTCTGC
IL17E (IL25)	Interleukin 17E	736F 862R	AGGCTGTACCGTGTTTCCTTAG CCTTCATGGCAAGTGGTTGTAC
IL17F	Interleukin 17F	259F 398R	ATGAAAACCAGCGCGTTTCC ATTGATGCAGCCCAAGTTCC
IL17RA	Interleukin 17 Receptor A	793F 923R	TGACCAGTTTTCCGCACATG ACAGCACCCTTTAAGGTTGC
IL17RC	Interleukin 17 Receptor C	877F 1000R	TGCAGTTTGGTCAGTCTGTG TGCTGTGTGTGGTTGAGTTC
IL22RA1	Interleukin 22 Receptor Subunit Alpha 1	652F 780R	TGGCACCATCATGATTTGCG AAGCCCATGGAGAACAGGAAG

2.5. Western Blotting

To determine whether IL17A stimulation led to FOXO1 phosphorylation, we examined the downstream pathways of IL17A. IL17A binds to the IL17RA homodimer, IL17RC homodimer, and/or IL17RA-IL17RC heterodimer [48] and activates intracellular ACT1 by changing its steric structure, subsequently transmitting the signal to IkB kinase alpha (IKKA)/IKKB [49]. In contrast, the downstream regulation of Akt and FOXO1 phosphorylation by IKKA has been previously described [50].

The soluble fraction was prepared from KC02-44D cells cultured with or without stimulation using MPER reagent (Thermo Fisher Scientific) and protease inhibitors (Roche Diagnostics K.K.). Western blotting was conducted to quantify IKKA and IKKB protein levels. Proteins were separated on a 10% sodium dodecyl sulfate (SDS)–polyacrylamide gel and transferred onto a PVDF membrane, which was then blocked using Blocking One (Nacalai Tesque Inc.). The membrane was then incubated with 1/1000 IKKβ (D30C6) rabbit mAb (#8943, Cell Signaling Tech, Danvers, MA, USA, RRID: AB_11024092), 1/1000 IKKα (D3W6N) rabbit mAb (#61294, Cell Signaling Tech, RRID: AB_2799606), or 1/10,000 mouse β-actin antibody (Sigma-Aldrich, RRID: AB_476743, Cat# A5316) in TBS containing 5% Blocking One and 0.1% Tween-20 overnight at 4 °C. After washing, the membrane was incubated with 1:5000 Goat anti-rabbit-HRP (RRID: AB_2336198, VECTOR Laboratories, Burlingame, CA, USA, Cat# PI-1000) or 1:10,000 sheep anti-mouse-HRP (RRID: AB_772210, GE Healthcare Life Science, Chicago, IL, USA, Cat# NA931). The resulting complexes were visualized using Chemi-Lumi One L (Nacalai Tesque Inc.) and LAS 4000 (GE Healthcare Life Science). ImageJ 1.54g software (National Institutes of Health, Maryland, MD, USA) was used to determine band intensity as previously described [51]. ACTB was used as a loading control.

2.6. Immunocytochemistry

The sterilized cover glass was laid on a 6-well plate, and 0.3 × 10^6 KC02-44D cells were seeded onto it. Three groups were studied: (I) control, (II) E2 + MPA + cAMP-treated, and (III) E2 + MPA + cAMP + 10 ng/mL IL17A-treated groups. After 6 days with medium change every 3 days, cultured KC02-44D were subjected to formaldehyde fixation for 5 min at 22 °C and were then permeabilized with methanol for 5 min at 4 °C. KC02-44D were then incubated with a primary antibody (Foxo1-rabbit-mAb (C29H4) #2880 diluted to 1/1000, Cell Signaling Tech, RRID: AB_2106495) overnight at 4 °C, followed by incubation with a goat anti-rabbit-Alexa488 (# A-11008, Molecular Probes, US-OR, RRID: AB_143165) diluted 1/3000 and 1 µg/mL propidium iodide. LSM700 (Carl Zeiss Co., Ltd., Tokyo, Japan) was used to observe the stained cells. Positive staining rates in the nucleus and cytoplasm were calculated and averaged by visually counting the cytoplasm-stained, nucleus-localized, and cytoplasm-localized FOXO1 in images obtained from two independent individuals. In total, 15 images (945 cells) were taken in group I, 15 images (552 cells) in group II, and 15 images (552 cells) in group III of an area covering 1 mm^2. These images were used for analysis.

2.7. Statistical Analyses

The normality of data was confirmed using the Shapiro–Wilk test. Welch's *t*-test was used to compare the means of the two groups with the Bonferroni correction. *p*-values of < 0.05 were considered as indicative of statistical significance. SPSS software was used for all statistical analyses (version 22.0; IBM Corp., Armonk, NY, USA).

3. Results

3.1. Elevations in IL1B, IL23A, and IL17D in Decidualized EnSCs

After 12 days of decidualization treatment of primary culture EnSCs, *IL1B* and *IL23A* were significantly upregulated compared with their levels in unstimulated cells (control) ($p < 0.05$) (Figure 1). Further examination of changes in the expression of IL17 gene family members revealed no expression of *IL17A*, *IL17E* (*IL25*), or *IL17F* in EnSCs. In contrast, *IL17B*, *IL17C*, and *IL17D* were detected, and a significant upregulation of *IL17D* was observed during decidualization ($p < 0.05$, Figure 1).

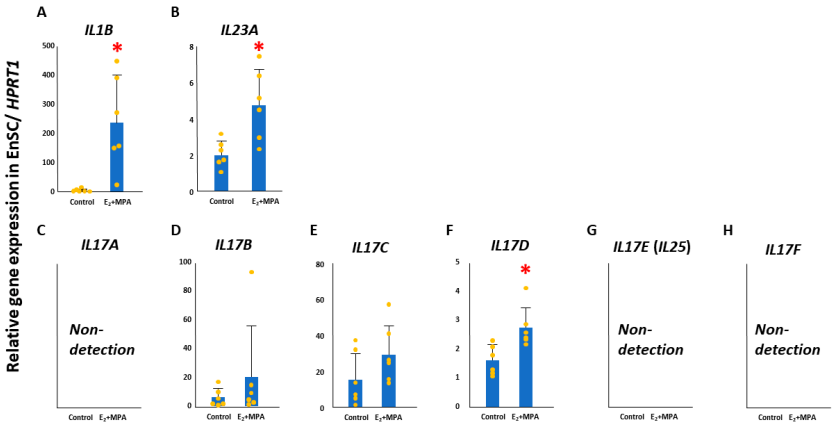

Figure 1. Changes in *IL1B*, *IL23A*, and *IL17D* expression in decidualized endometrial stromal cells (EnSCs). The significant increases in *IL1B* ((**A**), $p < 0.05$), *IL23A* ((**B**), $p < 0.05$), and *IL17D* ((**F**), $p < 0.05$) were observed in decidualized EnSCs. No significant differences in *IL17A* (**C**), *IL17B* (**D**), *IL17C* (**E**), *IL17E* (**G**), and *IL17F* (**H**). * significantly different from the control ($p < 0.05$). *HPRT1*, hypoxanthine phosphoribosyltransferase 1; E2, estradiol; MPA, medroxyprogesterone acetate; *IL*, interleukin; *IL1B*, interleukin 1 beta; *IL23A*, interleukin 23 subunit alpha.

3.2. Cytokines in KC02-44D Cells

Significant upregulation of *IL1B* and *IL23A* was shown in decidualized KC02-44D compared with their levels in control cells ($p < 0.05$, Figure 2). Meanwhile, there was no

change in *IL17A*, *IL17B*, *IL17C*, *IL17E*, or *IL17F* (Figure 2). *IL17D*, however, was significantly upregulated in KC02-44D cells during decidualization ($p < 0.05$; Figure 2).

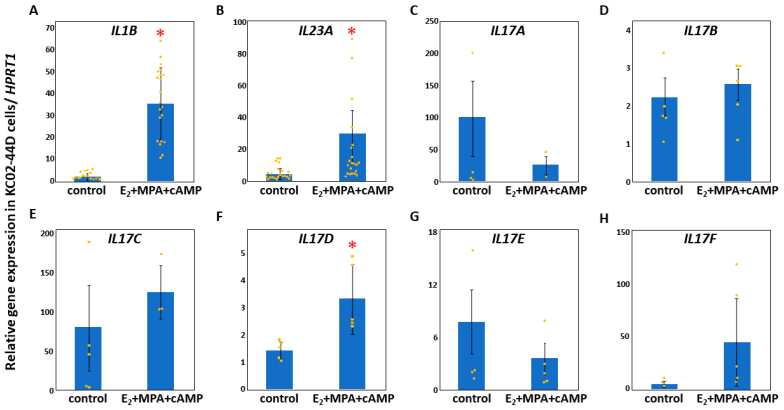

Figure 2. Cytokine expression in KC02-44D cells. Significant increases in *IL1B* ((**A**), $p < 0.05$), *IL23A* ((**B**), $p < 0.05$), and *IL17D* ((**F**), $p < 0.05$) were observed in decidualized KC02-44D cells. No significant differences were noted in *IL17A* (**C**), *IL17B* (**D**), *IL17C* (**E**), *IL17E* (**G**), and *IL17F* (**H**). * significantly different from control ($p < 0.05$). *HPRT1*, hypoxanthine phosphoribosyltransferase 1; E2, estradiol; MPA, medroxyprogesterone acetate; *IL*, interleukin; *IL1B*, interleukin 1 beta; *IL23A*, interleukin 23 subunit alpha.

3.3. Responsiveness of EnSCs to γδ17T and ILC3-Derived Cytokines

Activated γδ17T and ILC3 cells secrete IL17A and IL22. IL17A receptor-encoding genes *IL17RA* and *IL17RC* [48] were significantly upregulated in decidualized EnSCs (Figure 3, $p < 0.05$). Meanwhile, there was no expression of *IL22RA1*, which encodes an IL22 receptor, in the EnSCs (Figure 3). Receptor gene expression was confirmed in human endometrial tissue.

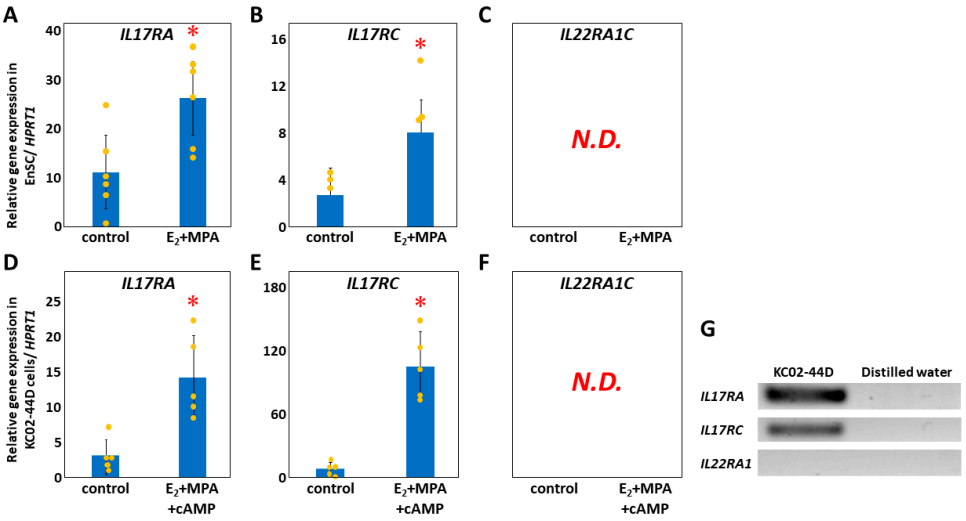

Figure 3. Responsiveness of decidualized endometrial stromal cells (EnSCs) and KC02-44D cells to ILC3-derived cytokines. Significant increases in *IL17RA* [(**A**,**D**), $p < 0.05$] and *IL17RC* [(**B**,**E**), $p < 0.05$] and no significant differences in *IL22RA1C* (**C**,**F**) were observed in decidualized EnSCs and KC02-44D cells. * $p < 0.05$ vs. control using Welch's *t*-test. PCR and agarose gel electrophoresis were performed using distilled water as a negative control to confirm their presence or absence, and *IL17RA* and *IL17RC* signals were con-firmed to have specific sample-dependent amplifications (**G**). *HPRT1*, hypoxanthine phosphoribosyltransferase 1; E2, estradiol; MPA, medroxyprogesterone acetate; *IL17RA*, interleukin 17 receptor A; *IL17RC*, interleukin 17 receptor C; *IL22RA1*, interleukin 22 receptor subunit alpha 1; *N.D.*, no detection.

Subsequently, KC02-44D cells were subjected to a 6-day decidualization treatment to examine changes in interleukin receptor expression. qPCR analysis confirmed a significant upregulation of *IL17RA* and *IL17RC* in decidualized KC02-44D ($p < 0.05$). *IL22RA1* expression was not detected (Figure 3).

3.4. IL17A Treatment of KC02-44D Cells

As *IL17RA* and *IL17RC* expression was detected in EnSCs, IL17A secreted from activated γδ17T and ILC3 cells might have some effect on decidualization. Therefore, we treated KC02-44D cells for 6 days with 0, 0.1, 1.0, 10, or 100 ng/mL IL17A, based on previous studies [37] to examine changes in *PRL*. A significant increase in *PRL* was observed in the decidualized group relative to the control group. However, no effect of IL17A was observed. Because there was no clear difference in the effect of IL17A concentration on *PRL*, we examined its effect on other genes using concentrations from previous studies (10 ng/mL) [52].

3.5. Effect of IL17A on Decidualization Markers in KC02-44D Cells

Next, we examined the decidualization markers [53] in decidualized KC02-44D cells treated with 10 ng/mL IL17A. Although *IGFBP1* was significantly upregulated in decidualized KC02-44D ($p < 0.05$), significant suppression of *IGFBP1* was observed in the IL17A-stimulated cells compared to levels in decidualized KC02-44D ($p < 0.05$, Figure 4). Further, significant upregulation of *PRL*, *IL15*, *HAND2*, and *IL17D* was noted in the decidualized KC02-44D ($p < 0.05$, Figure 4). There was no effect of additional IL17A stimulation in E2 + MPA+ cAMP-treated cells (Figure 4).

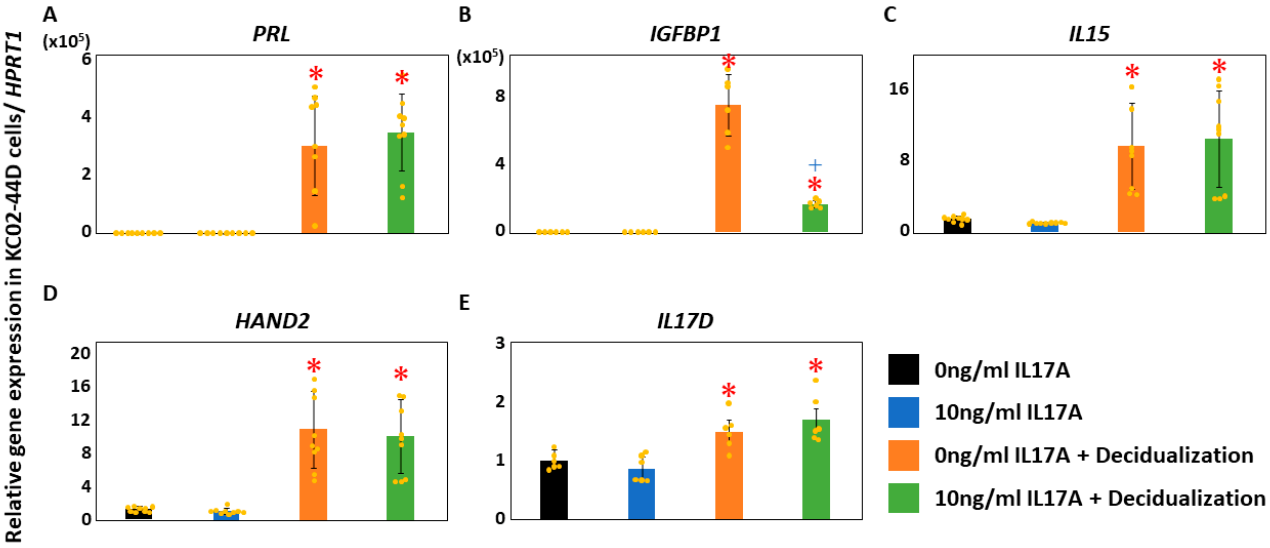

Figure 4. Effect of IL17A on decidualization markers in KC02-44D cells. Significant upregulation of PRL [(**A**), $p < 0.05$], IGFBP1 [(**B**), $p < 0.05$], IL15 [(**C**), $p < 0.05$], HAND2 [(**D**), $p < 0.05$], and IL17D [(**E**), $p < 0.05$] was found in the decidualized KC02-44D cells. Additional IL17A stimulation only affected IGFBP1 expression ((**B**), $p < 0.05$) in E2 + MPA+ cAMP-treated cells. * $p < 0.05$ vs. control using Welch's *t*-test with Bonferroni correction. +: $p < 0.05$ vs. 0 ng/mL IL17A + decidualization using Welch's *t*-test with Bonferroni correction. *HPRT1*, hypoxanthine phosphoribosyltransferase 1; *PRL*, prolactin; *IGFBP1*, insulin-like growth factor-binding protein 1; *IL15*, interleukin 15; *HAND2*, heart and neural crest derivatives expressed 2; *IL17D*, interleukin 17 D; IL17A, interleukin 17 A.

3.6. Effect of IL17A on FOXO1 Localization in KC02-44D Cells

In KC02-44D cells, IL17A stimulation causes the phosphorylation of decidualization marker FOXO1 and its subsequent migration out of the nucleus, resulting in a downregulation of *IGFBP1* [54,55]. After KC02-44D cells were stimulated with or without IL17A for

6 days, we examined the subcellular localization of FOXO1 [56]. We expected to stain the nucleus with propidium iodide, but the cytoplasm and nucleolus were also fluorescently labeled (Figure 5, purple). This localization suggested that the RNA molecules were labeled with propidium iodide. FOXO1 was fluorescently labeled with a FOXO1 antibody and Alexa488-secondary antibody (green). Increased localization of FOXO1 in the nucleus was found in the decidualized cells in comparison with that in the untreated cells, with changes in FOXO1 localization to the cytoplasm observed following additional IL17A stimulation (Figure 5A). FOXO1 localization in the nucleus or cytoplasm was determined, and the frequency of FOXO1 relative to the total cell number included in the entire image was calculated for each image, whereafter differences in frequency were assessed. A significant increase in the nuclear localization of FOXO1 was found in decidualized cells and IL17A additionally stimulated cells in comparison with that in untreated KC02-44D controls ($p < 0.05$). Furthermore, a significant increase in FOXO1 translocation from the nucleus to the cytoplasm was observed in the IL17A additionally stimulated cells in comparison to that in decidualized cells ($p < 0.05$) (Figure 5B).

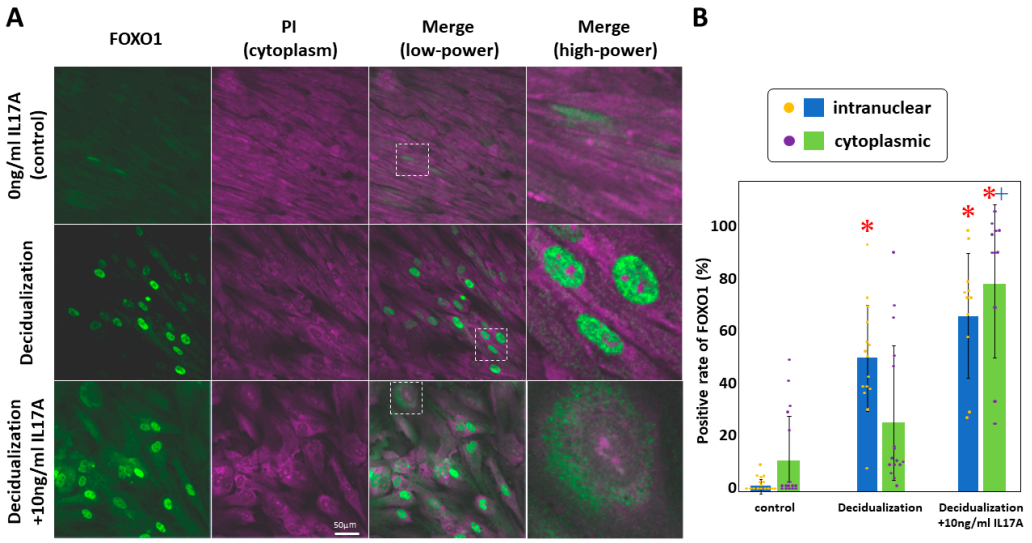

Figure 5. Effect of IL17A on FOXO1 in KC02-44D. The cytoplasm and nucleolus were stained with PI (purple). FOXO1 was fluorescently labeled using a FOXO1 antibody and an Alexa488-secondary antibody (green). The high-power images are magnified versions of the dashed squares in the low-power images (**A**). Localization in the nucleus or cytoplasm was determined, whereafter the frequency was calculated relative to the number of cells in the entire image covering an area of 1 mm^2 (**B**). * $p < 0.05$ vs. control using Welch's *t*-test with Bonferroni correction. + $p < 0.05$, vs. decidualization using Welch's *t*-test with Bonferroni correction. IL17A, interleukin 17 A; FOXO1, Forkhead box protein O1; PI, propidium iodide.

3.7. Mechanism of IL17A-Dependent FOXO1 Migration

To assess changes in the downstream signaling of IL17A, we detected IKKA and IKKB levels in KC02-44D cells after additional stimulation with IL17A. Normalized IKKA band densities were 2.59 ± 0.03 (mean ± SD, control), 1.09 ± 0.46 (decidualization), and 0.75 ± 0.14 (decidualization +IL17A) (Figure 6A). IKKB molecular densities were 0.13 ± 0.03 (control), 0.16 ± 0.02 (decidualization), and 0.16 ± 0.04 (decidualization +IL17A) (Figure 6A). We observed a significant decrease in IKKA ($p < 0.05$) and no change in IKKB in the IL17A-stimulated cells compared to control levels (Figure 6B).

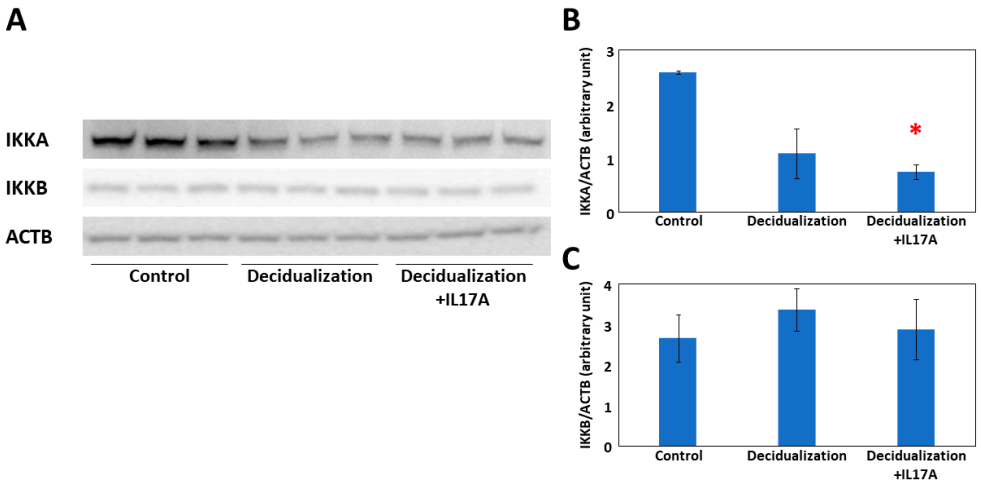

Figure 6. Mechanism of IL17A-dependent FOXO1 migration. After KC02-44D cells were stimulated with or without 10 ng/mL IL17A for 6 days, IKKA, IKKB, and ACTB levels were quantified (**A**). The obtained signals were analyzed using ImageJ and normalized to ACTB band intensity. Significant suppression of IKKA was noted with IL17A stimulation ((**B**), $p < 0.05$). There was no significant difference in IKKB among the three groups (**C**). * $p < 0.05$ vs. control using Welch's *t*-test with Bonferroni correction. IL17A, interleukin 17 A; IKKA (CHUK), component of inhibitor of nuclear factor kappa B kinase complex; IKKB (IKBKB), inhibitor of nuclear factor kappa B kinase subunit beta; ACTB, actin beta.

4. Discussion

In this study, decidualization of EnSCs isolated from the human endometrium and of the endometrial stromal cell line KC02-44D induced a significant upregulation of *IL1B*, *IL23A*, and *IL17D* expression [15,41], affecting the proliferation, differentiation, and functional regulation of γδ17T and ILC3 [30,33,38–40,57,58], which is involved in innate immunity and embryo implantation in the endometrium. This finding prompted us to explore the interactions between decidualized EnSCs and IL17A-producing immune cells. Therefore, we examined the expression of *IL17RA*/*IL17RC* and *IL22RA1*, which encode receptors for IL17A and IL22 [32] secreted from activated γδ17T and ILC3, observing the upregulation of *IL17RA* and *IL17RC*, but not *IL22RA1*, in both EnSCs and KC02-44D cells. Furthermore, IL17A stimulation during decidualization of KC02-44D cells suppressed *IGFBP1* expression. As the involvement of FOXO1 in this repression has been suggested [59], the intracellular localization of FOXO1 was examined. FOXO1 migration into the cytoplasm was observed in decidualized cells with additional IL17A stimulation. *IGFBP1* downregulation occurred following FOXO1 migration out of the nucleus. Furthermore, analysis of IL17A receptor downstream signaling revealed a reduction in the amount of IKKA, suggesting the involvement of IKKA in this localization change.

It has been suggested that γδT cells are the major source of IL17A in various non-lymphoid tissues, including the uterus [23,26–28]. The crucial role of IL17A in maintaining health in response to infection, physiological stress, and injury, as well as in autoimmune diseases, is well recognized [38,60–62]. γδT cells, which are thought to be a key source of IL17A in the uterus [23], may facilitate IL17A-induced trophoblast migration and thus play an important role in defense against infection, in addition to potential involvement in pregnancy in young women. Unlike γδT cells in other tissues, uterine immune cells need to both defend against infection and tolerate allogeneic fetuses during pregnancy. γδ17T cells in the endometrium do not express granzyme B and CD107a like cytotoxic γδT [23]. Thus, γδ17T cells are thought to tolerate the fetus, with cytotoxic γδT providing protection against infection. Comparison of data from allogenic and syngeneic reproductive pairs also indicates that γδT cells may play an important role in successful pregnancy [23]. Furthermore, γδT cells have been found to be abundant at the maternal–fetal interface during pregnancy as well as in the non-pregnant uterus [23], suggesting their involvement in

endometrial decidualization. In the decidualized EnSCs in this study, IL17A was implicated in the regulation of *IGFBP1* expression, a major decidualization marker. Overexpression of *IGFBP1* in the endometrium has been associated with impaired fetal growth and placental insufficiency and is thought to cause placenta accreta [63]. Our results suggest that in decidualized EnSCs, IL17A decreases IKKA protein levels, regulates downstream Akt and FOXO1 phosphorylation, and restores *IGFBP1* expression to a constant level. This suggests that IL17A secreted by γδ17T and ILC3 may contribute to the prevention of fetal growth defects caused by placental insufficiency and the development of placenta accreta by restricting the overexpression of *IGFBP1* in decidualized EnSCs.

ILCs are immune cells involved in innate defense and tissue remodeling. NK cells, lymphoid tissue inducer cells, and ILC1-3 belong to the ILC family [64], with all subsets observed within human uterine tissues [29–31,33]. The ILC3 fraction identified via flow cytometry also contains ILC precursors that can give rise to ILC1-3 [65,66]. ILC3 in the human decidua interact with decidualized EnSCs, suggesting that they contribute to innate immunity as well as vascular and tissue architecture [33]. Indeed, ILC3 in the decidua likely act on extravillous trophoblasts and modulate invasion by secreting granulocyte–macrophage colony-stimulating factor, X-C motif chemokine ligand 1, and macrophage inflammation protein 1α and 1β [31,67,68]. The results of this study suggest that their secretion from ILC3 [68] is dependent on activation by IL1B, IL23A, and IL17D secreted from decidualized EnSCs.

IL17A, from intrauterine γδ17T cells, which is thought to play an important role in EVT invasion, is highly abundant [23]. γδ17T cells are recruited into the uterus in response to estrogen, which increases in the blood with follicle development and induces IL17A production in γδ17T cells [69]. Therefore, their involvement in uterine function from the proliferative to secretory phases is also possible. In this context, it is interesting to follow the changes in expression and secretion of IL1B, IL23, and IL17D in EnSCs under estrogen stimulation conditions. ILC3 was reduced in eutopic endometrium during endometriosis [70] when compared to levels in normal endometrium. Therefore, we suggest the involvement of ILC3 in normal decidualization and the menstrual cycle. Further, dysregulation of ILC3 might contribute to endometriosis, consequent implantation failure, and recurrent pregnancy loss.

Limitations

This study demonstrated that the levels of *IL1B*, *IL23A*, and *IL17D*, which regulate γδ17T and ILC3 proliferation and differentiation, are elevated in decidualized EnSCs. Further, IL17A secreted from these cells affects EnSCs decidualization. However, because γδ17T and ILC3 samples derived from the human endometrium are difficult to obtain and these cell lines have not been established outside of mice [71,72], examining the effect of supernatant from decidualized human EnSC culture, which is thought to contain IL17D and other factors, on the growth and differentiation of human cells has not been possible. Since IL17D is also expressed in CD19(+) B cells and quiescent CD4(+) T cells [73,74], determining the localization of IL17D expression in EnSCs using actual uterine tissue sections is necessary. Furthermore, a comparison of *IL1B*, *IL23A*, and *IL17D* expression in decidualized EnSCs and the presence of γδ17T or ILC3 in the vicinity of decidualized EnSCs using endometrial tissue sections from healthy subjects and patients with infertility may lead to the discovery of a new category of obstetric diseases caused by γδ17T and/or ILC3. It is also necessary to measure cytokines, including IL17A and others, in the blood of patients experiencing preeclampsia, preterm labor, and miscarriage.

5. Conclusions

We found that EnSCs may regulate γδ17T and ILC3 in the endometrium and that *IGFBP1* is suppressed by IL17A secreted by the latter two cell types. This regulation occurs via the IL17A-IKKA-FOXO1 phosphorylation-*IGFBP1* axis.

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