



Controlled Ovarian Stimulation Protocols Alter Endometrial Histomorphology and Gene Expression Profiles

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Abstract

Although it is well appreciated that ovarian stimulation protocols for in vitro fertilization (IVF) alter endometrial receptivity, the precise cellular mechanisms are not known. To gain insights into potential mechanisms by which different ovarian stimulation protocols alter the endometrium, we compared histologic and gene expression profiles of endometrium from women undergoing conventional ovarian stimulation for IVF (C-IVF) with those undergoing minimal stimulation with clomiphene citrate (MS-IVF). Sixteen women undergoing MS-IVF ($n = 8$) or C-IVF ($n = 8$) were recruited for endometrial biopsy at the time of oocyte retrieval. Endometrial glands were large, tortuous, and secretory with C-IVF but small and undifferentiated with MS-IVF. Whereas RNA sequencing did not reveal changes in estrogen receptor or its co-regulators or classic proliferation associated genes in MS-IVF, together with immunohistochemistry, Wnt signaling was disrupted in endometrium from MS-IVF cycles with significant upregulation of Wnt inhibitors. Secreted frizzled-related protein 1 (sFRP1) was increased fourfold ($p < 0.01$), and sFRP4 was upregulated sixfold ($p < 0.01$) relative to C-IVF. Further these proteins were localized to subepithelial endometrial stroma. These data indicate that MS-IVF protocols with CC do not seem to impact endometrial estrogen signaling as much as would be expected from the reported antiestrogenic properties of CC. Rather, the findings of this study highlight Wnt signaling as a major factor for endometrial development during IVF cycles.

Keywords Clomiphene citrate · Minimal stimulation IVF · Endometrial gene expression · sFRP · Wnt signaling

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Introduction

Induction of follicular maturation for in vitro fertilization (IVF) is accomplished through administration of high-dose gonadotropins with the intention of retrieving as many oocytes as possible. In recent years however, milder ovarian stimulation protocols using a combination of oral agents (clomiphene citrate or aromatase inhibitors) and low-dose gonadotropins (≤ 150 IU/d) have been suggested to improve oocyte quality in women considered to be poor responders, especially those with diminished ovarian reserve (DOR) and advanced reproductive age (ARA)¹. In general, the advantages of minimal stimulation IVF (MS-IVF) include elimination of ovarian hyperstimulation syndrome and reduced gonadotropin consumption and overall expense^{2, 3}. One of the employed MS-IVF protocols utilizes the oral induction agent clomiphene citrate (CC) with low doses of human menopausal gonadotropins (hMG)⁴.

Since CC is a selective estrogen receptor modulator (SERM), it has variable agonistic-antagonistic effects on

estrogen receptor (ER)-rich tissues including the hypothalamus, pituitary, ovary, and uterus. Classically, CC is believed to be an endometrial antagonist and may negatively impact the endometrium as suggested by a thinner endometrial stripe observed by sonography^{5–7}. Recently, we reported that the use of prolonged daily CC in an MS-IVF cycle thins the endometrium significantly even in the setting of supraphysiologic estrogen levels⁸. Several biologic and treatment variables differ among MS-IVF and C-IVF patients, including age and AMH levels. Nevertheless, women undergoing MS-IVF with CC develop normal endometrium in subsequent embryo transfer cycles⁸, indicating that age and AMH levels do not have a striking impact on the endometrium. Studies using CC with or without gonadotropins confirm that CC has a negative impact on the endometrial lining^{5, 9–11}. Yet, there is little information regarding the cellular or molecular processes by which CC affects endometrial differentiation and development¹². Here, we tested the hypothesis that endometrial differentiation and gene expression are altered significantly after MS-IVF (with CC) compared with C-IVF.

Materials and Methods

Patient Recruitment and Stimulation Protocols

This study was approved by the Institutional Review Board of the University of Texas Southwestern Medical Center. Endometrial tissues were obtained after written informed consent from healthy women between 18 and 45 years of age undergoing in vitro fertilization. Women who were pregnant and had known uterine disease such as fibroids, adenomyosis, or endometrial polyps, known history of coagulopathy, and known or suspected cervico-vaginal or intrauterine infection were excluded. Patients with expected poor ovarian response per Bologna criteria¹³ and/or ARA > 40 years old underwent MS-IVF protocols. MS-IVF was comprised of daily CC until day of trigger with hMG 150 international units (IU) starting on the fifth day of stimulation every other day until trigger with hCG and leuprolide acetate 2 mg. C-IVF involved daily injection of high-dose recombinant follitropin-beta (> 200 IU, Follistim, Merck) with added hMG (75–150 IU) on the day of antagonist administration. In both stimulation protocols, a gonadotropin-releasing hormone antagonist was administered when the LH was >10 U/L to prevent premature ovulation or luteinization. Frequent monitoring of follicles and endometrial thickness was performed by transvaginal ultrasound. Dual trigger with hCG (5000–10,000 units) and leuprolide acetate 2 mg was used to induce final oocyte maturation when the two leading follicle sizes reached > 17 mm in average diameter. Patients undergoing C-IVF for elective oocyte cryopreservation or preimplantation genetic testing were recruited as controls. All patients recruited for this study were those who

choose to undergo oocyte/embryo freezing for embryo transfer in a later cycle.

Endometrial Tissue Collection

Endometrial tissue was collected after informed consent under general anesthesia using an endometrial biopsy (suction) cannula (Pipet Curet, CooperSurgical) at the time of oocyte retrieval (36 h after hCG and leuprolide administration). Endometrial tissue was either immediately snap frozen in liquid nitrogen and stored at –80 C or fixed in 4%paraformaldehyde and stored in 70% ethanol until further analysis.

Histopathology and Histomorphology

Slides were prepared with hematoxylin and eosin (H&E) staining. Endometrial histology was analyzed prospectively by Noyes criteria by two blinded independent gynecologic pathologists¹⁴. For assessment of endometrial maturation, the day of oocyte retrieval was considered the day of ovulation (day 0). Histomorphologic analysis was performed using digital images captured by a Nikon E600 microscope with a DXM1200C camera head, and calculations were made by NIS-Elements advanced research software v 3.2. The average of two nonoverlapping sections randomly chosen containing only endometrium, and no luminal epithelium was calculated per patient for each category. The number of glands per mm² was calculated for each sample. To avoid over counting, glands abutting the left and upper borders were not counted. Gland volume fraction was determined by dividing the total area of endometrial glands divided by the total area of the image expressed as a percentage. Average maximum gland diameter and average gland height were calculated per gland and averaged.

RNA Sequencing Power Analysis

A priori power analysis was performed to determine the number of samples per group needed to detect a threefold change in RNA gene expression. Using a mathematical equation described by Hart et al.¹⁵ where $\alpha = 0.05$, $\beta = 0.90$, and $\sigma = 0.4$, at least three samples were needed per group in order to detect a threefold change. Although these samples were precisely timed and hormonally controlled, potential biological variations could not be predicted. Sample size, therefore, was increased to five per group.

mRNA Library Preparation and Bioinformatics Analysis

RNA was obtained from endometrial tissue biopsy samples using RNAqueous 4PCR total RNA Isolation Kit (Thermo Fisher Scientific). Samples were run on the Agilent

Tapestation 4200 to determine the level of degradation, thus ensuring that only high-quality RNA was used (RIN Score 8 or higher). The Qubit fluorometer was used to determine the concentration prior to starting library prep. 4 µg of total DNase-treated RNA were then prepared using the TruSeq Stranded mRNA Library Prep Kit from Illumina. Poly-A RNA was purified and fragmented, before strand-specific cDNA synthesis. cDNA was then A-tailed, and indexed adapters were ligated. After ligation, samples were PCR amplified and purified with AmpureXP beads and then validated again on the Agilent Tapestation 4200. Before normalization and pooling, samples were quantified by Qubit and then run on the Illumina NextSeq 500 using V2 reagents. Raw data from machine was then de-multiplexed and converted to fastq files using bcl2fastq (v2.17, Illumina). The fastq files were checked for quality using fastqc (v0.11.2) and fastq_screen (v0.4.4) and were quality trimmed using fastq-mcf (ea-utils/1.1.2–806)¹⁶. Trimmed fastq files were mapped to hg19 (UCSC version from igenomes) using TopHat¹⁷, duplicates were marked using picard-tools (v1.127 <https://broadinstitute.github.io/picard/>), read counts were generated using featureCounts¹⁸, and differential expression analysis was performed using edgeR¹⁹. Additional analysis was performed with Ingenuity Pathway Analysis (IPA, Qiagen).

Immunofluorescence Staining

Paraffin-embedded endometrial samples were heated at 50°C for 15 min. Samples were rehydrated sequentially with xylene (mixed isomers), 100% ethanol, 90% ethanol, 75% ethanol, and 50% ethanol, rinsed in deionized H₂O and PBS × 2. Antigen retrieval was performed with heated citrate buffer and placed in water steamer for 30 min. After rinsing in PBS, the samples were blocked with 10% donkey serum in PBS for 1 h. Samples were incubated with anti-sFRP1 (Abcam, rabbit at 1:100) and anti-sFRP4 (Abcam, rabbit at 1:100) antibodies for 1 h at room temperature. After rinsing in PBS, samples were then incubated with donkey anti-rabbit secondary antibodies (1:1000) for 1 h at room temperature. Following rewashing with PBS, slides were counterstained with DAPI and mounted with Vectashield mounting medium for fluorescence (Vector Laboratories). Samples were visualized using the Nikon E600 confocal microscope. Tris-buffered saline with 0.5% Tween 20 (TBST) was used as diluent and wash buffer. Blocking steps, incubation of the primary and secondary antibodies, and washing steps were performed on a shaker. Antibodies were diluted in TBST containing 5% nonfat dry milk.

Statistical Analysis

In addition to analysis of RNA sequencing datasets described above, Student's t-test was used to compare two independent

groups and ANOVA for multiple groups. Results are presented as mean ± SEM or SD as indicated. For discrete variables, Mann-Whitney rank sum testing was used. *P* values ≤ 0.05 were considered statistically significant.

Results

Patient Demographics and Stimulation Outcomes

Baseline demographics and laboratory characteristics are provided in Table 1. Sixteen patients were recruited (eight in each protocol). As expected, women undergoing MS-IVF were older and had lower anti-müllerian hormone levels (AMH). MS-IVF protocols involved less gonadotropin units resulting in decreased peak E2 levels compared with C-IVF. It should be emphasized, however, that E2 levels were supraphysiologic even with MS-IVF relative to the typical values of 250–300 pg/ml with normal ovulation. Notably, endometrial thickness by sonography was decreased in MS-IVF relative to C-IVF despite high E2 levels. Prior studies indicate that decreased endometrial thickness in women undergoing MS-IVF is due to the protocol and is not related to other variables such as age or DOR or mean peak estradiol levels⁸. To confirm this observation, we report endometrial thickness for five patients in each group with subsequent frozen embryo transfer (FET) cycles available for review (Table 1). Endometrial thickness was statistically different between groups as determined by one-way ANOVA ($F(3, 12) = 12.81, p < 0.001$). Tukey post hoc testing revealed that endometrial thickness was significantly greater in C-IVF (12.23 ± 2.33 mm, $p < 0.01$), MS-IVF patients undergoing FET (9.46 ± 1.71 mm, $p < 0.05$) and C-IVF patients undergoing FET (9.46 ± 1.71 mm, $p < 0.01$) compared with MS-IVF stimulation cycle (5.9 ± 1.49 mm). Importantly, endometrial thickness returned to normal in cycles not affected by MS-IVF protocols. In the MS-IVF group ($n = 8$), all patients had decreased ovarian reserve and advanced reproductive age. One patient also had endometriosis. Data from this individual did not differ in terms of histology, gene expression, endometrial sFRP, or IHC. In the conventional stimulation group, infertility was due to male factor ($n = 4$), tubal factor ($n = 1$), and unexplained ($n = 1$), and two patients were undergoing fertility preservation.

Histomorphometry

H&E staining of endometrial tissues revealed dramatic changes between MS-IVF and C-IVF. After MS-IVF, small undifferentiated glands were dispersed in a sea of polygonal stromal cells. In contrast, large tortuous glands of secretory epithelium were the major component of endometrium after C-IVF (Fig. 1). Two gynecologic pathologists blinded to treatment groups agreed that the MS-IVF samples were early to mid-proliferative, and C-IVF biopsies

Table 1 Patient demographics, stimulation, and subsequent cycle outcomes

	MS-IVF (n = 8)	C-IVF (n = 8)	p	MS-IVF FET (n = 5)	C-IVF FET (n = 5)
Age (y)	39.9 ± 3.7	34.5 ± 3.1	<0.01		
BMI (kg/m ²)	25.0 ± 5.3	24.7 ± 4.5	NS		
AMH (ng/ml)	0.9 ± 1.1	3.7 ± 2.5	<0.01		
Gonadotropins (units)	656 ± 198	3646 ± 3714	<0.01	0	0
Endometrial stripe (mm)	5.4 ± 1.4	10.5 ± 3.1	<0.01	9.5 ± 1.8 ^δ	10.2 ± 2.7
Estradiol (pg/ml)*	851 ± 256	3107 ± 1318	<0.01	372 ± 178	376 ± 123
Progesterone (ng/ml)*	0.4 ± 0.2	1.2 ± 0.5	<0.01		
Progesterone (ng/ml)**	2.0 ± 0.7	9.7 ± 1.5	<0.01		
Oocytes retrieved (n)**	3.5 [3, 4.75]	13 [7.5, 24.5]	<0.01		
Diagnosis	DOR/ARA, 8 Endometriosis, 1	Male factor, 4 Tubal factor, 1 Unexplained, 1 Fertility preservation, 2			

± SD

*E2 and P4 levels were obtained the day of hCG administration (35 h prior to endometrial biopsy)

**P4 level obtained the day of biopsy

***median [25,75%]

^δ p < 0.01 compared with MS-IVF stimulation cycle

were early secretory post-ovulatory days 1–3 per Noyes criteria. Histomorphometric analysis revealed significant differences between the two stimulation protocols with an increased number of glands per cross-sectional area with

MS-IVF relative to C-IVF with decreased gland volume (Fig. 1E). Further, gland diameter and epithelial height were decreased significantly with MS-IVF suggesting decreased secretory differentiation (Fig. 1F, G).

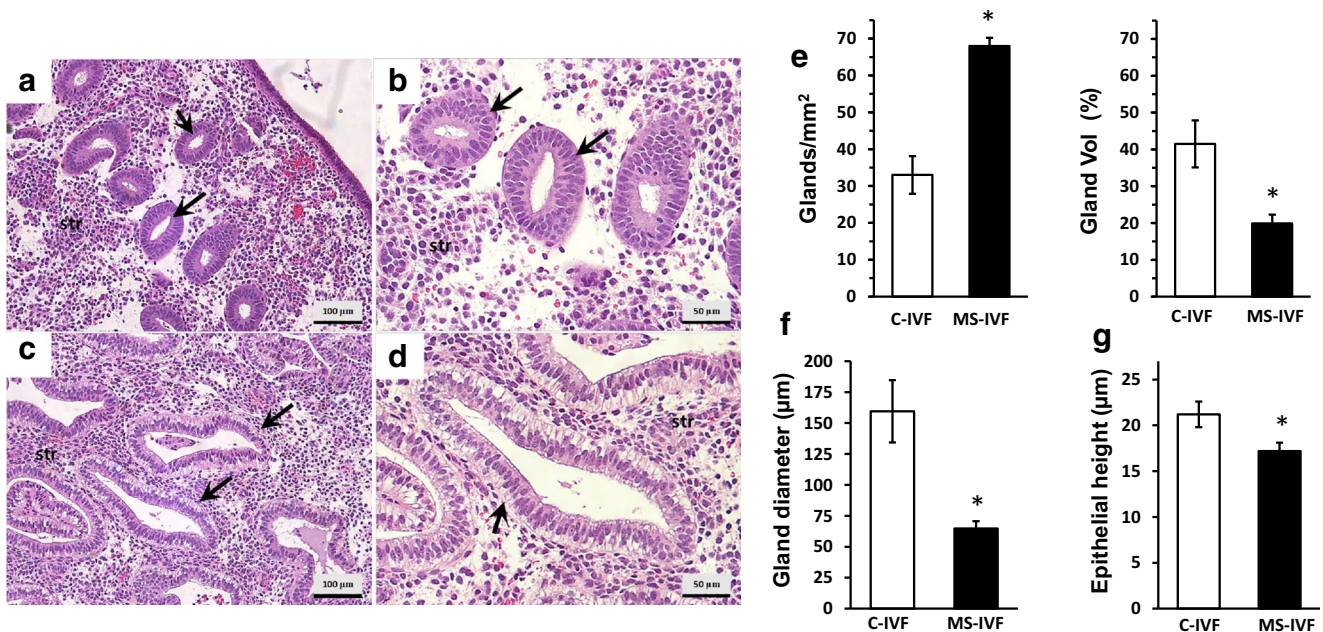


Fig. 1 H&E staining of endometrial tissues from women undergoing MS-IVF (A, B) or C-IVF (C, D). After MS-IVF, stroma (str) appears disrupted whereas glands (arrows) are small, straight, and undifferentiated with no mitotic bodies (low magnification (20×), A, high mag (40×), B). After C-IVF, tissue is defined as early secretory

endometrium (C, D) with enlarged tortuous glands, and epithelial sub-nuclear vacuolization. (A, C) Scale bar= 100 μm; (B, D) scale bar= 50 μm. (E) Quantification of endometrial gland number and glandular volume fraction. (F) Gland diameter and (G) epithelial height. Data represent mean ± SD. *P < 0.05

Gene Expression Profiling of Endometrial Tissues

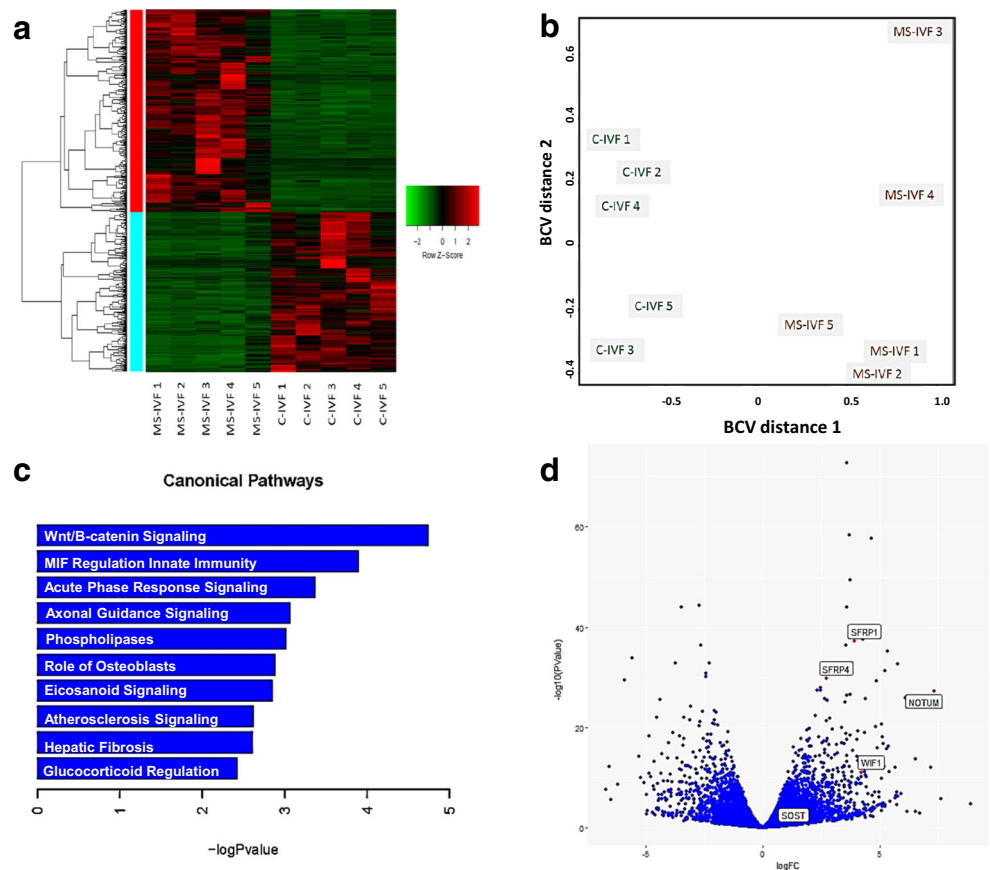
To determine the differences in endometrial gene expression between MS-IVF and C-IVF on a global scale, RNA sequencing of whole tissue endometrial tissue was conducted. Hierarchical clustering analysis and heat maps revealed that MS-IVF and C-IVF profiles clustered with dramatic changes in gene expression profiles (Fig. 2A and B, GEO accession number to be provided after manuscript acceptance). Specifically, 3.4% (723 of 20,764) genes were differentially expressed defined as false discovery rate (FDR) ≤ 0.05 and $|\log_2 \text{fold-change}| \geq 1.5$.

Ingenuity pathway analysis revealed several molecular pathways significantly different between MS-IVF and C-IVF (Fig. 2C). Based on endometrial histology and the role of estrogen-induced activation of estrogen receptor α in endometrial glandular proliferation, we suspected that estrogen receptor signaling including *ER α* , co-regulators, *SRC-1*, and *CREB-BP*, and *NCOR*, may be differentially expressed between MS-IVF with CC and C-IVF. Surprisingly, RNAseq did not reveal significant changes in ER or its co-regulators. Further, classic proliferation-associated genes in the endometrium such as *cyclin A* and *cMyc* or antiproliferative genes such as *p27Kip1* were not differentially expressed between MS-IVF and C-IVF

protocols. Serum progesterone levels were increased modestly in C-IVF (1.2 ng/ml) relative to MS-IVF (0.4 ng/ml, Table 1). RNAseq analysis, however, revealed increased progesterone receptor (PR) gene expression in MS-IVF relative to C-IVF (602 ± 44.9 vs 230.6 ± 26.6 fragments per kilobase of exon per million fragments mapped, $p < 0.0001$). Our RNAseq results indicated that IGF-1 mRNA was similar in C-IVF and MS-IVF. IGFBP4 and IGFBP5, but not other IGF binding proteins, were increased significantly (3.8- to 2.8-fold) in MS-IVF. Interestingly, expression of *17BHS2* was increased eightfold in C-IVF, not in CC-containing cycles (75.6 ± 11.6 vs 9.3 ± 4.4 FKPM). MIR regulation of innate immunity was differentially expressed between the two groups with downregulation of JUN (4-fold), FOS (18-fold), prostaglandin-endoperoxide synthase 2 (13-fold), and members of the phospholipase A2 group 4 (11-fold) in MS-IVF.

Perhaps, one of the most interesting pathways differentially expressed in endometrium from the two protocols was that of WNT/ β catenin signaling which is known to be involved in endometrial proliferation. Several WNT inhibitory genes were differentially expressed in minimal stimulation including *NOTUM* (161.67 FC), *WISE/SOST* (36.93 FC), *WIF-1* (18.46 FC), *sFRP1* (15.06 FC), and *sFRP4* (6.57 FC) highlighted on the volcano plot (Fig. 2D). Highly expressed

Fig. 2 RNA-Seq data analysis of endometrial tissues from MS-IVF and C-IVF. (A) Heatmap represents hierarchical clustering of the top 100 differentially expressed genes in 5 tissues from MS-IVF and 5 from C-IVF. (B) Hierarchical cluster analysis demonstrating biological coefficient of variation (BCV) of different IVF treatments (5 samples from MS-IVF and 5 from C-IVF). (C) Top 10 canonical pathways derived from ingenuity pathway analysis (IPA; Ingenuity Systems). (D) Volcano plot of mRNA expressions. Plotted along the x-axis is the mean of \log_2 fold-change, along the y-axis the negative logarithm of the p values. WNT inhibitors highlighted red demonstrate the upregulation of genes in the MS-IVF group



differentially regulated Wnt pathway regulators are listed in Table 2.

Protein Analysis and Immunolocalization

RNAseq revealed significant upregulation of *sFRP1* and *sFRP4* in endometrium from MS-IVF with transcripts for *sFRP4* more abundant than *sFRP1* (Fig. 3A, D). To confirm differential expression of these secreted frizzled-related proteins at the protein level, immunoblot analysis was conducted with homogenates of endometrial tissues from MS-IVF and C-IVF (Fig. 3B, E). In agreement with RNAseq analysis (Fig. 3A, D), sFRP1 was increased significantly in tissues from MS-IVF (fourfold, Fig. 3B, C). Likewise, sFRP4 was increased sixfold in MS-IVF (Fig. 3E, F).

Immunofluorescence was used to localize sFRPs in endometrial tissue. sFRP1 was localized to the endometrial stroma with notable accumulation directly underlying the glands (Fig. 4A, Fig. 1A). The intensity of stromal sFRP1 was increased strikingly in tissues from MS-IVF relative to C-IVF (Fig. 4A, C). sFRP4 also immunolocalized to stromal cells with a more homogeneous distribution relative to sFRP1 (Fig. 4B). With higher magnification, sFRP4 immunofluorescence intensity was increased in the periphery of the stromal cells, suggesting that this is likely secreted protein or anchored to the cell membrane (Fig. 1A). Consistent with immunoblot analysis, sFRP4 staining intensity was increased in stromal compartment from MS-IVF relative to C-IVF (Fig. 4B, D). To confirm the lack of sFRP expression in glandular epithelial cells, dual staining of sFRP1 and sFRP4 was conducted with that of pancytokeratin (Fig. 4E, F). The results confirmed strong subepithelial immunostaining of sFRP1 to be absent in epithelial cells (Fig. 4E). Similarly, stromal cell sFRP4 was absent in cytokeratin-positive glands (Fig. 4F).

Discussion

Mild and minimal ovarian stimulation protocols are based on the principle of optimal utilization of competent oocytes by using less medication thereby decreasing risks of ovarian hyperstimulation and cost while assuring at least comparable pregnancy outcomes as those of C-IVF¹. Whereas there is a growing trend toward frozen embryo transfer in conventional IVF due to embryo-endometrial desynchronization, there is little evidence regarding endometrial development with mild or minimal stimulation. Some postulate that minimal stimulation may improve fresh transfer rates due to more physiologic levels of hormones because a lower number of oocytes are stimulated. However, there are many types of mild stimulation, those that reduce the amount of gonadotropin (<150 IU) and others that further decrease the amount of gonadotropin by employing oral agents such as CC or aromatase inhibitors (MS-IVF)²⁰. Here, we compared endometrial gene expression profiles in MS-IVF cycles using CC with those in cycles using C-IVF.

Endometrial Thinning in MS-IVF

Based on the differences in demographics and laboratory profiles between the two groups of ovarian stimulation protocols used in this study, there are several potential explanations for abnormal histology and endometrial thinning observed in MS-IVF. First, women undergoing MS-IVF are likely to be older and have decreased ovarian reserve as indicated by decreased levels of AMH. We rejected these variables as causes of endometrial thinning because it is known that these women exhibit normal endometrial histology in subsequent cycles without gonadotropins and CC⁸, and we confirmed that patients enrolled in this study also generated normal endometrial thickness in the absence of gonadotropins + CC. Although single-agent aromatase inhibitors may decrease endometrial

Table 2 Differential expression of Wnt pathway regulators in endometrial tissues from MS-IVF and C-IVF protocols

GENE	FKPM MS-IVF	FKPM C-IVF	Fold Change	P Value	FDR
<i>sFRP1</i>	790.6 ± 112.1	52.5 ± 9.1	15.06	6.93E-38	1.60E-34
<i>sFRP4</i>	4088.4 ± 35.9	622.4 ± 58.7	6.57	1.77E-30	1.67E-27
<i>APCDD1</i>	725.1 ± 142.1	202.1 ± 41.4	3.59	1.45E-07	4.88E-06
<i>WNT4</i>	63.6 ± 12.8	18.6 ± 1.7	3.42	1.59E-10	1.14E-08
<i>AXIN2</i>	78.5 ± 14.0	28.2 ± 4.1	2.78	3.12E-08	1.25E-06
<i>WNT5A</i>	229.5 ± 48.7	88.0 ± 14.5	2.61	3.03E-06	7.12E-05
<i>WNT2</i>	75.6 ± 28.8	38.0 ± 3.8	1.99	0.005273	0.03775
<i>DKK3</i>	71.6 ± 9.7	47.1 ± 6.4	1.52	0.01097	0.066601
<i>FZD5</i>	70.8 ± 5.5	118.6 ± 13.5	-1.60	0.000129	0.001784

Data represent mean ± SEM fragments per kilobase of exon per million fragments mapped, minimum expression of 50 FKPM, and FDR < 0.07

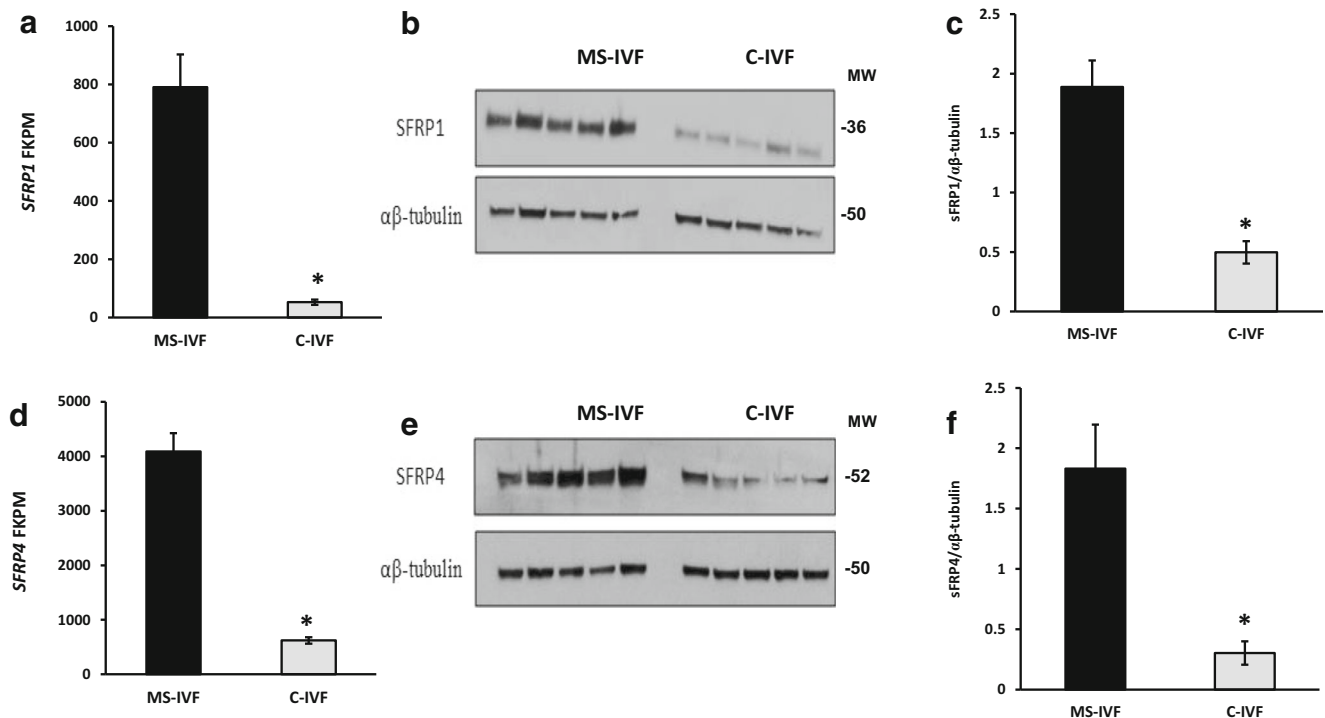


Fig. 3 Expression of *sFRP1* and *sFRP4* in endometrial tissues from MS-IVF and C-IVF. RNAseq data from endometrial tissues from MS-IVF ($n = 5$) or C-IVF ($n = 5$) for *sFRP1* (A) and *sFRP4* (D). Data expressed as mean \pm SEM fragments per kilobase of exon per Million fragments

mapped (FPKM). Immunoblot analysis of *sFRP1* (B) and *sFRP4* (E) in endometrium from five individual patients in each group. Quantification of *sFRP1* (C) and *sFRP4* (F) normalized to $\alpha\beta$ -tubulin in endometrium from MS-IVF or C-IVF. * $p \leq 0.05$

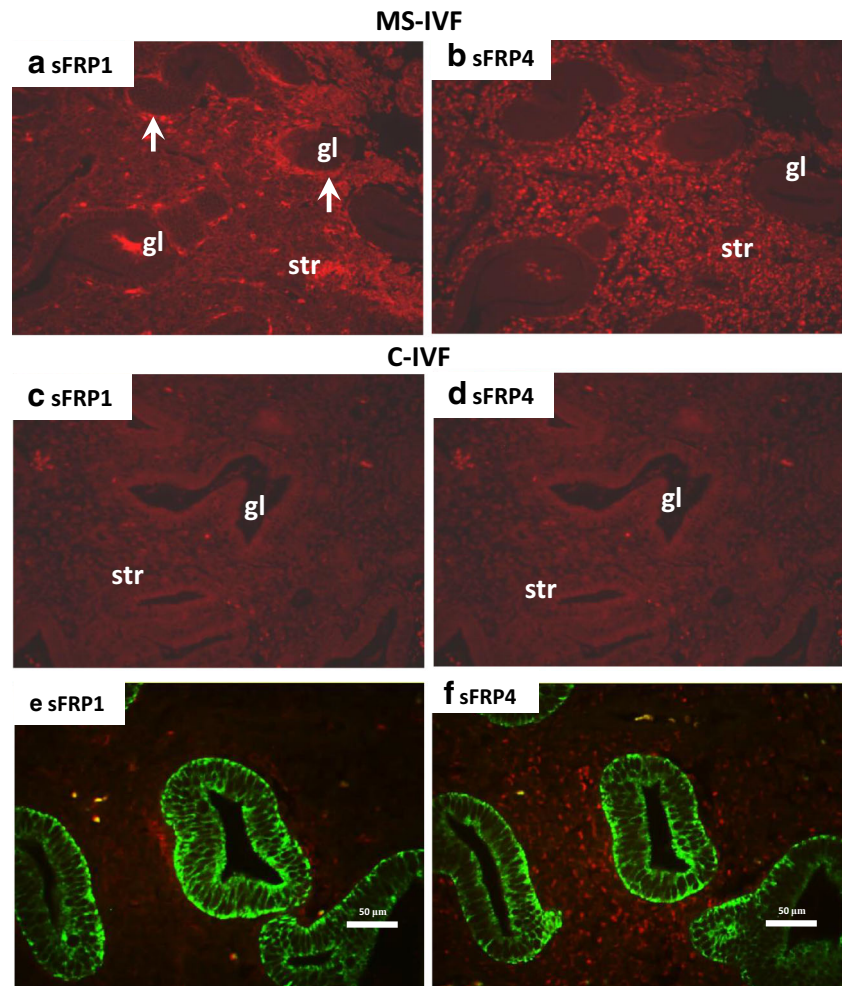
thickness depending on dose and duration, MS-IVF with aromatase inhibitors does not cause a thin endometrial stripe despite similar gonadotropin doses and E2 levels (26). Thus, it is logical to deduce that CC is the predominant cause of endometrial effects of MS-IVF used in this study.

CC, a mixture of estrogenic (zuclomiphene) and antiestrogenic (enclomiphene) geometric isomers, has been used for over 50 years for induction of ovulation. Although CC has been termed an antiestrogen, it is a SERM, all of which exert estrogenic or anti-estrogenic effects depending on cell type, gene of interest, and presence or absence of other cofactors or other transcription factors (such as PR) and E2 levels (21). As a member of the SERM family, its antiestrogenic effects cause release of GnRH by the hypothalamus and subsequent gonadotropins from the anterior pituitary. In 1981, Kokko et al. suggested that CC exhibits antiestrogenic effects on the endometrium through (i) competition with estrogen and (ii) reduced number of ERs. This hypothesis was supported by findings of Amita et al. in which CC inhibited recruitment of steroid receptor coactivator-1 and thereby ER α transactivation in human endometrial epithelial cells (12). Further, it has been shown that CC induces ubiquitination and degradation of ER α in Ishikawa cells (22). These limited investigations regarding the effect of CC on the endometrial cells, therefore, suggest that CC is a modest antagonist for E2-induced gene expression in human epithelial cells. It should be emphasized,

however, that the effects of CC on ER function in these studies were rather modest and may not explain the profound loss of epithelial differentiation and growth in endometrium from CC-treated cycles, especially considering that levels of E2 were far above physiological levels. Other studies have shown that E2-induced IGF-1 results in epithelial proliferation. Our RNAseq results indicated that IGF-1 mRNA was similar in C-IVF and MS-IVF. IGFBP4 and IGFBP5, but not other IGF binding proteins, were increased significantly (3.8- to 2.8-fold) in MS-IVF which may indicate that CC induces IGFBP4 and IGFBP 5 to attenuate IGF-1 action on glandular epithelium. We also evaluated the possibility that the gene encoding 17 β -hydroxysteroid dehydrogenase 2 (17 β -HSD2) which converts E2 to estrone (E1) may be differentially expressed in endometrium from the two groups. Interestingly, expression of *17BHS2* was increased 8-fold in C-IVF, not in CC-containing cycles (75.6 ± 11.6 vs 9.3 ± 4.4 FPKM).

At first glance, it may seem that the modest increase in progesterone levels in the C-IVF group may signal advanced secretory changes in the endometrium. The issue, however, is not a simple matter of serum progesterone levels (0.4 ng/ml in MS-IVF vs 1.2 ng/ml in C-IVF), both of which are above the Kd for PR (0.3–1 nM). Interestingly, sequencing data revealed significant increases in PR gene expression in the MS-IVF group (602 ± 44.8 compared with 230 ± 26.6 , $p < 0.0001$)

Fig. 4 Immunolocalization of sFRP1 and sFRP4 in endometrium. (A–B) MS-IVF samples at low magnification (20 \times) for sFRP1 (A) and sFRP4 (B). sFRP1 expression concentrated in stromal cells directly adjacent to the glands (arrows), whereas sFRP4 diffusely expressed in the stroma. (C–D) C-IVF samples at 20 \times for sFRP1 (C) and sFRP4 (D). gl (glands). str (stroma). Scale bar = 100 μ m. (E, F) Dual staining of minimal stimulation biopsies. MS-IVF samples were stained with anti-pan-cytokeratin (green) and anti-sFRP1 (E) or anti-sFRP4 (F) in red at 40 \times . Scale bar = 50 μ m



suggesting that progesterone effects may be more pronounced with MS-IVF, not C-IVF. Since progesterone is known to inhibit estrogen-induced proliferation of many ER-positive cells, it is possible that progesterone effects were more pronounced in MS-IVF. The “early secretory” phase interpretation by pathologists does not necessarily equal increased progesterone levels but may implicate increased PR complex formation, increases in cofactor expression, or lack of inhibitors or repressors. Both groups received GnRH antagonist prior to hCG trigger to prevent premature ovulation and progesterone release by the corpus luteum.

Taken together, the findings of this study do not support striking direct effects of CC-MS-IVF on classic estrogen receptor signaling as much as would be expected based on its antiestrogen properties. Rather, the results highlight Wnt signaling as a major factor for endometrial development during oocyte stimulation IVF protocols.

Wnt Signaling

WNT7a is a diffusible factor highly expressed in luminal endometrial epithelial cells that initiates cell proliferation. WNT

proteins bind and act through cell surface receptors known as frizzled^{23–25}. At the cell surface, Wnt/frizzled interactions activate Disheveled leading to inactivation of glycogen synthase kinase-3 β by phosphorylation and nuclear localization of β -catenin to regulate gene transcription²⁶. Secreted frizzled-related proteins (sFRPs) antagonize Wnt signaling at the receptor level^{27, 28}. Specifically, it has been shown that overexpression of sFRP4 and treatment with recombinant sFRP4 protein inhibited endometrial cancer cell growth in vitro²⁹. It was intriguing, therefore, that stromal-derived sFRP1 and sFRP4 were increased dramatically in endometrium from CC-treated cycles (six- to eightfold changes in tissue sFRP4 mRNA and protein). The findings herein suggest that at least some of the antiproliferative effects of CC on endometrial glands may be due to increased expression of Wnt inhibitors in the stroma that block Wnt-induced glandular proliferation.

Summary and Limitations

Next-generation RNA sequencing analysis of precisely timed endometrial tissues from MS-IVF and C-IVF discovered the

potential role of CC in MS-IVF protocols in dysregulation of endometrial glands through increased expression of Wnt inhibitors in endometrial stroma. The strengths of the study include sequencing of precisely timed hormonally controlled endometrial tissues with a number sufficient to demonstrate highly significant results with no overlap by hierarchical cluster analysis. The impact of the findings is limited because endometrial biopsies were conducted at the time of egg retrieval rather than the expected time of implantation, and all biopsies were from patients with future plans for FET. Thus, pregnancy outcomes cannot be deduced from this study. Endometrial biopsies at the time of implantation add risk and burden to patients. We suggest that endometrial development at the time of egg retrieval has a significant impact on endometrial development at the time of implantation. Although this investigation was focused on disruption of the Wnt pathway in endometrium, MIF regulation of innate immunity was also differentially expressed between the two groups. Work is ongoing to address the significance of this finding. Nevertheless, results of this work highlight the complexities of CC-MS-IVF-induced effects on the endometrium and involvement of Wnt signaling that was previously unrecognized and may play a crucial role in regulation of endometrial development in IVF protocols. Since patient demographics differ among women receiving MS-IVF compared with C-IVF, we cannot conclude with certainty that MS-IVF with CC is the direct cause of dysregulated Wnt signaling in the endometrium. The development of normal endometrium in subsequent transfer cycles in MS-IVF with CC patients, however, suggests that this ovarian stimulation protocol adversely affects the endometrium.

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Authors' Contribution J. W. conducted the experiments, analysis, and wrote the manuscript. P. K. contributed to experimental design. M.K. and C.X. provided bioinformatic and statistical analysis. S.B., J.W., and O.B. identified patients, obtained informed consent, and conducted endometrial biopsies. B.C., O.B., and R.A.W. contributed to experimental design, analysis, data interpretation, and writing of the manuscript. All authors gave final approval of the article.

Compliance with Ethical Standards

Conflict of Interests BC received research grants from AbbVie and Medicines360. JW, PK, MK, CX, SB, OB, and RW have nothing to declare. No other potential conflicts of interest relevant to this article were reported.

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