



Original Article

Ovulatory follicular fluid promotes the clonogenicity and invasion of ectopic and eutopic endometrial cells

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ABSTRACT

Objectives: Endometriosis is a chronic, estrogen-dependent disorder characterized by the ectopic growth of endometrial-like tissue. Despite its significant impact on fertility and quality of life, the pathogenesis of endometriosis remains poorly understood. This study investigates the potential role of ovulatory follicular fluid (FF) in driving disease progression. Based on the “double engines theory,” which posits that retrograde menstruation provides the “seeds” for ectopic lesions while ovulation fuels their growth, we hypothesize that FF enhances endometrial cell proliferation, clonogenicity, migration, and invasion. **Materials and Methods:** Primary eutopic and ectopic endometrial cells were isolated from infertile women and treated with 10% diluted FF collected from *in vitro* fertilization patients. Cellular morphology, proliferation, clonogenicity, migration, and invasion were evaluated using colony formation, transwell, and Matrigel assays. Statistical analyses were performed to compare cellular responses between eutopic and ectopic cells. **Results:** Morphological analysis revealed distinct adaptive changes, with ectopic cells predominantly adopting a more fibroblast-like phenotype. Ectopic cells exhibited higher clonogenicity (29.8-fold) capacities, lower proliferation (0.49-fold) and migration (0.11-fold) capacities, and similar invasion capabilities compared to eutopic cells. FF significantly enhanced proliferation (1.7-fold in eutopic and 1.3-fold in ectopic cells) and clonogenic capacity, with eutopic cells forming 31.3 times more colonies and ectopic cells showing a 1.3-fold increase. The clonogenic area expanded dramatically, increasing 261-fold in eutopic and 4.9-fold in ectopic cells. In addition, FF promoted migration (1.8-fold in eutopic and 2.9-fold in ectopic cells) and invasion (9.1-fold in eutopic and 4.8-fold in ectopic cells). These findings suggest that FF may play a pivotal role in the early establishment and progression of ectopic lesions. **Conclusion:** The results highlight the critical role of FF in enhancing endometrial cell survival, proliferation, and dissemination. This supports the “double engines theory” of endometriosis, emphasizing the significant contribution of ovulation to the pathogenesis of the disease.

KEYWORDS: Clonogenicity, Endometriosis, Eutopic and ectopic endometrial cells, Follicular fluid, Invasion

Submission : 12-Mar-2025
Revision : 30-Apr-2025
Acceptance : 05-May-2025
Web Publication : 04-Aug-2025

INTRODUCTION

Endometriosis is a chronic, estrogen-dependent gynecological disorder characterized by the ectopic implantation, proliferation, and, in severe cases, invasion of endometrial-like tissue outside the uterine cavity, primarily within the peritoneal cavity and ovaries. It affects approximately 6%–10% of women of reproductive age and is a leading cause of infertility and chronic pelvic pain [1,2]. Despite its prevalence, the pathogenesis of endometriosis remains incompletely understood. A well-established mechanism

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How to cite this article: Hsu CF, Seenan V, Manickam S, Wang LY, Chang YC, Chen YH, et al. Ovulatory follicular fluid promotes the clonogenicity and invasion of ectopic and eutopic endometrial cells. Tzu Chi Med J 2026;38(1):68-74.

Access this article online	
Quick Response Code: 	Website: www.tcmjmed.com
	DOI: 10.4103/tcmj.tcmj_90_25

contributing to the ectopic implantation of endometrial tissue is retrograde menstruation, which transports eutopic endometrial cells into the peritoneal cavity [3]. However, the factors that influence the survival, clonogenicity, and invasive potential of these ectopically implanted cells remain poorly defined.

Eutopic endometrial cells from patients with endometriosis exhibit distinct molecular and epigenetic alterations that enhance their migratory and invasive capabilities, predisposing them to ectopic implantation [4,5]. Furthermore, the peritoneal and ovarian microenvironments play a critical role in lesion development by fostering aberrant immune responses, inflammatory cytokine production, and oxidative stress, all of which promote the survival and proliferation of ectopic endometrial cells [6,7]. Within the ovarian cortex, endometriotic lesions (ovarian endometriomas) display enhanced clonogenicity, likely due to the influence of local estrogen production and paracrine signaling from ovarian stromal components [8-10]. Nevertheless, the precise molecular mechanisms underlying these processes remain elusive.

Given that endometriosis is a hormone- and ovulation-dependent disease, we have proposed a “double engines theory” to explain its pathogenesis. This theory posits that, in addition to retrograde menstruation providing the “seeds” for ectopic implantation, ovulation acts as the “fuel” that drives the growth and progression of ectopic endometrial tissue [11]. Ovulatory follicular fluid (FF), which is enriched with mutagens [12,13] and growth factors [14-17], exerts potent transforming effects on the fallopian tube fimbrial epithelium (FTE), which is exposed to FF during ovulation [18]. Notably, both FF and the FF-drained postovulatory peritoneal fluid promote stemness, clonogenicity, and peritoneal seeding of the transforming FTE [19,20]. Moreover, previous studies have demonstrated that FF can facilitate the peritoneal dissemination of ovarian cancer cells by promoting epithelial–mesenchymal transition (EMT) [18] and enhancing invasive properties [17,21]. A similar mechanism may contribute to the initial seeding and development of ectopic endometrial tissue.

In this study, we hypothesize that ovulatory FF exerts transforming effects on endometrial cells both before and after the establishment of endometriosis. Using primary cells isolated from eutopic and ectopic endometrial tissues, we investigated the effects of FF on clonogenicity, invasion, and other cellular phenotypes. We found that FF enhanced proliferation, migration, invasion, and clonogenic capacity in both cell types, with eutopic cells showing a particularly robust increase in colony formation and clonogenic area. These findings suggest that FF may play a pivotal role in promoting the survival, expansion, and invasive potential of ectopic endometrial cells, thereby contributing to the initiation and progression of endometriotic lesions.

MATERIALS AND METHODS

Cell isolation and culture

Samples were transported in serum-free Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12, Gibco, USA) supplemented with 100 U/mL penicillin and

100 µg/mL streptomycin at 4°C and processed within 6 h. Tissues were washed three times with phosphate-buffered saline (PBS, pH 7.4) to remove blood and debris, then minced into ~ 1 mm ≥ fragments under sterile conditions. Digestion was performed in 0.1% collagenase type I (1 mg/mL, Sigma-Aldrich, USA) and 0.01% DNase I (10 µg/mL, Sigma-Aldrich, USA) in DMEM/F-12 at 37°C for 30–60 min with intermittent shaking. The digested suspension was passed through a 100 µm cell strainer (Falcon, USA), centrifuged at 400 g for 5 min, and resuspended in serum-free DMEM/F-12. The isolated cells were cultured in DMEM/F12 medium supplemented with 10% FBS. To ensure experimental consistency, cell passages were limited to fewer than 10. Informed consent was obtained, and the study was approved by the Institutional Review Board. This study was approved by the Institutional Review Board of Tzu Chi Medical Center, Taiwan (IRB Approval Nos. IRB110-069-A and IRB113-056-A) and was conducted in accordance with the ethical principles of the Declaration of Helsinki.

Cell proliferation assay

Cell proliferation was assessed using the XTT Cell Proliferation Assay Kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. Briefly, cells were seeded in 96-well plates at a density of 5×10^3 cells per well in MCDB/M199 medium supplemented with 1% FBS and incubated overnight at 37°C in a humidified atmosphere with 5% CO₂ to allow for cell attachment. The next day, cells were treated with 10% FF for 24 h, while the control group was maintained in 1% serum-containing medium as a vehicle control (VC). Following treatment, 50 µL of XTT reagent was added to each well and incubated for an additional 4 h at 37°C. Absorbance was measured at 450 nm with a reference wavelength of 650 nm using a microplate reader (BioTek, USA). Cell proliferation was expressed as a percentage relative to the control group.

Clonogenicity assay

Cells were seeded at a density of 1000 cells per well in 2 mL of culture medium in 6-well plates. The vehicle group was maintained in DMEM/F12 supplemented with 1% FBS, while experimental groups were treated with medium containing or lacking 10% FF. The medium was refreshed every 3 days, and cells were incubated under standard culture conditions (37°C, 5% CO₂) for 12 days to allow colony formation. Colonies were then fixed with 4% paraformaldehyde and stained with 0.8 mM crystal violet (Sigma-Aldrich, St. Louis, MO, USA). The number of colonies was quantified using ImageJ software (NIH, Bethesda, MD, USA). All experiments were performed in triplicate.

Cell migration assay

Cell migration was assessed using a 24-well transwell chamber system (Costar 3422, Corning Inc.) with 8 µm pore polycarbonate membranes. Cells were serum-starved for 6 h, then suspended in 0.3 mL serum-free MCDB/M199 medium and seeded into the upper chamber at a density of 2×10^4 cells per well. After overnight of incubation at 37°C with 5% CO₂, migration was stimulated by adding 10% FF to both the upper and lower chambers, bringing the total volume to 0.5 mL per

well. Following 24 h of incubation, the membranes were fixed in 4% paraformaldehyde for 20 min and washed with PBS. Nonmigrated cells on the upper surface of the membrane were gently removed using a cotton swab, while migrated cells on the lower membrane surface were stained with Giemsa. The number of migrated cells was quantified by counting three random fields per membrane under a light microscope.

Invasion assay

For invasion analysis, 24-well transwell chambers were used with membranes precoated with 60 µL diluted Matrigel (Corning Inc.) overnight at 37°C to mimic the extracellular matrix. After serum starvation, 1 × 10⁴ cells were seeded in 0.3 mL of serum-free MCDB/M199 medium in the upper chamber. Both the upper and lower chambers were supplemented with 0.5 mL of medium containing 10% FF. After 48 h of incubation at 37°C with 5% CO₂, the membranes were fixed in 4% paraformaldehyde for 20 min and washed with PBS. Noninvaded cells on the upper surface were removed, and invaded cells on the lower surface of the membrane were stained with Giemsa. The number of invaded cells was counted in three random fields per membrane using a light microscope.

Statistical analysis

All data were expressed as the mean ± standard error. Statistical analyses were conducted using GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA, USA) and Microsoft Excel 2019 (Microsoft Corp., Redmond, WA, USA). Detailed descriptions of the statistical methods used for each analysis are provided in the respective figure legends.

Artificial intelligence usage statement

During the preparation of this work, the author (s) used chat and asked artificial intelligence to improve the readability of English. After using this tool/service, the authors have reviewed and edited the content as needed and take full responsibility for the publication’s content.

RESULTS

Morphological characteristics of eutopic and ectopic endometrial cells

Primary endometrial cells were isolated and cultured from eutopic and ectopic endometrial tissues obtained from women undergoing infertility evaluations [Table 1]. Eutopic endometrial cells, collected via brushing of the endometrium during the menstrual phase of the menstrual cycle, displayed a mixed population of epithelial-like and fibroblast-like morphologies by passage 3 [Figure 1a]. In contrast, ectopic endometrial cells, derived from the cyst wall of an endometrioma in *in vitro* fertilization (IVF) patients at the preovulatory phase of the menstrual cycle,

predominantly exhibited a fibroblast-like morphology by passage 3 [Figure 1b]. These ectopic cells demonstrated significantly higher capacities for clonogenicity (29.8-fold), while showing a lower capacity for proliferation (0.49-fold) and migration (0.11-fold). Both cell types demonstrate similar capabilities in cell invasion. These morphological and growth differences may reflect distinct cellular adaptations to their respective microenvironments and pathological conditions.

Follicular fluid enhances proliferation and colony formation of eutopic and ectopic endometrial cells

To evaluate the effect of FF on cell growth, the primary cells were cultured with 10% diluted FF pooled from 27 IVF patients. Under standard culture conditions with 10% FBS, eutopic and ectopic endometrial cells showed doubling times of 48 and 56 h, respectively, establishing a baseline to compare the proliferative effects of FF. The FF significantly enhanced the proliferation of both cell types, with eutopic endometrial cells showing a 1.7-fold increase and ectopic endometrial cells exhibiting a 1.3-fold increase [Figure 2a]. Clonogenicity is a critical factor for the ectopic seeding and establishment of endometrial cells. Using a low-density culture system (1000 cells per well), we assessed the clonogenic potential of both cell types [Figure 2b]. After 12 days of culture, colony formation increased by 31.3-fold in eutopic endometrial cells and by 1.3-fold in ectopic endometrial cells when treated with FF compared to VC [Figure 2c]. In the presence of 10% FF, the colony area% of eutopic endometrial cells increased significantly to 261-fold, and ectopic endometrial cells increased by 4.9-fold [Figure 2d]. These results suggest that FF not only promotes proliferation and colony formation but also enhances the migratory potential of endometrial cells, which may contribute to their ability to establish ectopic lesions.

Follicular fluid enhances the migration and invasion potential of eutopic and ectopic endometrial cells

The baseline migratory activity of the two cell types was assessed using a transwell migration assay. FF significantly enhanced the motility of both eutopic and ectopic endometrial cells, with eutopic cells showing a 1.8-fold increase and ectopic cells exhibiting a 2.9-fold increase [Figure 3a and b]. Given that deep-infiltrating endometriosis is characterized by cancer-like tissue invasion into the stroma and deeper tissues [22], we evaluated the invasive potential of both cell types using a Matrigel invasion assay. Eutopic endometrial cells exhibited a 4.3-fold higher baseline invasion capacity compared to ectopic cells. After treatment with FF, the invasiveness of eutopic and ectopic cells increased by 9.1-fold and 4.8-fold, respectively. These results indicate that FF promotes invasion regardless of baseline differences. Notably, eutopic endometrial cells showed a 9.1-fold increase in invasiveness following FF treatment, while ectopic endometrial

Table 1: Information of eutopic/ectopic primary endometrial cells

Cell type	Morphological characteristics	Source of tissue	Phase of menstrual cycle	Diagnosis	Age
Primary endometrial cells P3	Mix	Endometrium	Menstrualphase	Hypomenorrhea	43
Primary endometriosis cells P3	Fibro-like	Chocolate cyst wall	Preovulatory phase	IVF case with ovarian endometrioma	38

IVF: *In vitro* fertilization

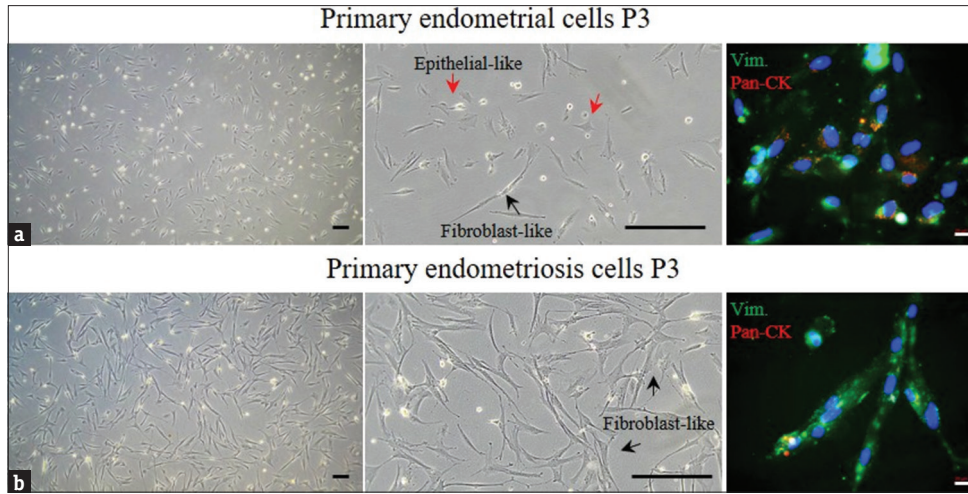


Figure 1: Morphological characteristics of eutopic and ectopic endometrium. (a) Phase-contrast micrograph of third-passage (P3) eutopic endometrial cells *in vitro*, displaying a characteristic cobblestone-like epithelial morphology (red arrows) with monolayer adherence. Mixed cell populations were observed, with a predominance of Pan-cytokeratin (Pan-CK)-positive epithelial cells and a relatively low expression of vimentin. (b) Phase-contrast micrograph of P3 ectopic endometriotic cells, exhibiting altered morphology characterized by elongated, fibroblast-like shapes (black arrows) and irregular cytoplasmic protrusions. The majority of cells were vimentin-positive, with only a small subset expressing Pan-CK. Images were acquired using an inverted phase-contrast microscope at $\times 40$ and $\times 200$, and fluorescence microscopy at $\times 400$. Scale bars: phase-contrast images, 200 μm ; immunofluorescence, 20 μm

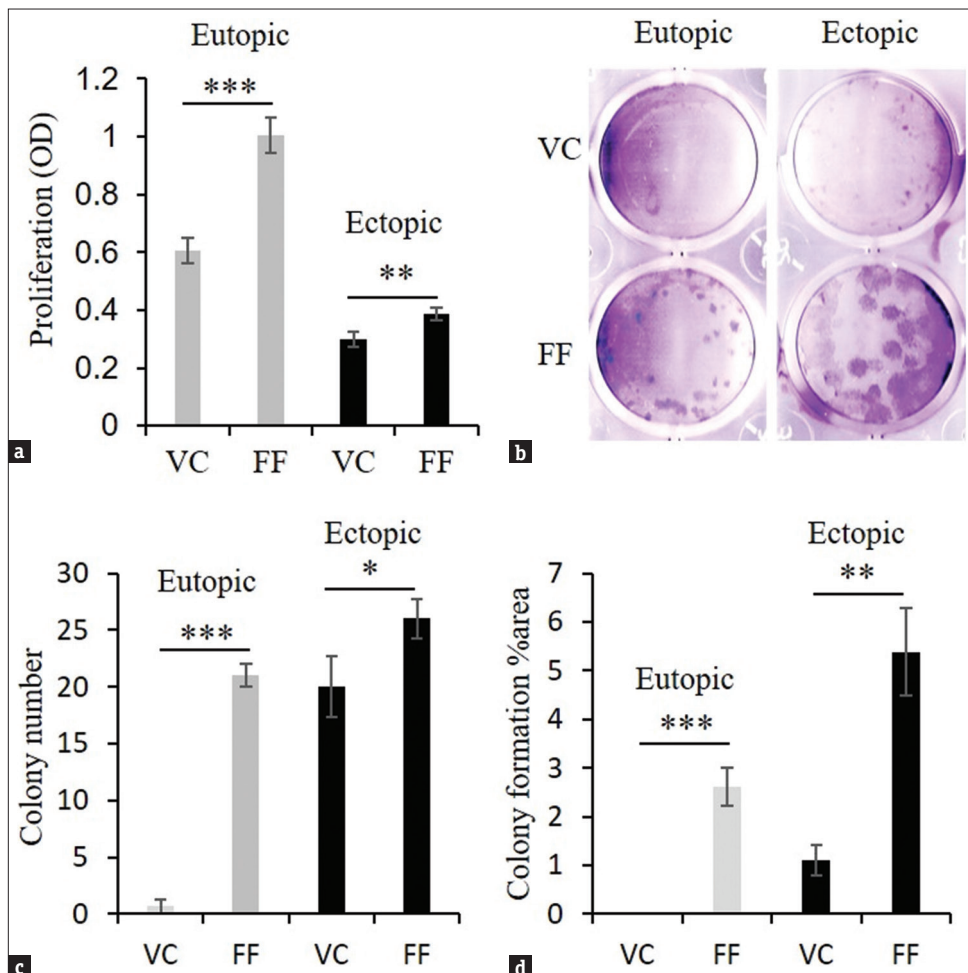


Figure 2: Follicular fluid (FF) enhances proliferative activity and stimulates clonogenic potential in eutopic and ectopic endometrial cells. (a) Quantification of cell proliferation by XTT assay following 24-h exposure to 10% FF versus vehicle control (VC; 1% fetal bovine serum), demonstrating significantly enhanced proliferation in both cell types ($n = 3$ independent experiments, performed in triplicate; mean \pm standard deviation [SD]). (b) Representative images of crystal violet-stained colonies after 12-day culture in 10% FF versus VC. (c) Quantification of colony-forming area (%) reveals a significant increase in clonogenic growth under FF stimulation. (d) Total colony counts per well confirm FF-mediated enhancement of clonogenic potential ($n = 3$ technical replicates; mean \pm SD). *Statistical significance was assessed using a two-tailed unpaired Student's *t*-test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

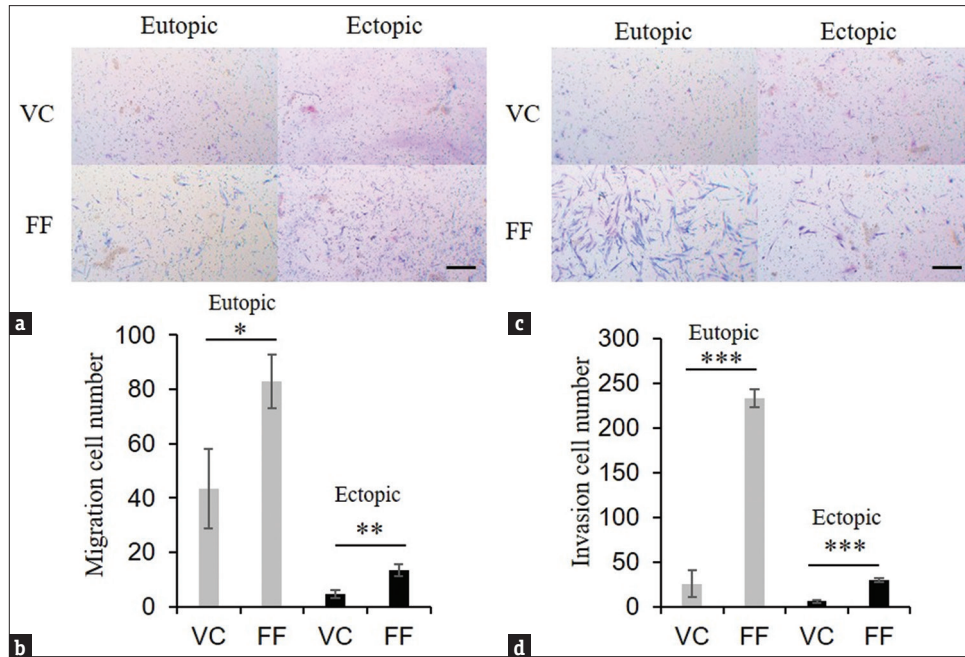


Figure 3: Follicular fluid (FF) promotes the migratory and invasive capacities of eutopic and ectopic endometrial cells. (a) Representative images from Transwell migration assays (8 μ m pore size) after 24-h incubation, with migrated cells stained using crystal violet (scale bar = 100 μ m). (b) Quantification of migrated cells per field shows significantly increased motility in response to 10% FF compared to vehicle control (1% fetal bovine serum). (c) Representative images from Matrigel-coated Transwell invasion assays (8 μ m pores, 48-h incubation), demonstrating enhanced invasion in the FF-treated group; cells were fixed and stained with 0.1% crystal violet (scale bar = 200 μ m). (d) Quantitative analysis of invasive cell numbers confirms FF-induced enhancement of invasiveness (n = 3 independent experiments; mean \pm standard deviation). *Statistical significance was evaluated using a two-tailed unpaired Student's *t*-test: **P* < 0.05, ****P* < 0.001

cells displayed a 4.8-fold increase [Figure 3c and d]. These findings suggest that FF exerts a more pronounced pro-invasive effect on eutopic endometrial cells, potentially facilitating their initial detachment and infiltration into surrounding tissues during the establishment and progression of endometriosis.

DISCUSSION

The findings of this study provide compelling evidence supporting the hypothesis that ovulatory FF plays a critical role in modulating the behavior of endometrial cells, both eutopic and ectopic, and contributes to the pathogenesis of endometriosis. Our results demonstrate that FF significantly enhances the proliferation, clonogenicity, migration, and invasion of endometrial cells, with notable differences observed between eutopic and ectopic cell populations [Table 2]. These findings align with our proposed “double engines theory,” which posits that retrograde menstruation provides the “seeds” for ectopic implantation, while ovulation, through the action of FF, acts as the “fuel” driving the growth and progression of endometriotic lesions [11].

Notably, while eutopic cells showed greater responses to FF in terms of proliferation, colony formation, and invasion, ectopic cells demonstrated a stronger response in cell migration. The ectopic endometrial cells exhibited a fibroblast-like morphology and expressed vimentin, suggesting that these cells have undergone significant adaptations in response to their microenvironment, making them more responsive to FF in terms of cell migration. This phenotypic shift may reflect the influence of local factors such as estrogen production, paracrine signaling from ovarian stromal components, and the inflammatory milieu on the ovary and within the peritoneal cavity [8-10].

The differentially enhanced clonogenicity and invasive potential of eutopic and ectopic endometrial cells in response to FF stimulation further highlight the potent effect of FF in promoting the establishment of primary endometriosis from ectopically located endometrial cells, while its effect on the propagation of established endometriotic cells is comparatively less potent. The proliferative and clonogenic effects of FF on endometrial cells are particularly noteworthy. FF is enriched with growth factors, such as IGF2, HGF, and EGF-like ligands [12-17], creating a permissive microenvironment that may support the survival and expansion of ectopic endometrial cells. It also contains abundant soluble extracellular matrix proteins, such as fibronectin and vitronectin [23], as well as a variety of adhesion molecules that may facilitate the attachment and outgrowth of both eutopic and ectopic cells. These components may collectively enhance the proliferative, migratory, and clonogenic potential of endometrial cells in response to ovulatory stimuli.

In our clonogenic assays, FF induced a 31.3-fold increase in colony formation in eutopic endometrial cells and a 1.3-fold increase in ectopic endometrial cells after 12 days of culture [Figure 2c], relative to their respective baseline (day 0) levels. Interestingly, despite the higher baseline clonogenic potential typically associated with ectopic cells, the relative increase upon FF stimulation was more robust in eutopic cells. This suggests that eutopic endometrial cells may retain a more plastic or stem-like phenotype that is more responsive to FF-derived stimuli. In contrast, the more modest increase in clonogenicity among ectopic cells may reflect their adaptation to the ectopic niche, potentially characterized by a more stable.

Table 2: Summary of phenotypic changes after follicular fluid treatment

Cell type	Proliferation (fold)	Colony formation (n)	Colony formation (% area)	Migration (fold)	Invasion (fold)
Primary endometrial cells P3	×1.7	×31.3	×261	×1.8	×9.1
Primary endometriosis cells P6	×1.3	×1.3	×4.9	×2.9	×4.8

These findings indicate that while FF promotes clonogenicity in both cell types, the magnitude and nature of the response are modulated by cellular origin and possibly epigenetic reprogramming associated with ectopic localization. This interpretation is in line with previous reports demonstrating that FF promotes EMT and enhances the invasive and stemness-associated properties of various cell types, including those involved in ovarian cancer progression [17,18,21] and may similarly influence pathological endometrial cell populations [19,20].

The migratory and invasive phenotypes induced by FF further highlight its role in facilitating the spread and establishment of endometriotic lesions. The pronounced pro-invasive effect of FF on eutopic endometrial cells, in particular, suggests that FF may play a critical role in the initial stages of endometriosis by enabling endometrial cells to detach, migrate, and infiltrate surrounding tissues. This is supported by the observation that FF enhances the motility and invasiveness of both eutopic and ectopic cells, albeit to varying degrees. The differential response of eutopic and ectopic cells to FF may reflect their distinct molecular and epigenetic profiles, which have been shown to influence their migratory and invasive capabilities [4,5]. We speculate that the higher baseline invasiveness observed in eutopic endometrial cells may reflect an inherent plasticity, enabling them to respond more readily to microenvironmental cues during the early stages of ectopic implantation. In contrast, ectopic endometrial cells may have undergone EMT and long-term adaptation to the ectopic niche, which could result in reduced basal invasive activity but enhanced stability and survival within established lesions. These observations support the notion that eutopic and ectopic endometrial cells are functionally distinct and subject to dynamic regulation throughout the progression of endometriosis.

These findings have important implications for understanding the pathophysiology of endometriosis and identifying potential therapeutic targets. The ability of FF to promote the survival, proliferation, and invasion of endometrial cells suggests that targeting FF-mediated signaling pathways or the factors within FF itself could offer novel strategies for disease intervention. For instance, inhibitors of growth factor signaling or agents that modulate the inflammatory and oxidative stress responses within the peritoneal microenvironment may hold promise for preventing or treating endometriosis [6,7]. Ultimately, inhibition of ovulation by combined (estrogen plus progesterone) or progesterone-only oral contraceptives can be the most cost-effective way of prevention of endometriosis [24].

CONCLUSIONS

This study provides novel insights into the role of ovulatory FF in the pathogenesis of endometriosis. By enhancing

the proliferation, clonogenicity, migration, and invasion of endometrial cells, FF acts as a key driver of disease progression. These findings support the “double engines theory” and highlight the importance of ovulation as a critical factor in the establishment and progression of endometriotic lesions. Further research is needed to elucidate the specific molecular mechanisms underlying FF’s effects and to explore the therapeutic potential of targeting FF-mediated pathways in endometriosis.

Acknowledgments

The authors acknowledge the core facilities provided by the Advanced Instrumentation Center of the Department of Medicine Research, Hualien Tzu Chi Hospital, and the Buddhist Tzu Chi Medical Foundation, Hualien, Taiwan.

Data availability statement

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

Financial support and sponsorship

This study was supported by the National Science and Technology Council, Taiwan, ROC (NSTC 113-2314-B-303-009-MY3, and MOST 110-2314-B-303-008), and the Tzu Chi Medical Foundation (TCRD113-071).

Conflicts of interest

Dr. Dah-Ching Ding, an editorial board member at *Tzu Chi Medical Journal*, had no role in the peer review process of or decision to publish this article. The other authors declared no conflicts of interest in writing this paper.

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