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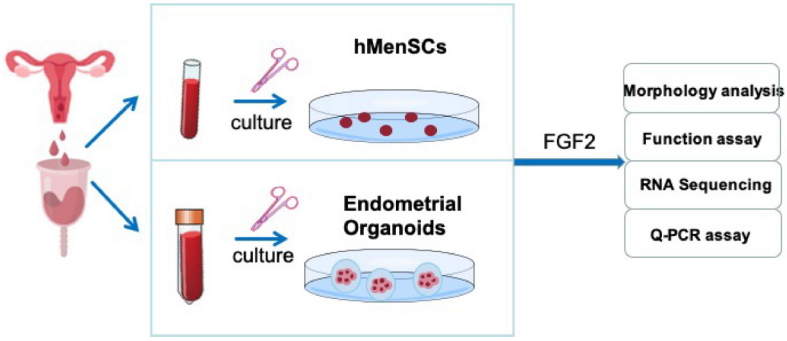
Graphical abstract



In Brief

Our study explores the unexplored potential of FGF2 in boosting the regenerative capacities of hMenSCs and endometrial organoids. We've devised a robust cultivation method and revealed that FGF2 enhanced hMenSC & endometrial organoid regenerative properties via modulating genes related to growth, proliferation, metabolism, and signaling. FGF2's dual effects on hMenSCs & organoids hold promise for regenerative medicine & endometrial diseases.

Highlights

- Establishment of a rapid and high-quality culture platform for hMenSCs & endometrial organoids
- Gene network regulated by FGF2 in hMenSCs & endometrial organoids
- The divergent effects of FGF2 on hMenSCs & endometrial organoids

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The utiliity of FGF2 in the rapid and high-quality cultivation of hMenSCs and endometrial organoids

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ABSTRACT

Human menstrual blood-derived stem cells (hMenSCs) show promise in regenerative medicine and endometrial disease treatments. Organoids, three-dimensional (3D) tissue replicas, provide accurate disease models. Fibroblast growth factor 2 (FGF2), with established roles in stem cell functions, remains underexplored in its impact on hMenSCs and endometrial organoids. Our study aims to explore these unexplored effects of FGF2. In our study, we have developed a method for rapid and high-quality cultivation of MenSCs and organoids. Using this novel platform, we demonstrate that FGF2 alters hMenSC morphology, enhancing growth and proliferative, migratory, and anti-aging capabilities. RNA sequencing suggests this is linked to downregulated cell adhesion genes. FGF2 promotes proliferation via ribosomal biogenesis and ATP metabolism, possibly delaying senescence. In organoids, FGF2 reverses compaction and volume reduction, promoting growth and fibroblast release. q-PCR analysis shows FGF2 regulates genes related to stemness, proliferation, metabolism, and PI3K-AKT signaling, with distinct patterns in hMenSCs and organoids. This research advances regenerative medicine and reproductive biology by optimizing the cultivation of hMenSCs and organoids with FGF2 for potential therapeutic applications.

KEYWORDS

hMenSCs, endometrial organoids, FGF2, rapid and high-quality cultivation, RNA sequencing, regenerative medicine

Introduction

In recent years, menstrual blood-derived stem cells (MenSCs) have garnered significant research attention due to their outstanding properties. These cells exhibit robust self-renewal capabilities, ease of accessibility, and remarkable directed differentiation potential. Additionally, MenSCs possess strong regenerative abilities and display low immunogenicity, making them a promising candidate for regenerative medicine applications[1,2]. When compared to mesenchymal stromal cells (MSCs), MenSCs demonstrate superior extraction efficiency, extended passaging capacity, and improved proliferative capacity[3]. These advantageous characteristics enhance the feasibility and efficacy of using MenSCs in transplantation procedures or *in vitro* experiments, thereby advancing the field of regenerative medicine and paving the way for novel therapeutic strategies for various diseases and injuries.

MenSCs exhibit remarkable differentiation potential, transforming into cardiomyocytes, pulmonary epithelial cells, neurons, muscle cells, and endothelial cells *in vitro*[1]. Preclinical studies show promising therapeutic outcomes in intrauterine adhesions[4], myocardial infarction[5], diabetes[6], and liver fibrosis[7]. However, limited clinical trials have been conducted due to insufficient knowledge of MenSC cellular properties. Systematic investigations are needed to identify mediators of MenSC unique properties, facilitating the development of effective MenSC-based therapeutic strategies for diseases.

The FGF protein family, encompassing 23 members, plays a pivotal role in regulating stem cell regeneration, tissue repair, and metabolic functions. FGF2, a well-studied member, has been thoroughly examined in various stem cell processes[8]. FGF2's key function includes inducing stem cell migration, crucial for cell regeneration. FGF2 can trigger bone marrow mesenchymal stromal cell (BMMSC) migration via the Akt/PKB pathway, even at low doses, while higher concentrations may have ambivalent

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effects[9]. FGF2's impact on dental pulp stem cell (DPSC) migration has also been reported[10]. FGF2's diverse roles in stem cell biology highlight its potential in regenerative medicine.

Furthermore, FGF2 in human umbilical vein endothelial cells (HUVECs) acts as a chemotactic factor, promoting anti-senescence and proliferation[11,12]. Limited stem cell proliferation hinders their application in tissue regeneration. Recent studies reveal bFGF's mitogenic properties, enhancing dental pulp stem cell growth via ERK and FGFR/PI3K signaling[12]. FGF2 regulates cell cycle genes, including Ki67, CDC2, and cyclin B1, initiating cell proliferation[13]. These findings support FGF2's potential as a strategy to amplify stem cells, crucial for tissue regenerative treatments.

FGF2 plays a crucial role in maintaining pluripotency in hESCs, primarily through secreted proteins from MEF feeder layers. These proteins support pluripotency[14]. FGF2 also enhances expression of pluripotent markers (NANOG, OCT4, and REX1) in various stem cells (sterol regulatory element binding proteins cleavage-activating proteins (SCAPs), DPSCs, and stem cells from human exfoliated deciduous teeth (SHED)), preserving self-renewal, and differentiation potential[15,16]. While FGF2's roles in MenSCs remain undefined, its importance in stem cell biology is undeniable. FGF2 is a key factor in sustaining pluripotency and regenerative potential *in vitro* and *in vivo*.

Our research delved into FGF functions in MenSCs & organoids. *In vitro* MenSC experiments & RNA-seq analysis revealed FGF2's crucial role in promoting proliferation, self-renewal, & pluripotency marker expression. FGF2 also prevented senescence & enhanced migration. Comparable effects were observed in organoid experiments. FGF2's dual effects on MenSCs & organoids hold promise for tissue engineering & regenerative medicine.

Materials and Methods

MenSCs culture

Human menstrual blood samples were collected from a healthy female donor for primary culture. Volunteers signed a written informed consent before participating in the study. Isolated hMenSCs were maintained under sterile conditions, following our established protocols[17]. Subsequently, the samples were washed ≥ 6 times in 1× Hank's balanced salt solution (HBSS, Gibco, USA) and minced into small fragments using ophthalmic scissors. The tissue fragments were then transferred to centrifuge tubes containing 3 mL of α-MEM medium (supplemented with 1 U/mL Dispase II) and incubated at 37 °C for 30 min. The cell suspensions were centrifuged at 1000× *g* for 3 min, twice, at room temperature. The supernatant was discarded, and the cell pellet was resuspended in α-MEM medium supplemented with 10% fetal bovine serum (FBS), 1× GlutaMAX, 1% pyruvate, and 20 ng/mL penicillin and streptomycin). The cells were cultured in 6-well plates and maintained in a 37 °C incubator with 5% CO_2. The adherent cells were continued until up to 70% confluency for 6–7 days. Throughout the experiment, the stem cells were passaged 4–6 times. FGF2 (recombinant human, HumanZyme, USA) was stocked at 50 000× and subsequently diluted 10× with α-MEM to use.

Organoid culture

Endometrial organoids with Matrigel-free was established from the shedding of human endometrium as previously described[18]. We ensure that written informed consent was obtained from the human donors and the entire operation adheres stringently to the principles of clinical ethics. After thorough washing (Washing Buffer LSNO00100201; LiShengBiotech, Shanghai, China) and mechanically digesting the tissue into fragments no larger than 3 mm in size, the fragments were transferred to a 10-cm dish 15 mL of endometrial organoid culture medium (LSTO01300401; LiShengBiotech, Shanghai, China) was added. The dish was then incubated at 37 °C in a 5%CO_2 environment. The following day, organoids of suitable size as well as an appropriate number of dispersed single cells were selected and transferred to a 96-well plate, with 200 μL of endometrial organoid culture medium per well. Five wells were designated as control groups, while another five wells received an additional 20 nmol/L FGF2 (recombinant human, HumanZyme, USA). On days 0, 1, 2, 3, 5, and 7 of the drug testing, the organoids were tracked and recorded at fixed positions using a dissecting microscope and an inverted phase contrast microscope. Furthermore, on days 3 and 5, a medium exchange was performed, where 80 μL of old medium was removed and replaced with 100 μL of fresh culture medium.

Cell growth assay

MenSCs were inoculated in 96-well plates, and 10 μL of CCK8 solution and various dose of FGF2 (0, 5, 10, and 20 ng/mL) were added to each well. Three different time points (48, 72, and 144 h) were selected for each group. After the incubation period, the absorbance at 450 nm was measured using a microplate reader.

Immunofluorescence staining

Cells grown on coverslips in 24-well plates were fixed with freshly prepared 4% paraformaldehyde for 10 min. After rinsing with phosphate buffered saline (PBS), the cells were blocked with 3% bovine serum albumin (BSA) for 1 h at room temperature. Then, cells were incubated with the following primary and secondary antibodies: rabbit anti-goat Oct4 (1:1000), mouse anti-goat CD9 (1:1000), mouse anti-goat CD44 (1:1000), rabbit anti-goat ki67 (1:1000), goat anti-mouse 488 antibody (1:1000), goat anti-rabbit 594 antibody (1:1000). Finally, slides were mounted in antifade mountant with 4',6-diamidino-2-phenylindole (DAPI). Immunofluorescence photos were captured by fluorescence microscope and the means of the percentages of positive cells at each time point were analyzed statistically using the software ImageJ (Version 1.53m).

Cell migration analysis

hMenSCs were inoculated in a six-well plate and scratched with a 10ul short shotgun tip after 24 h. The scratched cells were removed by washing with PBS and incubated with medium. The cell gaps were captured at 0, 24, 72, and 144 h after the scratches were made.and the migration was observed after. The cells were analyzed by ImageJ software. ImageJ software was used for quantify and analyze the migratory behavior of the MenSCs.

SA-β-gal staining

Following 144 h of FGF exposure, MenSCs were washed with PBS. Following this, they were fixed for 15 min at room temperature. Subsequently, the cells were exposed to the SA-β-gal solution (pH 6.0) overnight at 37 °C. Finally the stained MenSC samples were viewed under a microscope.

Gene expression analysis

RNA was reverse-transcribed using FastKing gDNA Dispelling RT SuperMix (KR118, Tiangen, China). For quantitative real-time PCR (qPCR), gene-specific forward and reverse primers were used to amplify the samples with LightCycler 96 Instrument (Roche). The primer sequences are listed in the Electronic Supplementary Material (ESM). The target gene expression levels were normalized to those of the housekeeping genesL: GAPDH and 18S. Relative gene expression levels were calculated as 2−(ΔΔCt) values. The following specific primers were synthesized by Invitrogen and showed in the ESM.

RNA extraction and identification of DEGs

Cells were incubated with FGF2 (0, 10, and 20 ng/mL) for 0, 24, and 48 h and all RNA samples were isolated using the Trizol for RNA-seq. High-throughput sequencing was performed using the DNBSEQ platform. RNA-seq fastq files with three biological replicates were aligned to the Human Reference Genome. Differentially expressed genes (DEG) were obtained by analyzing the number of TPM counts. $P < 0.05$, |Log2Fold Change (FC)| ≥ 1 were included in further analysis as significant differential expression.

GO enrichment and KEGG pathway analysis

The online tool for Hiplot Pro (https://hiplot.com.cn)was used to perform the gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of DEGs. $P < 0.05$ were considered statistically significantly enriched GO terms and KEGG pathways. For the time-series data, DEG based on |Log2FC| > 1.5 (up or down) were detected at 2 timepoints (24 or 48 h) with respect to time 0 h. A protein–protein interaction (PPI) network analysis was conducted using the STRING database (https://www.string-db.org/)and DEGs associated with proliferation, senescence, migration, were performed by PPI network analysis.

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM) in independent experiments. Statistical significance was determined by two-tailed Student's *t*-tests between two groups using GraphPad Prism version 8. $^*P < 0.05$ is considered statistically significant.

Results

FGF inducing the severe changes in cell morphology of MenSCs without affecting stemness maintenance

We initially aimed to elucidate the impact of varying doses of FGF2 on MenSCs *in vitro*. MenSCs were isolated from menstrual blood collected from healthy adult women (Fig. 1). Cells were cultured in medium supplemented with FGF2 at concentrations of 5, 10, and 20 ng/mL for 144 h, followed by morphological and growth assessments using light microscopy (Figs. 1a and 1b).

In standard culture conditions, MenSCs exhibited a rounded morphology and exhibited indefinite outgrowth. Exposure to low-dose FGF2 (5 ng/mL) resulted in notable morphological changes, including a more oval shape, decreased cell area, and altered length/width ratio (Figs. 1b–1e). Additionally, this low-dose FGF2 treatment modestly enhanced cell proliferation in MenSCs (Figs. 1c and 1d), suggesting that FGF2 can modulate cell morphology and promote growth. Notably, treatment with higher doses of FGF2 (10 and 20 ng/mL) induced more robust morphological changes and a significant increase in cell number, nearly doubling the cell population (Figs. 1b–1e).

To further investigate the mechanisms underlying the effects of FGF2, we examined whether exposure to FGF2 altered the maintenance of stemness and induced cell differentiation in MenSCs. Immunofluorescence analysis revealed that MenSCs cultured under standard conditions primarily expressed CD9 and OCT4, markers associated with MSCs (Fig. 1f). However, upon exposure to FGF2 for 144 h, $CD44^+$ cells were detected, indicating the presence of a distinct cell population (Fig. 1g). Interestingly, $CD44^+$ positive ratio exhibited uniformity, with no significant differences was observed regardless of the FGF2 dose (Fig. 1h), suggesting that the stemness of MenSCs is relatively insensitive to FGF2 treatment.

Collectively, these results demonstrate that FGF2 exerts a profound influence on cell morphology and growth in MenSCs while maintaining their capacity to express stem cell markers. However, FGF2 exhibits a substantial propensity to sustain the stemness feature characteristics of hMenSCs.

FGF2 comprehensively regulates hMenSCs growth, proliferation, migration, and senescence

To further explore the role of FGF2 in inducing cell growth in MenSCs, we conducted a detailed analysis of the effects of different concentrations of FGF2 on MenSCs during a 144-h culture period. Cell growth was monitored periodically using light microscopy, and a time-phase analysis revealed that the most significant changes in cell number occurred within 72 h. Specifically, high (20 ng/mL) and medium (10 ng/mL) doses of FGF2 significantly promoted cell growth, resulting in a marked increase in cell numbers compared to the control group during the 144-h culture period (Figs. 2a and 2b). Low-dose FGF2 (5 ng/mL) did not exhibit a similar stimulatory effect.

To further validate these observations, we employed the CCK8 assay to quantitatively assess cell growth. Consistent with our previous findings, there was no significant change in cell growth in any of the FGF2 groups after 48 h. However, after 72 h of incubation, both the 10 and 20 ng/mL doses of FGF2 led to a dramatic increase in optical density (OD), indicating enhanced cell growth. Notably, the 20 ng/mL dose of FGF2 had the most pronounced effect on cell growth in MenSCs (Fig. 2c).

To gain further insights into the potential role of FGF2 in MenSCs, we evaluated cell migration using a wound healing assay. This assay measures the ability of cells to migrate and close a scratch wound created in a cell monolayer. At 24 h after inducing the scratches, there were no significant differences in cell migration among the different groups. However, after 72 h, MenSCs treated with high-dose FGF2 (20 ng/mL) exhibited significantly faster migration rates compared to control cells (Fig. 2d, left panel). Furthermore, cell migration rates increased with higher concentrations of FGF2, while low- or medium-dose FGF2 did not significantly differ from the control group at this time point. Finally, at 144 h, both high- and medium-dose FGF2-treated cells migrated faster than control cells, while low-dose FGF2 had no significant effect on cell migration (Figs. 2d and 2e).

FGF has been previously implicated in promoting self-renewal and inhibiting cell senescence in stem cells. To further investigate this potential role in MenSCs, we employed β-Galactosidase staining to monitor senescence cells. The staining process allowed us to visualize senescent cells, which were marked by a blue color.

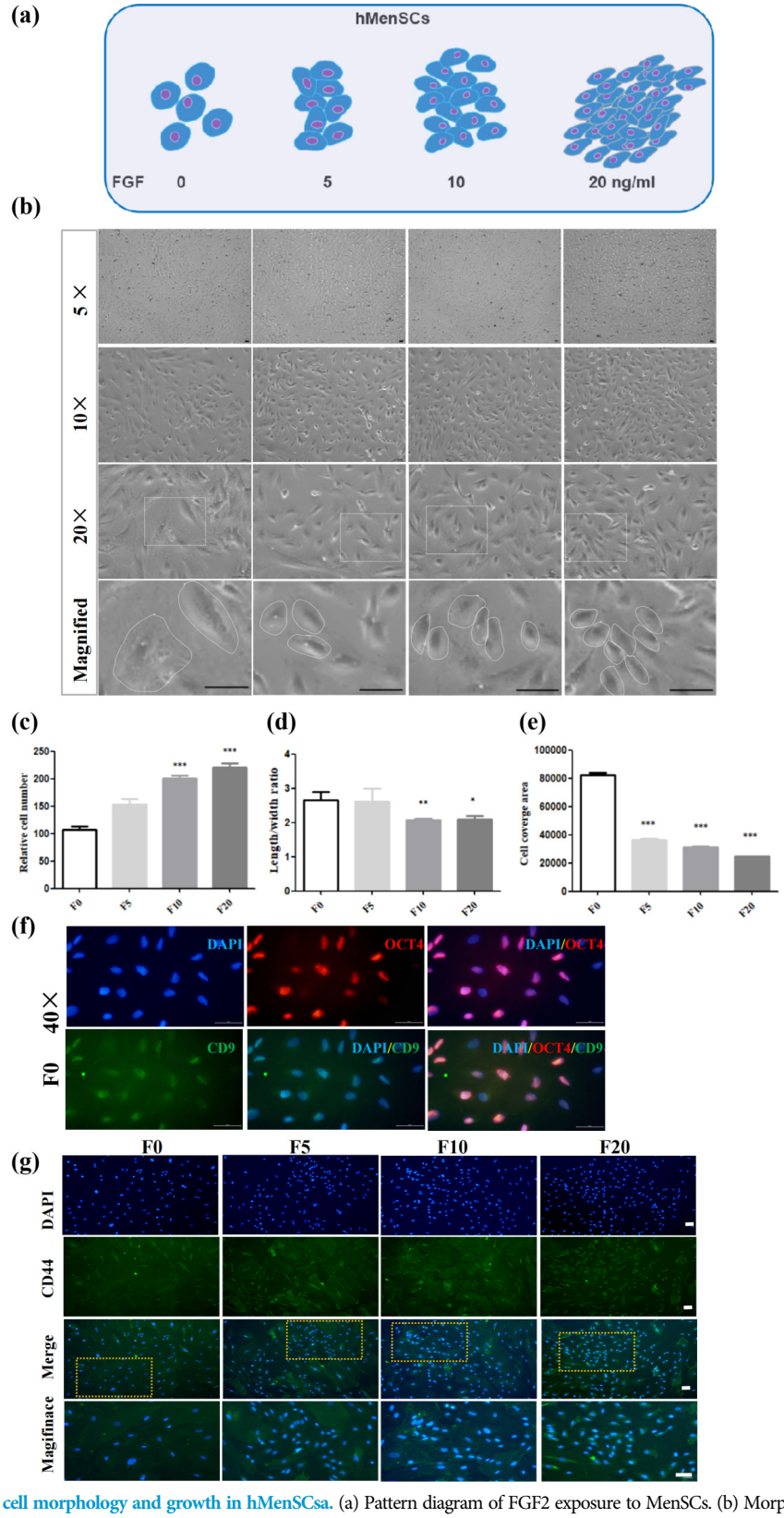

Figure 1. Role of FGF2 on cell morphology and growth in hMenSCsa. (a) Pattern diagram of FGF2 exposure to MenSCs. (b) Morphological changes in MenSCs treat with FGF2were analyzed by light photo. (c)(d)(e) Cell coverage area, length/width ratio and cell number in Menscs treat with FGF2 for 144h. (f) OCT, CD9 positive cell in MenSCs were monitored by fluorescence microscopy. (g) Representative immunofluorescence images of CD44 were obtained after vary dose of FGF2 treatment. (h) Column statistical chart of CD44 positive rate scale bar = 50 μm. The data are presented as the mean ± SEM.

Our analysis revealed that the intensity of β-galactosidase-positive cells was significantly decreased after treatment with FGF2. Specifically, low-dose FGF2 treatment resulted in an average reduction of senescent cells to 14% (Figs. 2f and 2g). Notably,

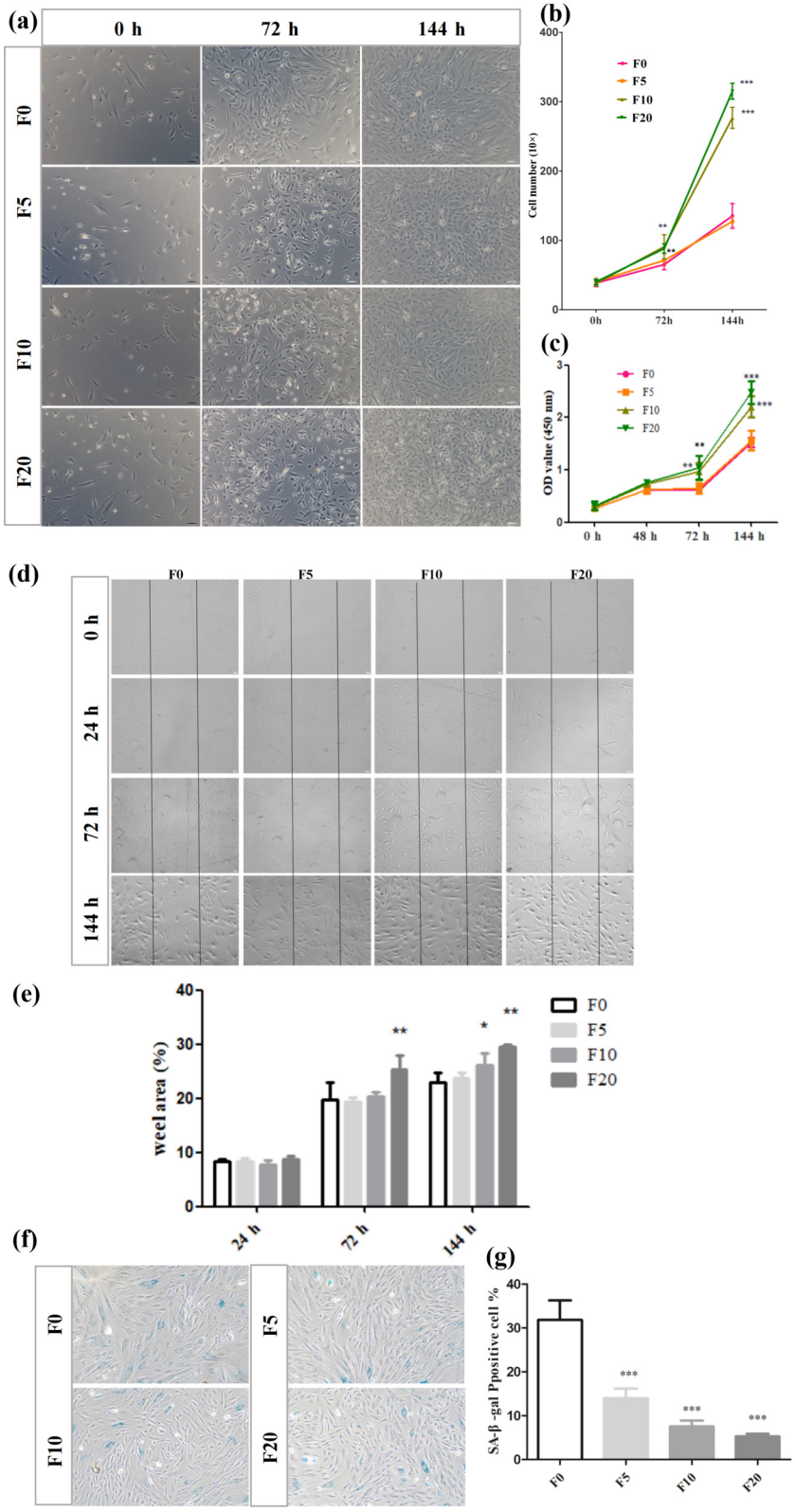

Figure 2. Effects of FGF2 on cell growth, migration and senescence in hMenSCs. (a) light photo of MenSCs during FGF2 culture for 144 h. (b) Time-phase analysis of cell number change in FGF2-treated MenSCs. (c) Cell growth of MenSCs treat with FGF2 (5, 10, and 20 ng/mL) was evaluated by CCK8 assay for 48 and 72 h. (d) Cell migration is accelerated in the presence of FGF2. Cell monolayers were scratched (bounded by black line) and stimulated with low (5 ng/mL), medium (10 ng/mL), or high (20 ng/mL) FGF2. Images of the scrath wounds were acquired at 24, 72, 144 h after stimulation. (e) Column statistical chart of migrated area. (f) and (g) Senescence cells level were measured after treatment with FGF2 as indicated. The values are expressed as the mean ± SEM of results from three independent experiments, with three parallel samples for each group in each experiment. * $P < 0.05$ vs. FGF20 group, * *$P < 0.01$ vs. FGF20 group.

medium and high doses of FGF2 exerted even more profound effects, further decreasing the number of senescent cells. These results clearly demonstrate that FGF2 treatment results in a significant loss of senescence cells in MenSCs.

Given the well-documented relationship between cell growth and proliferation, we further investigated the impact of FGF2 on the proliferation of hMenSCs. Immunofluorescence staining was performed at 72 and 144 h to detect the expression of Ki67, a marker of cell proliferation. Low FGF2 conditions modestly increased the percentage of Ki67-positive cells compared to the control group, while medium and high FGF concentrations robustly enhanced proliferation at 72 h (Fig. 3a and 3c). Consistent results were observed after 144 h of culture (Figs. 3b–3d). Our results suggest that FGF2 significantly increases cell

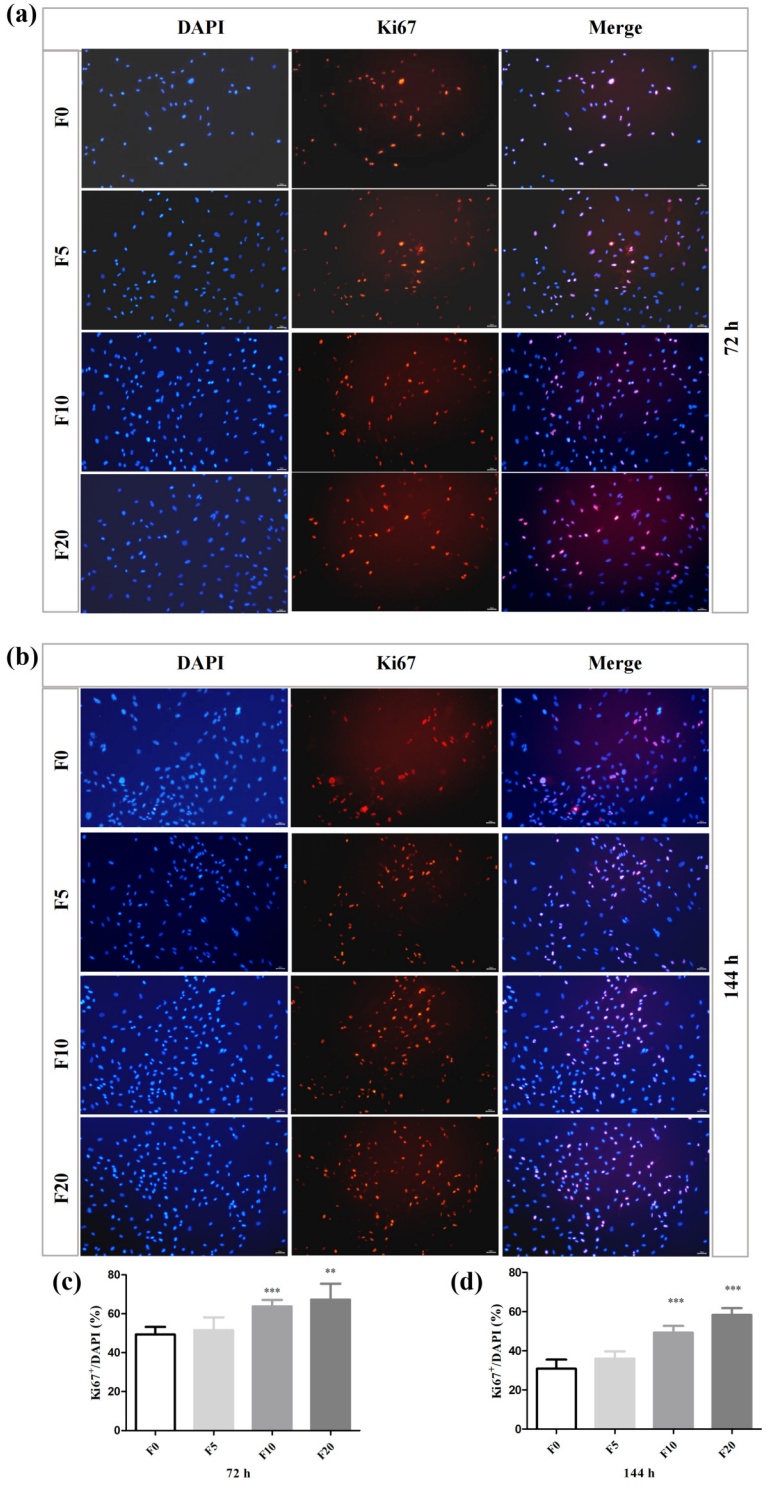

Figure 3. FGF22 induced cell prolifaration in MenSCs. (a) Representative fluorescent images of ki67 level showed red using immunofluorescence for 72 h. The nucleus showed blue using DAPI, Scale bar = 50 μm. (b) Fluorescent images of ki67 expression showed red in FGF2 culture for 144 h. (c) and (d) The expression of ki67 Positive rate in MenSCs using histogram analysis at 72 and 144 h. Data are showed as mean ± SEM of 3 independent experiments. (*P < 0.05, **P < 0.01, ***P < 0.001 vs. control).

numbers in hMenSCs, primarily by activating cell proliferation. FGF2 appears to be a rapid regulatory factor promoting cell growth in MenSCs.

Collectively, our data highlight the profound effects of FGF2 on hMenSCs, underscoring its pivotal role in enhancing cellular growth, migration, and anti-senescent, thereby pointing to its expansive role in modulating the functional landscape of stem cells.

Whole transcriptome analysis of MenSCs in the presence of FGF2

To further elucidate molecular mechanisms, transcriptional activity of FGF2 in MenSCs was mapped via RNA-seq over 48 h. Clustering analysis revealed distinct patterns for medium (10 ng/mL) and high (20 ng/mL) FGF2 treatments (Figs. 4a and 4b). Differential gene expression analysis identified 944 upregulated and 1253 downregulated genes in the F10 group, compared to controls (Fig. 4c). Similarly, the F20 group showed 1016 upregulated and 1274 downregulated genes (Fig. 4d), with 754 genes commonly upregulated in both groups. Notably, 1732 genes exhibited significant differential expression ($|\log2FC| > 1$, FDR < 0.05), including 754 genes upregulated in both F10 and F20, and 978 genes downregulated in both (Fig. 4e). The top 20 upregulated and downregulated genes in response to FGF2 are highlighted in Fig. 4f. The top genes likely play pivotal roles in mediating the cellular responses to FGF2 in MenSCs and offer potential candidates for further study.

GO and KEGG pathway analysis of DEGs in MenSCs treat with FGF2

Based on GO enrichment, the top 7 BP, CC, and MF terms for upregulated DEGs in FGF2-treated MenSCs were identified, emphasizing ribonucleoprotein complex biogenesis, ribosome biogenesis, ncRNA processing, serine/threonine kinase activity, cadherin binding, and RNA catalysis (Fig. 5a). Cellular component analysis highlighted cell–substrate junctions, focal adhesion, and preribosome. KEGG analysis revealed enrichment in MAPK signaling and ribosome biogenesis (Fig. 5b). RNA-seq data concurred with morphological observations, with heatmaps showing upregulated genes in ribonucleoprotein biogenesis and cell proliferation (Fig. 5c). Downregulated DEGs associated with extracellular matrix organization, cell-substrate adhesion, and collagen-rich matrix (Fig. 5d). KEGG implicated HPV infection, PI3K-Akt signaling, proteoglycans in cancer, and focal adhesion (Fig. 5e). FGF2's known effects on cell proliferation, differentiation, migration, and survival were consistent with our findings.

Next, we delved deeper into the expression patterns of the 55 downregulated genes related to cell-substrate adhesion in response to FGF2. The heatmap clearly demonstrated a higher expression

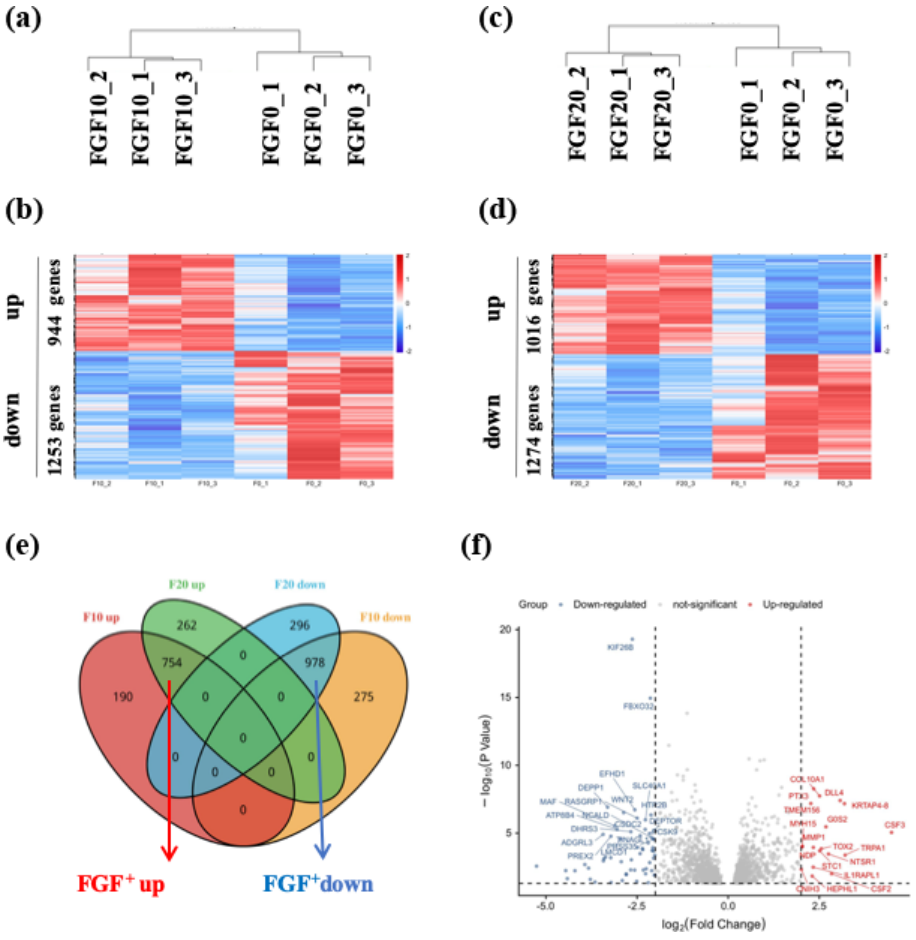

Figure 4. DEGs in hMenSCs under the influence of FGF2. (a) and (b) Cluster and heatmap representing the expression of DEG between FGF210 group and FGF20 group. (c) and (d) Cluster and heatmap profiles for gene expression of DEG between FGF220 group vs FGF20 group. (e) Venn map shows FGF2 up+ genes and FGF2 down+ genes number in MenSCs following treatment with FGF2 for 48h. (f) List of top 20 genes up (red point) and down (blue point) regulated with FGF2 treatment.

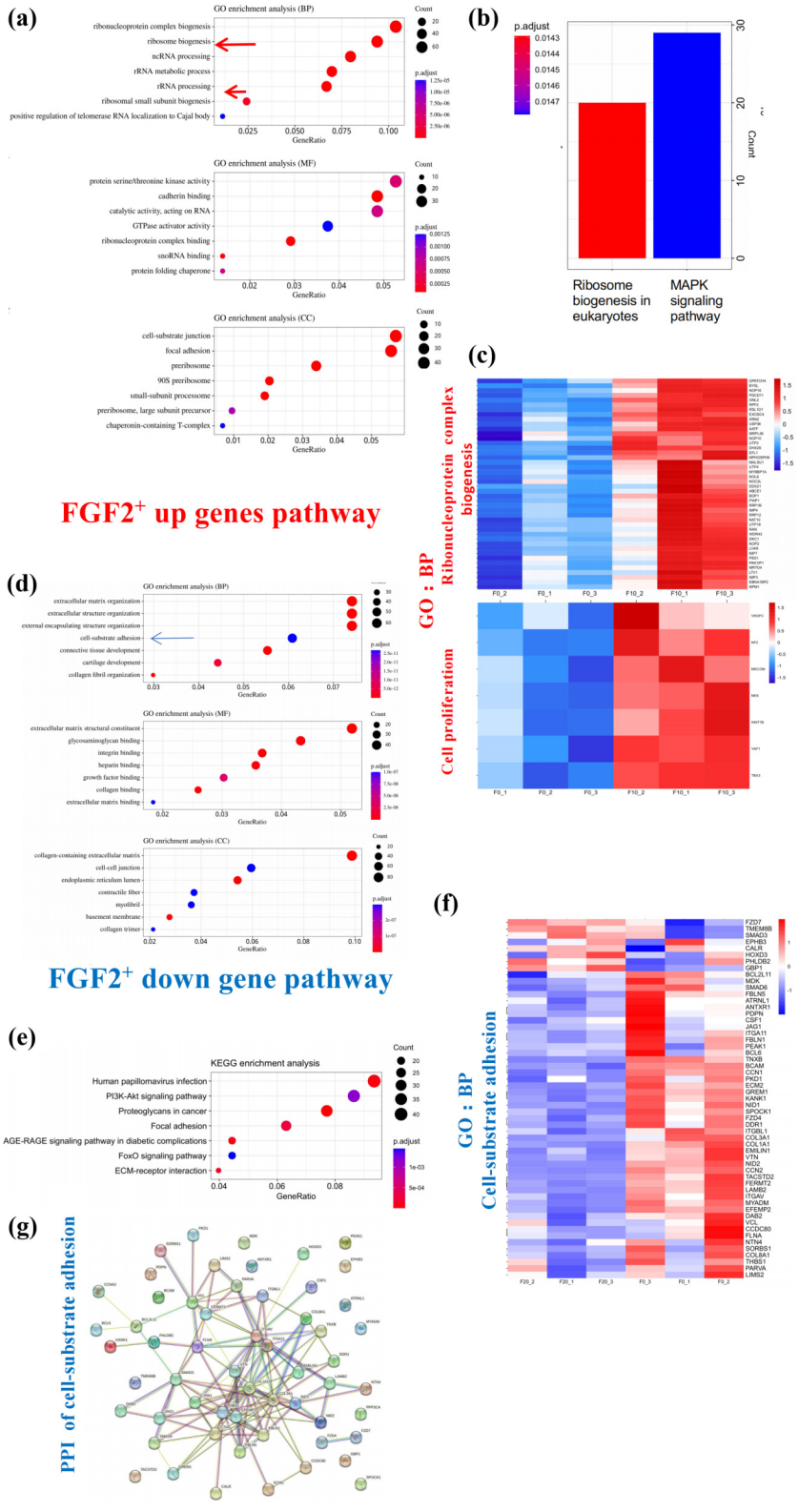

Figure 5. GO analysis and KEGG analysis for the DEGs of MenSCs upon FGF2 stimulation. (a) and (d) The top 7 enriched GO terms (biological process, molecular function, cellular component) analysis for the up-regulated DEGs or the down-regulated DEGs. (b) and (e) The top 7 enriched KEGG pathway analysis for the up-regulated DEGs or the down-regulated DEGs. (c) Heatmap for common genes enriched in ribonucleoprotein complex biogenesis. (f) Heatmap for common genes enriched in cell-substrate adhesion. (g) PPI analysis of core genes in cell-substrate adhesion term.

of key cell adhesion genes such as COL1A1, COL3A1, THBS1, VTN, and EFEMP2 in normal culture conditions. However, upon FGF2 stimulation, regardless of the dose, a significant decrease in the expression of these genes was observed (Fig. 5f). To further explore the interactions among these genes, we selected the core genes of cell-substrate adhesion with a degree beyond 10 and

visualized them in a protein-protein interaction (PPI) network.

Collectively, these results indicate that FGF2 serves as a potent inducer of MenSCs growth and survival. The diverse range of pathways associated with the genes exhibiting differential expression in response to FGF2 suggests that it regulates a complex molecular network to fulfill its role.

Temporal transcriptome analysis of MenSCs following FGF2 application

In a dose-dependent study, we discovered functional modules modulated by FGF2 exposure in MenSCs. These modules were consistently observed in the high FGF2 group across the 0, 24, and 48 h time points. To probe the temporal mechanisms underlying FGF2's effects, we analyzed transcriptome data and signaling pathways.

Comparing F20 0h with F20 24h, 1101 differentially expressed genes (DEGs) were identified, with 716 upregulated and 385 downregulated. Similarly, between F20 0h and F20 48h, 3173 DEGs were found, including 2117 upregulated and 1056 downregulated genes (Fig. 6a). This underscores the significant role of gene expression alterations in response to high FGF2 levels over 48 h. Cluster analysis confirmed distinct cell states (Fig. 6b).

Venn diagram analysis revealed that 1555 DEGs were uniquely upregulated in the F20 48h group compared to no change in the F20 24h group (F20 time up+ genes). Conversely, 743 DEGs were uniquely downregulated in the F20 48h group (F20 time down+ genes) (Fig. 6c). Heatmaps visualized the expression patterns of these genes across the 0-48h dataset (Fig. 6d and 6e).

To gain insights into the underlying mechanisms, we conducted gene ontology enrichment analysis. The upregulated DEGs were primarily associated with precursor metabolite and energy generation, ATP metabolic processes, and other energy-related functions (Fig. 6f). A circular diagram visually represented the upregulated genes linked to energy metabolism (Fig. 6g).FGF2 downregulation in F20 time down+ genes impacts key players in HPV infection, PI3K-Akt, MAPK signaling, and cellular senescence pathways, as revealed by KEGG analysis (Fig. 6h). Visual representations of gene interactions in these pathways are presented in circle graphs (Fig. 6i), while protein-protein interaction networks offer insights into the intricate web of senescence-related genes (Fig. 6j).

Overall, our findings provide a comprehensive understanding of the temporal and dose-dependent effects of FGF2 on MenSCs, particularly in terms of gene expression alterations and energy metabolism.

FGF2 treatment influences morphology and growth of Organoids

With volunteer consent, endometrial samples were obtained and meticulously processed for organoid culture. Initially, organoids were obscured by residual red blood cells (Fig. 7a). However, during a 9-day culture period, the organoids underwent morphological changes, exhibiting clearer boundaries and decreased volumes (Figs. 7b and 7c). Some organoids developed translucent vesicles with enhanced optical transmittance (Fig. 7b), while others displayed appendages or circular structures (Fig. 7c).

Notably, the organoids were sensitive to FGF2 treatment (Fig. 8). Control organoids continued to shrink and release cells. Fibroblasts adhered to the walls and spread out, while released red blood cells formed a radial pattern known as a plaque (Fig. 8). Conversely, FGF2-treated organoids exhibited vesicle-like structures with superior optical transmittance and robust growth, with some maintaining or even slightly increasing their volumes (Figs. 8a and 8c). Furthermore, fibroblasts released from the FGF2-treated organoids exhibited faster growth and frequently formed plaques (Fig. 8b). Given these promising results, we propose that FGF2 could potentially serve as a clinically effective therapeutic strategy for endometrial repair.

FGF2 affected gene expression of stem cells biomarker, cell proliferation, ATP metabolism, cell senescence in oragnoids

To gain a deeper understanding of gene modulation in FGF2-exposed organoids during culture, we conducted Q-PCR to analyze gene expression signatures. We selected a subset of relevant pathway genes, validated the identified representative DEGs on hMenSCs, and then analyzed their expression patterns in organoids. Consistent with the results from hMenSCs experiments, the expression of stemness genes, including CD44, OCT4, and CD9, remained stable over time following FGF2 treatment during organoid development (Fig. 9a).

Among cell proliferation-related genes, the expression of VEGFC, NES, and TBX3 generally increased in both control and FGF2-treated groups, with the exception of Wnt7b (Fig. 9b). However, upon further analysis, we found that only VEGFC expression was upregulated specifically in response to FGF2 treatment, corroborating our RNA-seq analysis (Fig. 9b). In contrast, NES and TBX3 expression decreased following FGF2 treatment.

ATP metabolism-related genes, such as AKD1, C11ORF83, IMMP2L, and EFTUD1, were highly expressed in organoids during the 21-h cultivation process. Notably, the changes in expression of these genes after FGF2 treatment differed significantly from those observed in hMenSCs (Fig. 9c). This suggests that the molecular pathways targeted by FGF2 to regulate organoid growth and proliferation are not entirely analogous to those in hMenSCs.

Intriguingly, SIRT1, an anti-senescence gene also known as a lifespan gene, was identified as highly expressed in FGF2-treated organoids (Fig. 9d). Therefore, the upregulation of SIRT1 following FGF2 exposure may hold significant biological implications. Additionally, we examined senescence-related pathway genes, including MAPK signaling pathway components RELA, MAP2K1, and EIF4E2, and found that their expression patterns in organoids closely resembled those in hMenSCs (Fig. 9d).

Collectively, these findings provide insights into the complex gene modulation networks underlying the response of organoids to FGF2 treatment, highlighting both conserved and distinct regulatory mechanisms compared to hMenSCs.

A schematic diagram of FGF2 function in hMenSCs and organoids

FGF2 plays a pivotal role in regulating diverse aspects of hMenSCs development, repair, and regeneration, encompassing stemness maintenance, migration, proliferation, as well as cellular senescence (Fig. 10). Concurrently, genetic alterations occur in various signaling pathways, including ribosome biogenesis, cell-substrate adhesion, ATP metabolic processes, and cell senescence pathways.

Upon continuous stimulation with FGF2, we observed organoids gradually diminishing in size and exhibiting less dense structures. Fibroblast-like cells emigrated from the organoids and

adhered to the culture plate. Notably, FGF2 treatment led to the emergence of more radial plaques due to the release of red blood cells (Fig. 10). This morphological transformation may be intimately linked to alterations in gene expression patterns across multiple pathways, differing from those observed in hMenSCs.

The complexity of these interactions underscores the need for

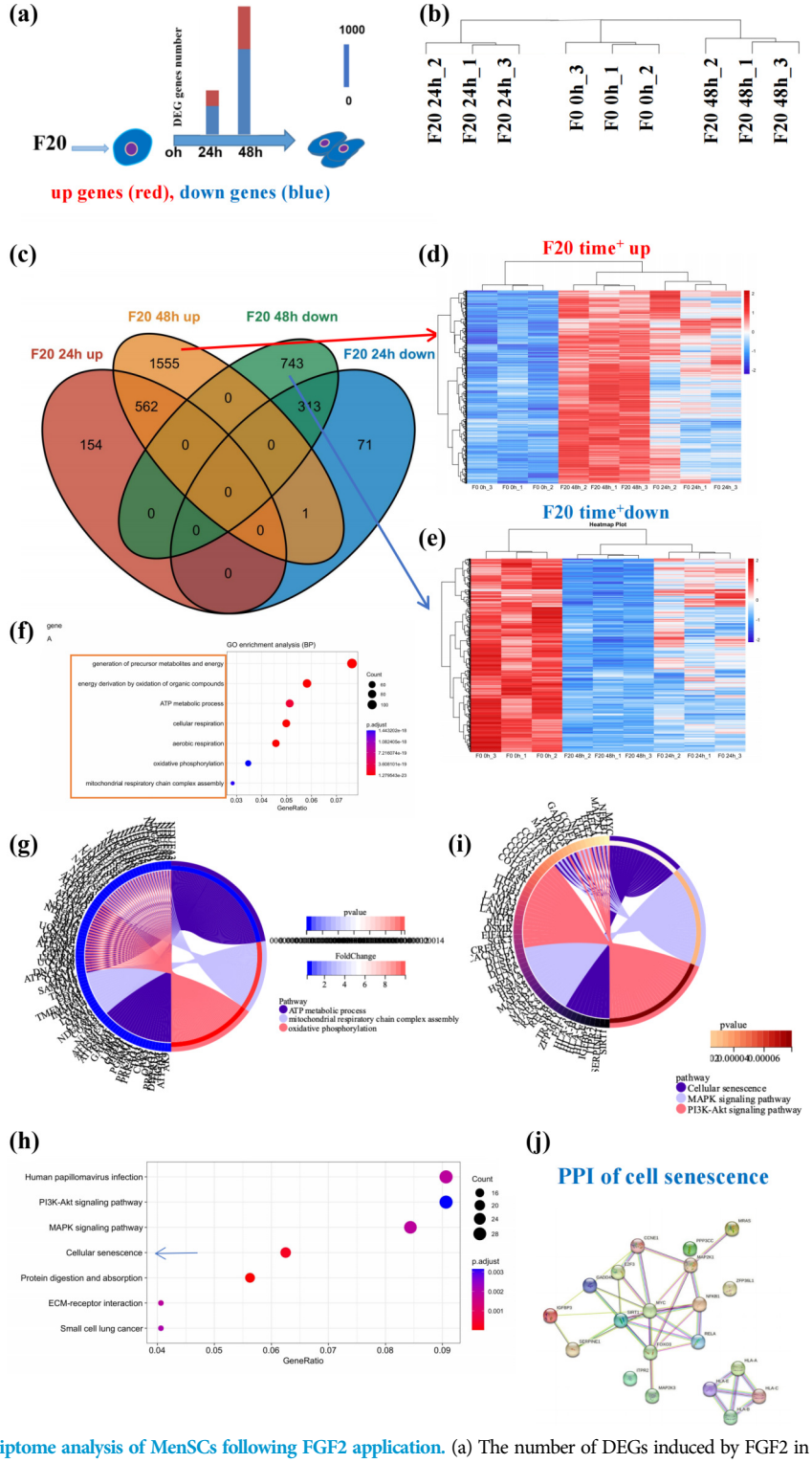

Figure 6. Temporal transcriptome analysis of MenSCs following FGF2 application. (a) The number of DEGs induced by FGF2 in MenSCs in 0–48 h. The up- and down-regulated genes in the heat map relative to the levels in F0 0h group are shown in red and blue, respectively. (b) Hierarchical clustering of DEGs among F20 0h group, F20 24h group, and F20 48h group. (c) Venn diagram shows F20 time up+ genes and F20 time down+ genes number among the F20 group in 0–48 h. (d) and (e) Heatmaps show F20 time up+ genes and F20 time down+ genes in the 0-48h dataset. Data are represented as fold change up (red colored squares) or down (blue colored squares). (f) The top biological GO terms in BP enriched genes in F20 48h group. (g) circular diagram of energy metabolite-related three terms in F20 time up+ genes. (h) KEGG enrichment analysis of F20 time down+ genes were showed. (i) Circular diagram of three KEGG pathway in F20 time down+ genes. (j) PPI analysis of celluar senescence term within the KEGG pathway in F20 time down+ genes.

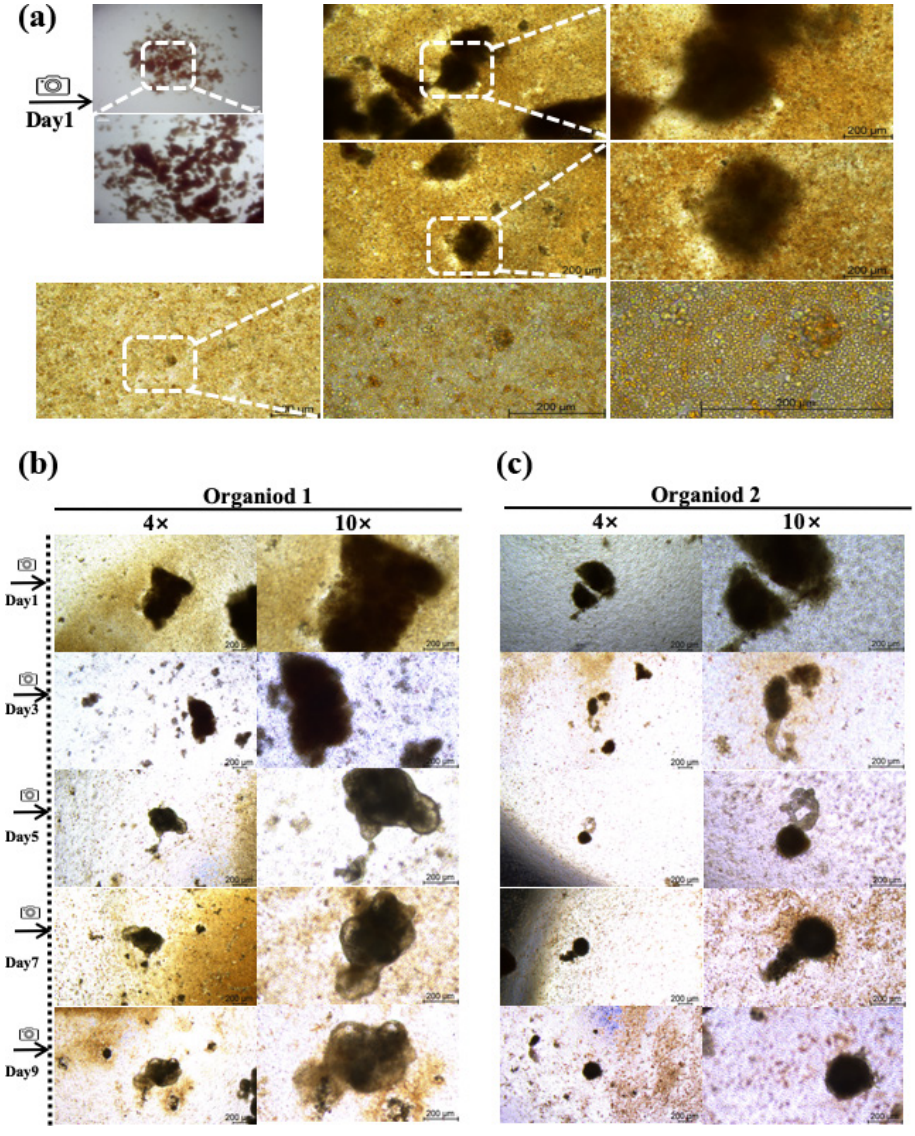

Figure 7. Morphological image of human endometrium-based organiod over time. (a) Light image of co-culture of organoids with single cells on day1. (b) and (c) Light images of different forms of organoids during cultivation 9 day.

further investigation to elucidate the precise molecular mechanisms underlying the FGF2-mediated effects on organoid development and senescence. Such insights could hold significant implications for the therapeutic application of FGF2 in endometrial repair and regenerative medicine.

Discussion

As the therapeutic promise of MenSCs as seed cells continues to expand, our understanding of their cellular characteristics remains limited. In this study, we investigated the impact of various FGF2 doses on MenSCs and human endometrium organoids *in vitro*. FGF2 is known to promote tissue regeneration and repair by modulating key biological processes such as proliferation, differentiation, and migration[9–12,19–21]. Here, we not only confirmed these findings but also delved deeper into the mechanistic role of FGF2 in MenSCs and organoids using RNA-seq analysis, Q-PCR.

After exposure to FGF2, notable changes were observed in the cellular morphology of MenSCs, transitioning from a flat and stretched appearance to a more three-dimensional structure. Both the cell area and the size of the cell nucleus decreased.

Surprisingly, the expression of canonical MSC marker genes, including CD44, CD9, and OCT4, remained unchanged following FGF2 treatment. CD9, a core transcription factor, regulates self-renewal, cell adhesion, movement, differentiation, proliferation, and chemotherapy resistance in stem cells[22–24], while OCT4 is a key guardian of embryonic stem cell pluripotency[25,26]. This observation suggests that despite exposure to FGF2, the fundamental capacity for multiple differentiations in these cells remained unaffected.

Our findings further revealed that various doses of FGF2 elicited similar growth-promoting effects over time in MenSCs. FGF2 induced a dose-dependent increase in cell growth and proliferation during a 144-h *in vitro* period. This aligns with previous reports indicating that FGF2 enhances the growth potential and lifespan of human MSCs[27,28]. Additionally, FGF2 at high concentrations (100 ng/mL) appears to activate cell self-renewal by inhibiting BMP signaling in hESCs[29]. Therefore, our data suggest that the growth-promoting effects of FGF2 on MenSCs occur in a dose-dependent manner over time, potentially due to its ability to stimulate cell proliferation and maintain stemness.

Per Douglas et al. found smaller cells display accelerated metabolism and mass transfer due to their elevated surface-to-

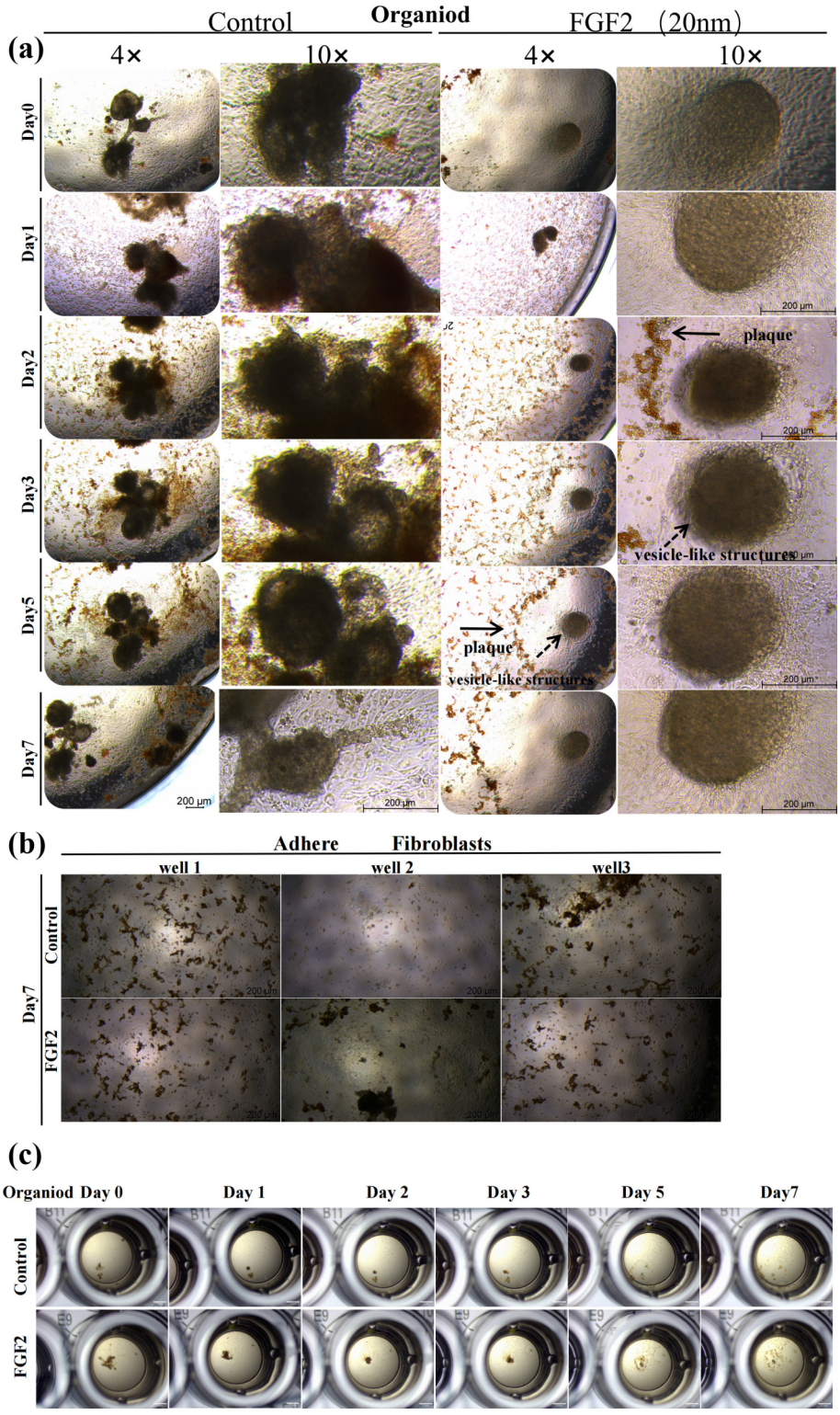

Figure 8. Effects of FGF2 on the morphology of organoids. (a) Effects of FGF2 on the morphology of organoids during 7 days of cultivation. (b) Effects of FGF2 on adhere fibroblasts from organoids on day 7. (c) Panoramic photography of FGF2 on the growth of organoids.

volume ratio, enhancing nutrient uptake, waste clearance, and gas exchange, vital for high-flux cells like intestinal and alveolar epithelia[30]. Our findings indicate that FGF2-induced nuclear reduction may signify a metabolic shift, with altered gene expression, enhancing cellular responsiveness and fostering growth/proliferation. In conclusion, morphological and functional changes in hMenSCs post-FGF2 treatment are intimately linked.

Collectively, our study provides novel insights into the cellular characteristics and response of MenSCs to FGF2, offering valuable information for future therapeutic applications in endometrial diseases.

Based on the RNA-sequence analysis, a comprehensive gene expression profile of MenSCs in response to different concentrations of FGF2 was obtained. Under control, FGF2 10,

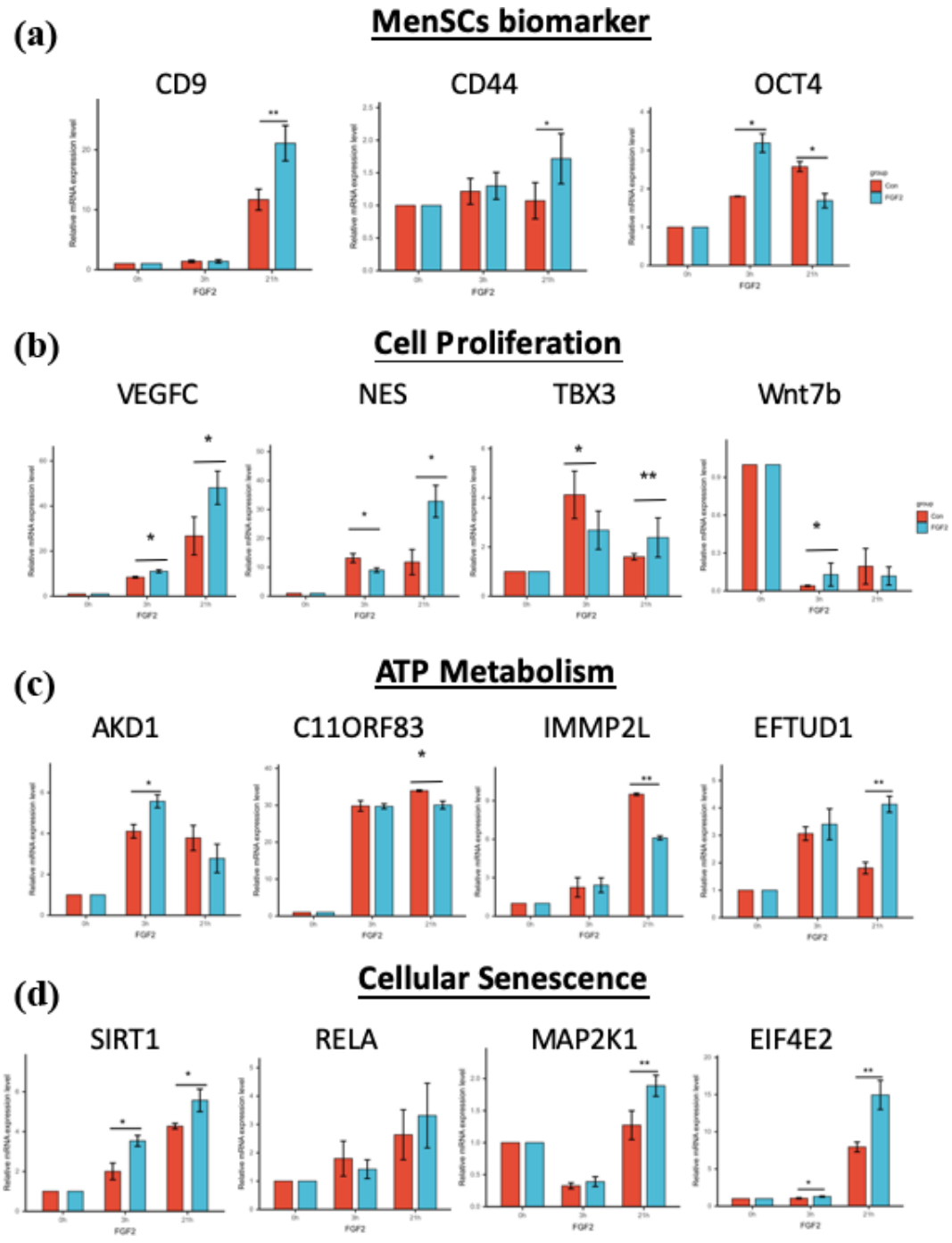

Figure 9. Gene expression profiles of organoids upon treatment with FGF2. (a) Expression level of MenSCs biomarker-related genes after 21h with FGF2 treatment. (b) Expression level of cell Proliferation-related genes with FGF2 treatment. (c) Expression level of ATP metabolism-related genes with FGF2 treatment. (d) Expression level of cellular senescence-related genes with FGF2 treatment. Data are presented as the mean ± SEM (n = 3; ns, no significance; *P < 0.05; **P < 0.01; ***P < 0.001; Student's t-test).

and FGF2 20 groups, a total of 754 up-regulated and 978 down-regulated differentially expressed genes (DEGs) were identified. Among the top 20 up-regulated genes, several genes encoding proteins previously associated with regulating stem cell proliferation, differentiation, and self-renewal stood out, including TrpA1 and DLL4[31,32]. These findings suggest that FGF2 actively modulates the expression of genes critical for maintaining the stem cell phenotype.

Functional enrichment analyses further revealed that the up-regulated DEGs were primarily involved in ribosome biogenesis processes. Ribosome biogenesis is a crucial aspect of cellular physiology, linked to various physiological phenomena and diseases. In the context of stem cells, it is known to maintain high protein synthesis efficiency, essential for stem cell homeostasis[33,34]. Therefore, it is plausible that FGF2 promotes cell growth and proliferation in MenSCs by regulating protein synthesis, thereby facilitating the necessary signal transduction mechanisms.

On the other hand, the down-regulated DEGs in the FGF2 groups were primarily enriched in cell-substrate adhesion activities. This suggests that FGF2 might inactivate genes related

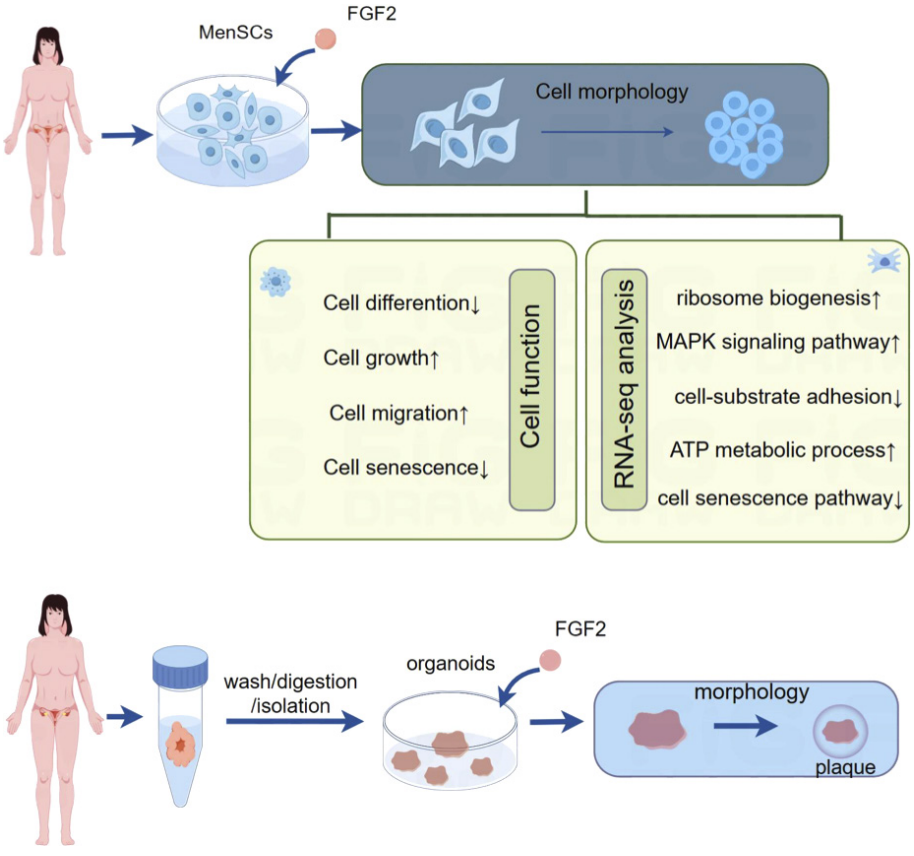

Figure 10. Role of the FGF2 in hMenSC and human endometrium organiod. FGF2 regulates the growth of both hMenSC and endometrium organoid, exerting its influence on both morphological and genetic levels. Upon treatment with FGF2, notable changes were observed in the cells and nuclei of hMenSC, with a decrease in size, as well as a reduction in the volume of organoids. These morphological alterations are indicative of the dynamic nature of cellular responses to FGF2 stimulation. RNA-seq analysis and q-PCR were employed to identify changes in genetic patterns across multiple pathway genes.

to cell-substrate adhesion, leading to alterations in cell morphology and enabling MenSCs to perform their biological functions effectively. Cytoskeleton-dependent cell adhesive processes play a pivotal role in regulating cell shape changes, migration, and proliferation[35,36]. Our findings align with this, as we observed that higher concentrations of FGF2 exhibited a greater potential to induce cell migration in MenSCs compared to lower concentrations. The downregulation of cell adhesion-related genes could result in changes in cell shape and structure, ultimately influencing cell migration.

Additionally, KEGG pathway analysis revealed that FGF2 downregulates a broad set of genes associated with the PI3K-Akt signaling pathway. This pathway is known to regulate various cellular processes, including cell growth, survival, and metabolism[37]. The downregulation of these genes suggests that FGF2 might modulate the activity of the PI3K-Akt pathway in MenSCs, potentially influencing their proliferative and migratory capabilities.

Collectively, our findings provide a deeper understanding of the molecular mechanisms underlying the response of MenSCs to FGF2. By regulating gene expression patterns related to ribosome biogenesis, cell-substrate adhesion, and PI3K-Akt signaling, FGF2 appears to orchestrate a complex network of interactions that promote cell growth, proliferation, and migration in MenSCs. These insights hold promise for the development of novel therapeutic strategies utilizing MenSCs in the treatment of endometrial diseases and other related conditions.

To further delve into the temporal conversion mechanisms underlying FGF2's actions over a 48-h culture period, we conducted comprehensive bioinformatics analyses, encompassing GO term annotation, KEGG pathway enrichment, and PPI network construction, all focused on differentially expressed genes.

Previous studies have underscored the pivotal role of ATP metabolism in governing the multilineage potential of mESCs[38]. Consistent with these findings, our data revealed that energy-related processes, particularly ATP metabolism, are intricately involved in FGF2's functional modulation of MenSCs at the 48-hour timepoint. Notably, these metabolic pathways were absent in FGF2-treated MenSCs at the 24-h mark, suggesting a temporal regulation of energy metabolism critical for enhancing proliferative capacity. Furthermore, studies have demonstrated that FGF2 intricately regulates cellular metabolic processes, encompassing the synthesis and degradation of amino acids, fatty acids, and nucleotides[39], thereby providing the energetic foundation for cellular proliferation. Concurrently, FGF2 modulates mitochondrial function[40], maintaining a delicate balance between energy homeostasis and metabolic stability. These are also the directions we intend to continue exploring and deepening our research on.

Furthermore, our KEGG enrichment analysis of downregulated genes over time revealed that the PI3K-AKT signaling pathway and cellular senescence emerged as the most significant biological processes. Cellular senescence, a state characterized by permanent cell cycle arrest, is recognized for its role in regulating cellular turnover and eliminating damaged or dysfunctional cells. Interestingly, key senescence-associated pathway was downregulated in the FGF2-treated group at the 48-h timepoint compared to the untreated control. The results are exactly consistent with those recent reported in the literature[41]. This

observation aligns with our experimental findings and suggests that FGF2 may promote cellular growth and attenuate senescence through modulation of senescence-related genes.

We have successfully cultured endometrial organoids from normal human tissues. With the prolongation of culture time, it was observed that the organoids contracted and shrunk due to autonomous aggregation, exhibiting diverse growth patterns. The organoids exhibited high sensitivity to FGF2, resulting in relatively good growth. Fibroblasts released from the organoids grew rapidly and frequently formed plaques, consistent with previous literature reports[18]. Further q-PCR analysis revealed that FGF2 treatment regulated the expression of multiple pathway genes. Genes involved in stemness maintenance exhibited stable expression and increased over time. However, the expression patterns of genes related to proliferation were not completely identical to those observed on the hMenSCs platform. Exploring differential FGF2 expression in hMenSCs vs. organoids, we hypothesize this arises from organoid heterogeneity, comprising complex cell type populations. Their intricate structure demands holistic consideration of drug impacts, transcending single-cell interactions. Thus, our study underscores the functional intricacies of FGF2. Given that organoids closely resemble natural endometrial cells, they provide a novel platform for better studying the pharmacological effects of FGF2. The discovery of the role of FGF also offers feasibility for autologous and allogeneic cell therapies, holding tremendous potential in the treatment of endometrial-related diseases in the future.

Conclusions

Collectively, our findings reveal that FGF2 profoundly impacts the entire lifecycle of hMenSCs, encompassing proliferation, differentiation, migration, and senescence. Notably, similar effects are observed in endometrium-derived organoids. This approach offers a valuable means to generate high-quality stem cells for transplantation. Key biological processes, including ribosome biogenesis, ATP metabolism, cell-substrate adhesion, cellular senescence, and PI3K-AKT signaling, are integral to the response of MenSCs and organoids to FGF2, promoting cell growth and survival. In conclusion, our platform demonstrates utility and provides compelling evidence for elucidating the mechanisms underlying the effects of FGF2, offering insights into potential therapeutic strategies for endometrial diseases.

Research ethics and patient consent

The studies involving human participants were reviewed and approved by Medical Ethics Committee of Shanghai Stomatological Hospital, Fudan University ([2020]0025). The participants provided their written informed consent to participate in this study.

Availability of data and material

The data and materials that support the findings of this study are available from the corresponding author upon reasonable request.

Declaration of conflicting interests

No competing financial interests exist.

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Author contributions

Min Wang and Hui Chen: Performing the biological experiments, investigation, and writing the draft. Xinyue Feng: Provider of endometrial sample. Jinqiu Feng and Tongyan Jin: biological samples collecting. Peiyi Wum and Tongyan Jin: Assisting the biological experiments. Min Wang, Jinqiu Feng, and Xiao Huang:Reviewing- editing, funding acquisition.

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