



Single-cell profiling and machine learning identify cuproptosis-related fibroblast subpopulations and fibrogenesis modulator AEBP1 in endometriosis

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Abstract

Endometriosis is characterized by progressive fibrosis and limited therapeutic options. Cuproptosis, a copper-dependent form of regulated cell death, has been implicated in multiple pathological conditions, but its relevance to fibroblast-mediated fibrotic progression in endometriosis remains unclear. Single-cell RNA sequencing data from normal, eutopic, and ectopic endometrial tissues were analyzed to assess cuproptosis-related gene (CRG) activity and fibroblast heterogeneity. Pseudotime analysis, cell–cell communication analysis and high-dimensional weighted gene co-expression network analysis were performed to identify disease-associated fibroblast states and candidate fibrosis-related genes. Machine learning approaches were applied to prioritize candidate hub genes. Functional validation was conducted in endometrial stromal cells, and a mouse model of endometriosis was used to assess the effects of tetrathiomolybdate (TTM), a copper chelator. Elevated CRG activity was enriched in a distinct fibroblast subpopulation with profibrotic transcriptional features. Network and machine learning analyses consistently prioritized AEBP1 as a candidate fibroblast-associated hub gene linked to cuproptosis-related signatures. In vitro, CuCl₂ plus elesclomol treatment was associated with increased AEBP1 and fibrosis-related marker expression, accompanied by changes in β -catenin pathway-related proteins, whereas FDX1 or AEBP1 knockdown attenuated these effects. In vivo, TTM treatment reduced lesion burden, fibrotic marker expression and collagen deposition in ectopic lesions. Cuproptosis-related molecular alterations are associated with fibroblast activation and fibrotic progression in endometriosis. Targeting copper metabolism may have therapeutic potential in limiting lesion fibrosis.

Keywords Endometriosis · Cuproptosis · Fibroblasts · AEBP1 · Single-cell RNA sequencing (scRNA-seq) · Fibrosis · β -catenin pathway

Background

Endometriosis (EMS) is defined as the presence of endometrium-like tissue outside the uterus, a disorder associated with severe pelvic pain and infertility [1–3]. Ectopic lesions exhibit high invasiveness, often inducing intra-abdominal fibrosis and adhesions that impair fertility and mental health [4]. While its exact pathogenesis remains unclear, core pathological features have been identified: immunoinflammatory responses, neurogenesis, estrogen dependence with progesterone resistance, and fibrosis [5]. Histologically, endometrial glands and stroma are surrounded by dense fibrous tissue—fibrosis itself arises from excessive extracellular matrix (ECM) accumulation, a process normally

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critical for tissue repair but dysregulated in EMS [6]. Despite growing recognition of these pathological hallmarks, the molecular mechanisms driving EMS-associated fibrosis remain poorly characterized. This also leads to therapeutic limitations. Current therapies, including hormonal drugs and surgical resection, alleviate pain and lesion burden but fail to address the underlying fibrotic process, resulting in elevated rates of adhesion recurrence post-treatment and persistent infertility. Myofibroblasts (MFBs) are recognized as the principal effector cells in endometriosis-associated fibrosis (EMS-associated fibrosis). Since their initial identification in ectopic lesions in 1996, research has suggested various origins for myofibroblasts, including fibroblast-myofibroblast transition (FMT), epithelial-to-mesenchymal transition (EMT), endothelial-to-mesenchymal transition (EndoMT), mesothelial-to-mesenchymal transition (MMT), and differentiation of mesenchymal stem cells [7]. However, the signals governing MFB differentiation in EMS are currently undetermined.

A prospective yet insufficiently investigated avenue in this context is cuproptosis—a recently characterized form of programmed cell death induced by intracellular copper accumulation, lipoylated tricarboxylic acid (TCA) cycle protein aggregation, and mitochondrial impairment [8]. Copper homeostasis is essential for cellular function; however, abnormal copper accumulation is linked to fibrosis in multiple organs. For instance, copper-induced mitochondrial reactive oxygen species (ROS) facilitate myofibroblast differentiation in cardiac fibrosis [9, 10], whereas copper chelation mitigates liver fibrosis in Wilson's disease [11, 12]. Nonetheless, the significance of cuproptosis in EMS-associated fibrosis has not been comprehensively investigated. Previous cuproptosis research in EMS is restricted to indirect correlations and does not include analysis of cuproptosis-specific events or their effects on stromal cell function. While MFBs drive ECM deposition, the specific fibroblast subpopulations that differentiate into MFBs and the role of cuproptosis in regulating this transition remain unclear. Traditional bulk transcriptomics, long the mainstay of EMS research, cannot resolve cell-type-specific cuproptosis responses or fibroblast–cuproptosis interactions, hindering the identification of fibroblast-specific therapeutic targets.

In this study, we discovered a candidate fibrosis-associated gene, Adipocyte Enhancer-Binding Protein 1 (AEBP1), associated with endometriosis fibroblasts. AEBP1 has been implicated in fibrotic regulation in several diseases, such as cardiac fibrosis and fibrosis within the pancreatic tumor microenvironment [13–15]. However, its expression, role, and regulatory mechanisms in EMS fibrosis remain uncharacterized. It remains unclear whether AEBP1 is enriched in endometriosis-associated fibroblast states and whether it

is associated with cuproptosis-related or copper-dependent profibrotic changes in this disease.

To address these existing knowledge gaps, we employed an integrated approach combining single-cell RNA sequencing (scRNA-seq), high-dimensional weighted gene co-expression network analysis (hdWGCNA), machine-learning analysis, and experimental validation. Our study aimed to characterize cuproptosis-related fibroblast states in endometriosis, prioritize candidate fibrosis-associated genes including AEBP1, and explore their potential association with profibrotic signaling. Furthermore, we used *in vitro* functional assays and an *in vivo* mouse model to evaluate whether cuproptosis-related molecular alterations were accompanied by changes in AEBP1 expression, fibrotic responses, and β -catenin pathway-related proteins. Collectively, these findings raise the possibility of a cuproptosis-related regulatory network associated with fibrotic progression in endometriosis and highlight AEBP1 as a candidate molecule for further study.

Methods

Patients and sample collection

This research was approved by the Ethics Committee of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology. Informed written consent was acquired from patients before to the collection of human tissues, in compliance with the Declaration of Helsinki principles. Normal endometrial tissue specimens were obtained from 15 women devoid of endometriosis who underwent hysteroscopy and endometrial biopsy. The post-procedure pathology analysis verified that these samples originated from normal endometrium ($n=15$). Paired eutopic and ectopic endometrial specimens were obtained from the same 15 patients diagnosed with stage III or IV endometriosis. Pathological histopathological analysis confirmed that all collected endometrial tissues were in the proliferative phase. All individuals exhibited regular menstrual cycles, were neither pregnant nor nursing, had not utilized hormonal drugs within six months preceding surgery, and presented no indications of serious medical or surgical disorders or associated complications.

Data collection

Single-cell RNA sequencing (scRNA-seq) data of endometrial tissues were obtained from the Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/>). Two GEO datasets, GSE179640 and GSE5572238, were acquired by using the “GEOquery” package in R

software. Four normal control samples, five eutopic endometrial samples, and five ectopic endometrium samples were chosen for following analytical processes. A total of 16 cuproptosis-related genes (CRGs) were systematically enrolled for bioinformatic analyses. These genes were identified and compiled according to the core molecular machinery of cuproptosis as originally characterized in the pivotal Cell study [8] and supplemented by previously published work on cuproptosis [16]. The complete list of the 16 CRGs is detailed in Supplemental Table S1. The bulk transcriptome dataset used for machine learning was sourced from the merged datasets of GSE7305 and GSE11691 from GEO (<https://www.ncbi.nlm.nih.gov/geo/>), which includes 19 ectopic endometrial tissue samples and 19 normal control endometrial samples. The *comBat* function from the “*sva*” R package was utilized for batch correction to mitigate potential batch effects across the two datasets.

Comprehensive processing of single cell datasets

Raw scRNA-seq count matrices were processed using Seurat (version 4.2.2) in R. Cells expressing fewer than 500 genes or more than 4000 genes were excluded, and genes expressed in fewer than 3 cells were removed. Cells with a high proportion of mitochondrial transcripts were also excluded using a cutoff of 15%. Quality-control metrics, including the distributions of *nFeature_RNA*, *nCount_RNA*, and *percent.mt*, are shown in Supplementary Fig. S1.

After quality filtering, data were normalized using SCTransform. Principal component analysis (PCA) was then performed on the scaled data (Supplementary Fig. S2). To correct for batch effects and integrate samples from different datasets, Harmony (version 0.1.1) was applied to the PCA embeddings using sample identity as the batch variable. Batch correction performance and sample integration are shown in Supplementary Fig. S3. A shared nearest-neighbor graph was constructed using FindNeighbors, and clustering was performed using FindClusters with a resolution of 0.5. Cell clusters were visualized by uniform manifold approximation and projection (UMAP). The clustering results for each sample and the numbers of retained cells per sample are provided in the Supplementary Figs. S4–S6. Cell-type annotation was performed according to canonical marker genes and previously published endometrial single-cell references. Marker genes for each cluster were identified using FindAllMarkers with the Wilcoxon rank-sum test. Cell annotation was performed according to a prior study [17, 18]. The unique expression patterns of the identified genes at the single-cell level were demonstrated using the “*scRNAtoolVis*” package (version 0.1.0).

Calculation of cuproptosis score

To estimate cuproptosis-related activity at the single-cell level, we used AUCell based on a predefined 16-gene cuproptosis-related gene (CRG) set derived from the seminal study by Tsvetkov et al. The *calcAUC* function from the AUCell package was used to calculate enrichment scores for the predefined CRG set in each cell. The *aucMaxRank* parameter was set to 10% of the ranked gene list for each cell. According to the distribution of AUCell scores, cells with an AUC score > 0.025 were defined as the CRG-high group, whereas the remaining cells were classified as the CRG-low group. PCA was performed as an orthogonal analysis to examine whether AUCell-based CRGs stratification was associated with broader transcriptomic divergence (Supplementary Fig. S7). The resulting score was used for downstream subgroup comparison and functional analyses. In this study, the AUCell-derived score was interpreted as a transcriptome-based indicator of cuproptosis-related activity rather than direct evidence of canonical cuproptotic cell death. To illustrate the distribution of cuproptosis scores among groups and enable subsequent comparison studies, the grouped cuproptosis scores were visualized using the “*ggplot2*” program in R software.

Enrichment analysis

The Seurat package’s “FindMarkers” function was used to determine the differentially expressed genes (DEGs) unique to each cell subcluster. Strict filtering criteria were used to define significant DEGs: only genes with an absolute log2 fold change ($|\log_2FC|$) > 0.25 and an adjusted *P*-value < 0.05 were deemed statistically significant. In single-cell transcriptome research, these criteria are frequently used to minimize false-positive results while balancing the identification of physiologically significant changes. Following the identification of significant DEGs, two forms of functional enrichment analyses—gene set enrichment analysis (GSEA) and gene ontology (GO) enrichment analysis—were carried out to investigate the possible biological roles of these genes across cell subgroups. The “clusterProfiler” package (version 4.0.1) in R software, a thoroughly tested instrument for functional annotation and enrichment analysis in omics research, was used for all enrichment studies [19].

Cell–cell communication analysis of fibroblasts stratified by CRG activity

Cell communication modulates target cell function by initiating a sequence of physiological and biochemical alterations through cell signal transduction, resulting in the target cell’s comprehensive biological effects. Intercellular

communication, facilitated by interactions between cell surface ligands and receptors, coordinates diverse cell types during development and is essential for numerous biological activities [20]. The “CellChat” tool (version 1.6.1) was utilized to detect and compare potential interactions between fibroblasts and other cell populations within cuproptosis-related gene (CRG) groupings. All analyses adhered to the package’s prescribed pipelines with default configurations, assuring alignment with conventional techniques for inferring cell–cell communication in single-cell data.

Trajectory analysis of fibroblast populations

An unsupervised pseudo-temporal analysis was conducted using the “Monocle” program (version 2.24.0) with the DDR-Tree technique and default parameters to investigate the trajectory of fibroblasts in the high score group of CRGs in endometriosis. Subsequently, the “plot_cell_trajectory,” “plot_genes_in_pseudotime,” and “plot_genes_branched_heatmap” were utilized to generate plots that graphically represent the dynamic expression of module genes along the pseudotime trajectories of high fibroblasts in CRGs. The differentiation status of cell subpopulations was evaluated alongside the pseudotime trajectory of cells to determine the extent of differentiation among cell subtypes.

High dimensional weighted gene co-expression network analysis

High-dimensional weighted gene co-expression network analysis (hdWGCNA) was used to identify important genes linked with fibroblasts in the high group of CRGs among endometriosis samples. A correlation matrix of gene expression, weighted gene co-expression networks, and module identification were performed. Module-trait connection research revealed modules highly correlated with high groupings of CRGs, and hub genes within these major modules were determined based on their intra-module connectivity. The first 120 hub genes were regarded as the principal genes.

Screening of EMS fibroblasts biomarkers by machine learning techniques

To identify robust fibroblast-associated candidate genes, the top 120 genes from the fibroblast-related blue module identified by hdWGCNA were subjected to machine-learning analysis using the merged bulk transcriptomic dataset (GSE7305 and GSE11691), which included 19 ectopic endometrial samples and 19 normal control endometrial samples. Three independent algorithms were applied, including random forest (RF), least absolute shrinkage and

Fig. 1 Single-cell RNA-sequencing analysis identifies a fibroblast-enriched CRG-high state with profibrotic transcriptional features in endometriosis. **A** Uniform manifold approximation and projection (UMAP) plots showing the distribution of the major cell populations in control (Con), ectopic, and eutopic endometrial samples after integration of the single-cell RNA-sequencing dataset. Annotated cell types include B cells, endothelial cells, epithelial cells, fibroblasts, monocytes, NK cells, smooth muscle cells, and T cells. **B** Histogram of AUCell scores for the predefined cuproptosis-related gene (CRG) signature across all cells. Cells with an AUC score > 0.025 were classified as the CRG-high group (17,977 cells), whereas the remaining cells were classified as the CRG-low group. **C** UMAP plot showing AUCell-derived CRG scores across all cells. Brighter colors indicate higher CRG activity. **D** Stacked bar plot showing the proportions of CRG-high and CRG-low fibroblasts in control, ectopic, and eutopic endometrial samples. Percentages are shown within the bars. **E** UMAP plots showing the distribution of CRG-high cells in control, ectopic, and eutopic samples. High-score cells are highlighted in yellow. **F**, **G** Gene set enrichment analysis (GSEA) of differentially expressed genes between CRG-high and CRG-low fibroblasts, showing enrichment of fibrosis-related biological processes, including collagen fibril organization and extracellular matrix organization. The normalized enrichment score (NES), adjusted P value, and false discovery rate (FDR) are indicated in each plot. CRGs, cuproptosis-related genes; UMAP, uniform manifold approximation and projection; GSEA, gene set enrichment analysis; AUC, area under the curve; FDR, false discovery rate

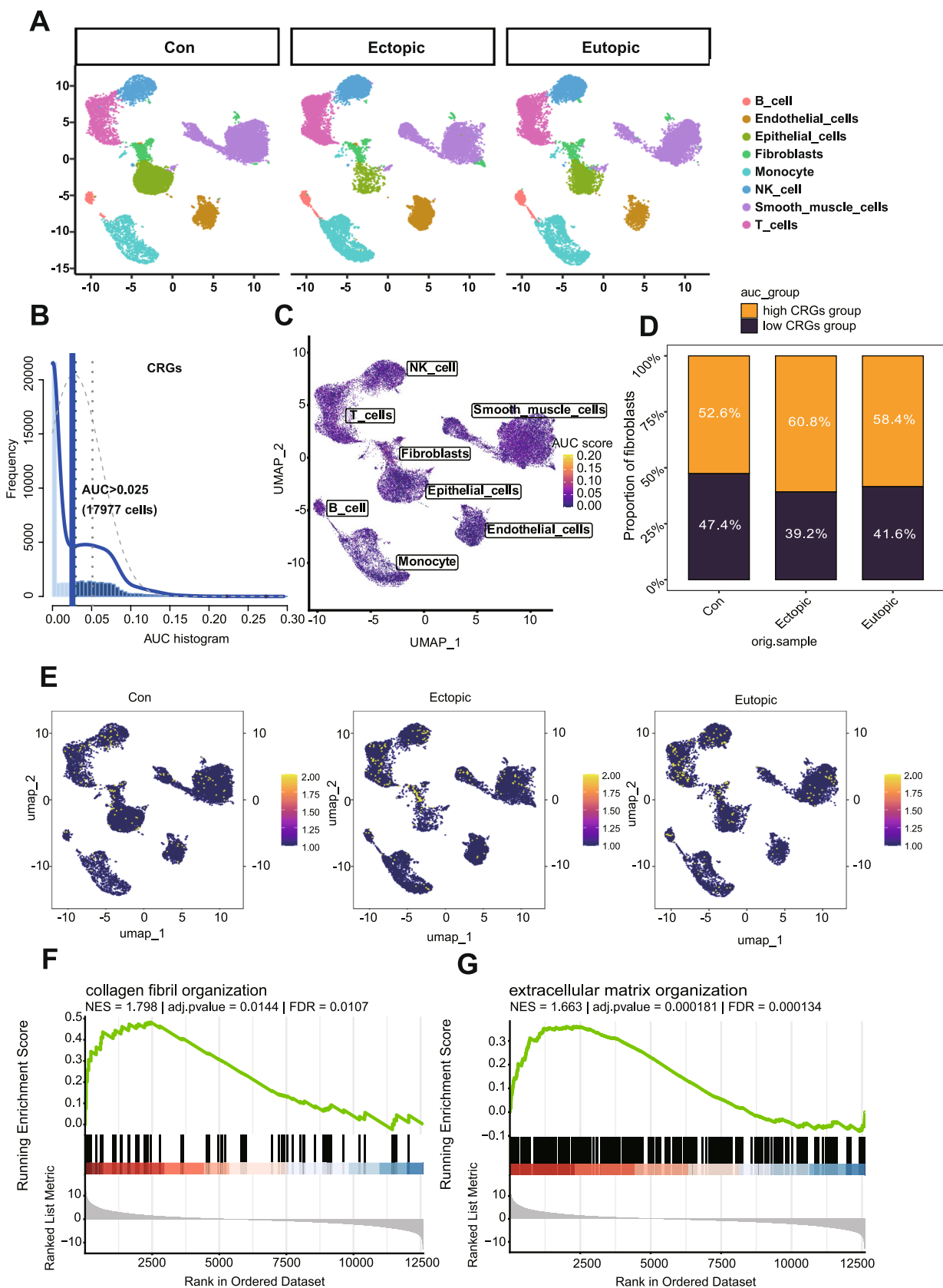
selection operator (LASSO), and support vector machine-recursive feature elimination (SVM-RFE).

For RF analysis, the randomForest package was used with 500 trees. Model stability was assessed using the out-of-bag error rate, and variables were ranked according to MeanDecreaseGini. Genes with higher importance scores were considered RF-selected candidate features. For LASSO analysis, the glmnet package was used, and the optimal penalty parameter (λ) was selected by tenfold cross-validation using the cv.glmnet function. Genes with non-zero coefficients at the selected λ value were retained as candidate features. For SVM-RFE analysis, candidate subsets with different numbers of variables were evaluated by cross-validation, and the subset with the lowest cross-validation RMSE was selected as the optimal model.

To improve robustness and minimize model-specific bias, genes identified by the three independent algorithms were intersected, and the overlapping genes were defined as candidate hub genes for subsequent analyses and experimental validation.

Isolation and culture of primary endometrial stromal cells

Primary ectopic endometrial stromal cells (EESCs) were extracted from ectopic endometrial tissues of ten individuals diagnosed with ovarian endometriosis, adhering to the designated protocol: Recently obtained tissues were washed with PBS, chopped with scissors, and thereafter incubated with preheated 0.1% type II collagenase (Sigma-Aldrich, St.



Louis, MO) in a shaker at 37 °C for 45 min. The mixture was filtered in succession through sterile sieves with hole sizes of 150 µm and 38 µm to exclude epithelial cells and undigested tissues, followed by centrifugation at 1000 rpm for 5 min. A red blood cell lysis buffer was introduced and mixed, subsequently followed by a second centrifugation to isolate primary endometrial stromal cells. Normal endometrial stromal cells (NESC) and EESC were cultivated in DMEM/F12 media enriched with 20% fetal bovine serum (FBS) in a 5% CO₂ incubator at 37 °C. Cells utilized for experimentation were subcultured no more than three times. The purity of isolated endometrial stromal cells (ESCs) was confirmed using immunofluorescence, which identified the expression of the epithelial marker E-cadherin (Abcam, ab40772, 1:50) and the mesenchymal marker vimentin (Abcam, ab92547, 1:50).

Cell culture and transfection assay

Human endometrial stromal cells (ThESCs) were obtained from the American Type Culture Collection (ATCC; catalog no. CRL-4003) and cultured in Dulbecco's Modified Eagle Medium (DMEM) enriched with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA). Cells were cultivated in a humidified incubator at 37 °C with 5% carbon dioxide (CO₂). The AEBP1 overexpression plasmid and its corresponding empty control plasmid, small interfering RNAs (siRNAs) directed against FDX1 and AEBP1, along with non-targeting negative control siRNAs, were chemically produced by DianJun Biotechnology Co., Ltd. (Shanghai, China). The minor interfering sequences of FDX1 siRNA#:sense, GC AAGUAGAGAUCUGGAATT; antisense, UUC CAGG AUCUCUACUUGCTT; AEBP1 siRNA#: sense, CCACA CUGGACUACAAUGATT; antisense, UCAUUGUAGU CCAGUGUGGTT. Cell transfection was conducted with the jetPRIME transfection reagent (Polyplus-transfection, Illkirch, France) in strict adherence to the manufacturer's prescribed methodology. Post-transfection, the transfection efficiency was assessed using western blot analysis to verify the overexpression of AEBP1 or the knockdown of FDX1/AEBP1. Subsequent functional studies were conducted only after confirming adequate transfection efficiency, in accordance with pre-established experimental criteria.

Protein extraction and western blotting analysis

Protein from tissues and cells was extracted using RIPA buffer (Beyotime, Shanghai, PR China) supplemented with PMSF (Sigma-Aldrich, St. Louis, MO) to inhibit protein breakdown. Proteins were denatured at 95 °C for 10 min and subsequently kept at -80 °C until required. For Western blot analysis, 30 µg of protein per sample was resolved using

Fig. 2 Cell-cell communication analysis reveals stronger incoming and outgoing profibrotic signaling in CRG-high fibroblasts. **A, B** Circle plots showing inferred incoming communication patterns received by CRGs.Low_Fibroblasts and CRGs.High_Fibroblasts, respectively, from other cell populations in ectopic endometrial tissue. **C, D** Circle plots showing inferred outgoing communication patterns sent by CRGs.Low_Fibroblasts and CRGs.High_Fibroblasts, respectively, to other cell populations. In the circle plots, line thickness reflects the inferred communication strength. **E, F** Bar plots showing the top ligand-receptor pairs associated with CRGs.Low_Fibroblasts and CRGs.High_Fibroblasts, respectively. Salmon bars denote signaling from fibroblasts to other cells, whereas turquoise bars denote signaling from other cells to fibroblasts. Total communication probability is shown on the x-axis. CRGs, cuproptosis-related genes; TGFβ1, transforming growth factor beta 1

12% SDS-PAGE and subsequently transferred to PVDF membranes (Millipore, MA, USA). Membranes were incubated with 5% skim milk in TBST (0.05% Tween-20) at room temperature for 1 h to minimize nonspecific binding. Primary antibodies against AEBP1(ab168355; Abcam), α-SMA (14395-1-AP; Proteintech), CTGF (25474-1-AP; Proteintech), β-catenin (M7A19; Selleck), c-myc (343250; Zenbio), β-actin (20536-1-AP; Proteintech), LIAS (11577-1-AP, Proteintech), FDX1 (12592-1-AP; Proteintech), Lipoic Acid (for Lip-DLAT) (ab58724; Abcam) were incubated with membranes overnight at 4 °C in the refrigerator. On the following day, membranes were subjected to three washes with TBST (5 min each) and subsequently incubated with goat anti-rabbit HRP secondary antibody (1:400; Proteintech, Wuhan, PR China) at room temperature for one hour. Following three more TBST washes (5 min each), protein bands were detected using ECL solution and subsequently photographed. Western blot quantified from three independent experiments. Band intensities were assessed using ImageJ.

Immunofluorescence (IF) staining

Endometrial stromal cells were fixed in 4% paraformaldehyde at 25 °C for 30 min, followed by permeabilization with PBS containing 0.1% Triton X-100 at 25 °C for 10 min. Non-specific binding sites were obstructed using 1% bovine serum albumin in PBS at 37 °C for one hour. Cells were then incubated with primary antibodies against AEBP1 (ab168355; Abcam, 1:100), α-SMA (14395-1-AP; Proteintech, 1:200), β-catenin (M7A19; Selleck, 1:200) and FDX1 (12592-1-AP; Proteintech, 1:100) at 25 °C for 1 h. Immuno-signals were detected using fluorescence-conjugated secondary antibodies (1:4000; Proteintech). Nuclei were stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) for a duration of 10 min. Ultimately, pictures were obtained by fluorescence confocal microscopy and analyzed using Image Pro Plus 6.0 software.



IHC, HE and Masson's trichrome staining

All tissues were immediately fixed in 4% buffered formalin to preserve tissue morphology. Subsequent paraffin

embedding, tissue sectioning (5- μ m thickness), and IHC, HE, and Masson's trichrome staining procedures were performed by Biosciences Biotechnology Co., Ltd. (Wuhan, China).

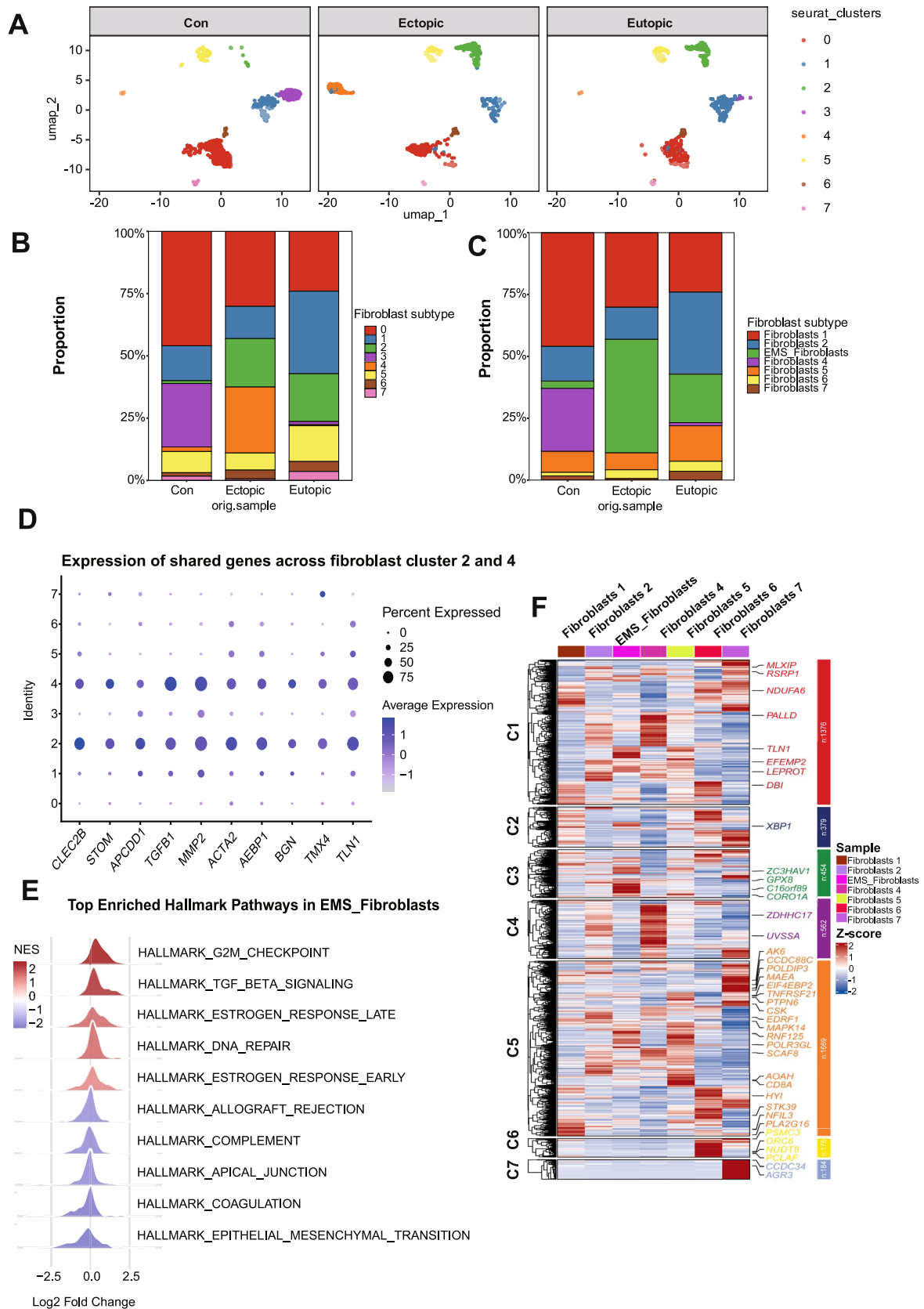


Fig. 3 Identification of an endometriosis-associated fibroblast population with distinct transcriptional and pathway features. **A** UMAP plots showing fibroblast subclustering in control, ectopic, and eutopic samples. Eight fibroblast clusters were identified. **B** Stacked bar plot showing the relative proportions of the eight fibroblast clusters in control, ectopic, and eutopic samples. **C** Stacked bar plot showing fibroblast subtype composition after integrating fibroblast clusters 2 and 4 as EMS_fibroblasts. **D** Dot plot showing the expression of representative shared genes across fibroblast clusters 2 and 4. Dot size indicates the percentage of cells expressing each gene, and dot color indicates the average expression level. **E** Ridge plot showing the top enriched Hallmark pathways in EMS_fibroblasts. **F** Heatmap showing differentially expressed genes across fibroblast subclusters. Genes were grouped into expression clusters (C1–C7) by unsupervised clustering. EMS_fibroblasts, fibroblast populations enriched in endometriosis samples and characterized by shared profibrotic transcriptional features; UMAP, uniform manifold approximation and projection

Assessment of mitochondrial membrane potential and ROS

Mitochondrial membrane potential was assessed using the JC-1 assay according to the manufacturer's instructions. After the indicated treatments, cells were incubated with JC-1 working solution (5 µg/mL) for 15 min at 37 °C, washed with buffer, and analyzed by flow cytometry. The proportion of cells with increased green fluorescence was used as an indicator of reduced mitochondrial membrane potential.

Intracellular mitochondrial ROS levels were assessed using MitoSOX Red (Beyotime Biotechnology, catalog no. S0061) according to the manufacturer's protocol. After treatment, cells were incubated with MitoSOX working solution (5 µM) for 30 min at 37 °C, counterstained with DAPI, and imaged by fluorescence microscopy. Fluorescence intensity was quantified using ImageJ from three independent fields per group.

Animal model and treatment

Experiments with C57BL/6 mice received approval from the Institutional Animal Care and Use Committee of Tongji Medical College, Huazhong University of Science and Technology (HUST), and adhered to applicable regulatory standards. Female C57BL/6 mice, aged 6 to 8 weeks and weighing 18 to 20 g, were acquired from BIONT Biotechnology Co., Ltd. in Beijing, China. A total of 40 mice were randomly allocated into three groups: the donor group (n=10), the negative control endometriosis group (Control EMS, n=15), and the TTM treated endometriosis group (TTM, n=15). Mice were anesthetized with pentobarbital sodium at a dosage of 60 mg/kg. To create the endometriosis model, the uterus of a single donor mouse was sectioned into 2–3 mm fragments, which were subsequently implanted onto the abdomen walls of two recipient mice. Each mouse received one endometrial fragment sutured

onto each side of the abdominal wall. Three weeks post-establishment of the surgical model, mice received 50 mg/kg TTM (HY-128530, MCE, China) via oral gavage. TTM was solubilized in a solvent comprising 5% DMSO, 40% PEG300, 5% Tween80, and 50% water, and subsequently diluted to a final concentration of 10 mg/ml. The control group of mice was administered the identical solvent devoid of TTM. At the end of treatment, mice were euthanized, and ectopic lesions were harvested for lesion measurement, protein extraction, histology, immunohistochemistry, and Masson's trichrome staining.

Statistical analysis

All statistical analyses and data visualization were conducted using R software (version 4.2.2). Data are presented as mean±SD unless otherwise specified. For comparisons between two groups, a two-tailed unpaired Student's t-test was used. For comparisons among three or more groups, one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparisons test was applied. A *P* value<0.05 was considered statistically significant. Statistical significance was indicated as follows: **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

Results

Single-cell RNA sequencing reveals cuproptosis-related genes (CRGs) groups in endometriosis

To identify genes predominantly indicative of cuproptosis alteration, we performed an in-depth study of single-cell sequencing data from normal, eutopic, and ectopic endometrial tissues. Following quality screening, 44,597 high-quality cells were carefully selected for further examination. The principal component analysis (PCA) reduction plot revealed no significant variations in cell cycles. After Harmony-based integration, cells from different samples showed improved mixing in low-dimensional space, suggesting that major batch-driven separation was reduced. The distribution of eight distinct cell clusters was illustrated using Uniform Manifold Approximation and Projection (UMAP) (Fig. 1A). Subsequently, utilizing the AUC score>0.025, all cells were allocated an AUC score for CRGs and classified into high-cuproptosis AUC and low-cuproptosis AUC groups (Fig. 1B). Cells exhibiting a greater quantity of cuproptosis-related genes (CRGs) were predominantly characterized by lighter-colored fibroblasts and smooth muscle cells (Fig. 1C). Since the occurrence of fibrosis in endometriosis is mainly associated with the

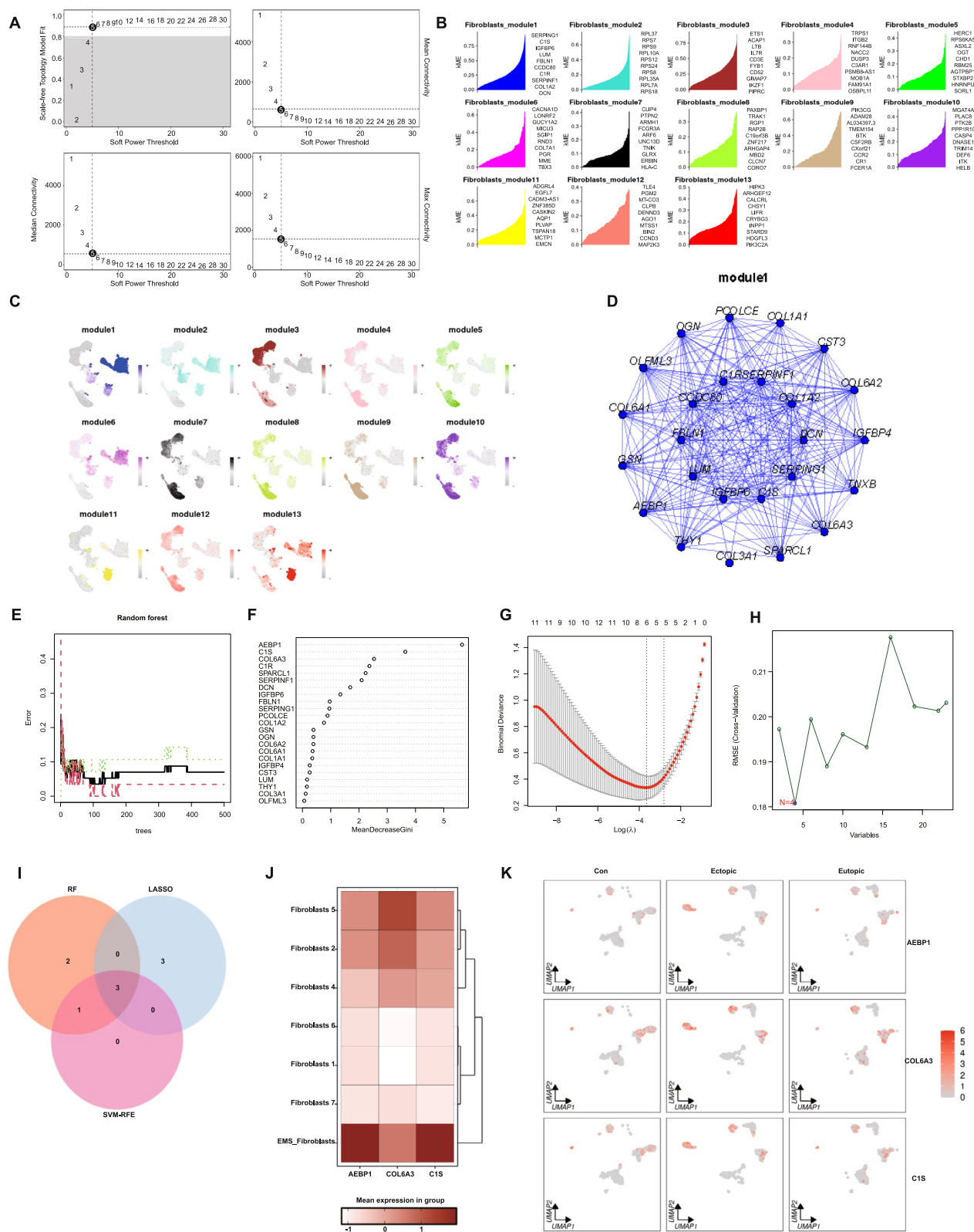


Fig. 4 Identification of fibroblast-associated hub genes by hdWGCNA and machine-learning analysis. **A** Scale-free topology analysis used to determine the optimal soft-thresholding power for high-dimensional weighted gene co-expression network analysis (hdWGCNA). A soft-thresholding power of 5 was selected for network construction. **B** Module eigengene (ME) plots showing 13 fibroblast-associated co-expression modules identified by hdWGCNA, together with representative genes within each module. **C** UMAP feature plots showing the spatial distribution of the 13 module scores across fibroblast populations. **D** Protein–protein interaction (PPI) network constructed from representative genes in the fibroblast-associated module. **E** Random forest error curve showing model stability as the number of trees increases. **F** Random forest variable-importance plot showing candidate genes ranked by MeanDecreaseGini. **G** LASSO regression analysis showing cross-validation results for feature selection and determination of the optimal penalty parameter. **H** Support vector machine-recursive feature elimination (SVM-RFE) analysis showing the relationship between the number of variables and model error. **I** Venn diagram showing overlap among candidate genes identified by random forest (RF), LASSO, and SVM-RFE analyses. **J** Heatmap showing the mean expression of the three overlapping hub genes (AEBP1, COL6A3, and C1S) across fibroblast subpopulations. **K** Feature plots showing the distribution of AEBP1, COL6A3, and C1S in control, ectopic, and eutopic fibroblast populations. hdWGCNA, high-dimensional weighted gene co-expression network analysis; PPI, protein–protein interaction; RF, random forest; LASSO, least absolute shrinkage and selection operator; SVM-RFE, support vector machine-recursive feature elimination; UMAP, uniform manifold approximation and projection

function of fibroblasts, we focused primarily on the proportions of fibroblasts with high and low cuproptosis scores among normal, eutopic, and ectopic endometrial fibroblasts. We found that the proportion of cells with high cuproptosis scores was significantly higher in ectopic and eutopic fibroblasts compared to normal fibroblasts (Fig. 1D). This suggests that cuproptosis-related genes or pathways in endometriosis may be involved in certain processes of fibrosis. We further visualized the distribution of fibroblasts with high cuproptosis scores in the clustering map using UMAP plots, which revealed a marked increase in yellow-colored cells (representing high cuproptosis scores) among ectopic fibroblasts (Fig. 1E). To further explore the specific functions of fibroblasts with high cuproptosis scores, we performed Gene Set Enrichment Analysis (GSEA) on the differentially expressed genes between fibroblasts with high and low cuproptosis scores. The results showed that the functions of fibroblasts in the high cuproptosis score group were significantly enriched in “collagen fibril organization” and “extracellular matrix organization” (Fig. 1F, G). This may indicate that cuproptosis-related or copper-dependent signaling is associated with fibroblast activation and fibrotic progression in endometriosis.

Comparative cell–cell communication analysis between CRG-high and CRG-low fibroblasts

The availability of a single-cell dataset afforded us a distinctive chance to examine cell–cell communication facilitated

by ligand–receptor interactions. To clarify the cell–cell communication network between fibroblasts and other cell types in ectopic endometrial tissues, we conducted an analysis utilizing CellChat, which is founded on established ligand–receptor pairings and their cofactors.

Subsequently, in the cell communication analysis, we classified fibroblasts into “CRGs.Low_Fibroblasts” and “CRGs.High_Fibroblasts” and compared the intensity of communication signals they received from and sent to other cell types. We found that CRGs.High_Fibroblasts may receive more communication signals from epithelial cells, endothelial cells, and monocytes (Fig. 2A, B). The transformation of epithelial cells and endothelial cells into fibroblasts and myofibroblasts is widely recognized as one of the mechanisms underlying fibrosis formation in endometriosis, and numerous studies have also elaborated on the role of the mononuclear phagocyte system in the immune microenvironment of endometriosis. Given the well-established involvement of these cell types in endometriosis-associated fibrosis, the increased signal reception from them by CRGs.High_Fibroblasts may imply a functional link between cuproptosis and fibrotic signaling cascades. Additionally, CRGs.High_Fibroblasts tend to emit stronger communication signals to other cells, which is reflected by thicker arrows in the circle plot (Fig. 2C, D). This suggests that cuproptosis-high fibroblasts may exhibit enhanced intercellular communication capacity, potentially integrating pro-fibrotic signals from epithelial cells, endothelial cells, and the mononuclear phagocyte system to facilitate fibroblast activation and subsequent fibrotic progression in endometriosis.

Subsequently, we compared the significantly activated receptor–ligand pairs between the two groups of fibroblasts. We found that COL1A1-related receptor–ligand pairs were present in both groups (Fig. 2E). As a well-recognized fibrosis marker, the expression of COL1A1 often indicates the activation of fibrotic processes. Interestingly, CRGs.High_Fibroblasts exhibited higher levels of TGFβ1 and Wnt related signals emitted by other cell types (Fig. 2F). This suggests that, distinct from CRGs.Low_Fibroblasts, CRGs.High_Fibroblasts may be preferentially regulated by TGFβ1 and Wnt signaling axes—two classical pathways implicated in fibroblast proliferation, differentiation, and extracellular matrix deposition. The enhanced crosstalk via these pro-fibrotic pathways might further reinforce the pro-fibrotic phenotype of CRGs.High_Fibroblasts, potentially amplifying the fibrotic cascade in endometriosis.

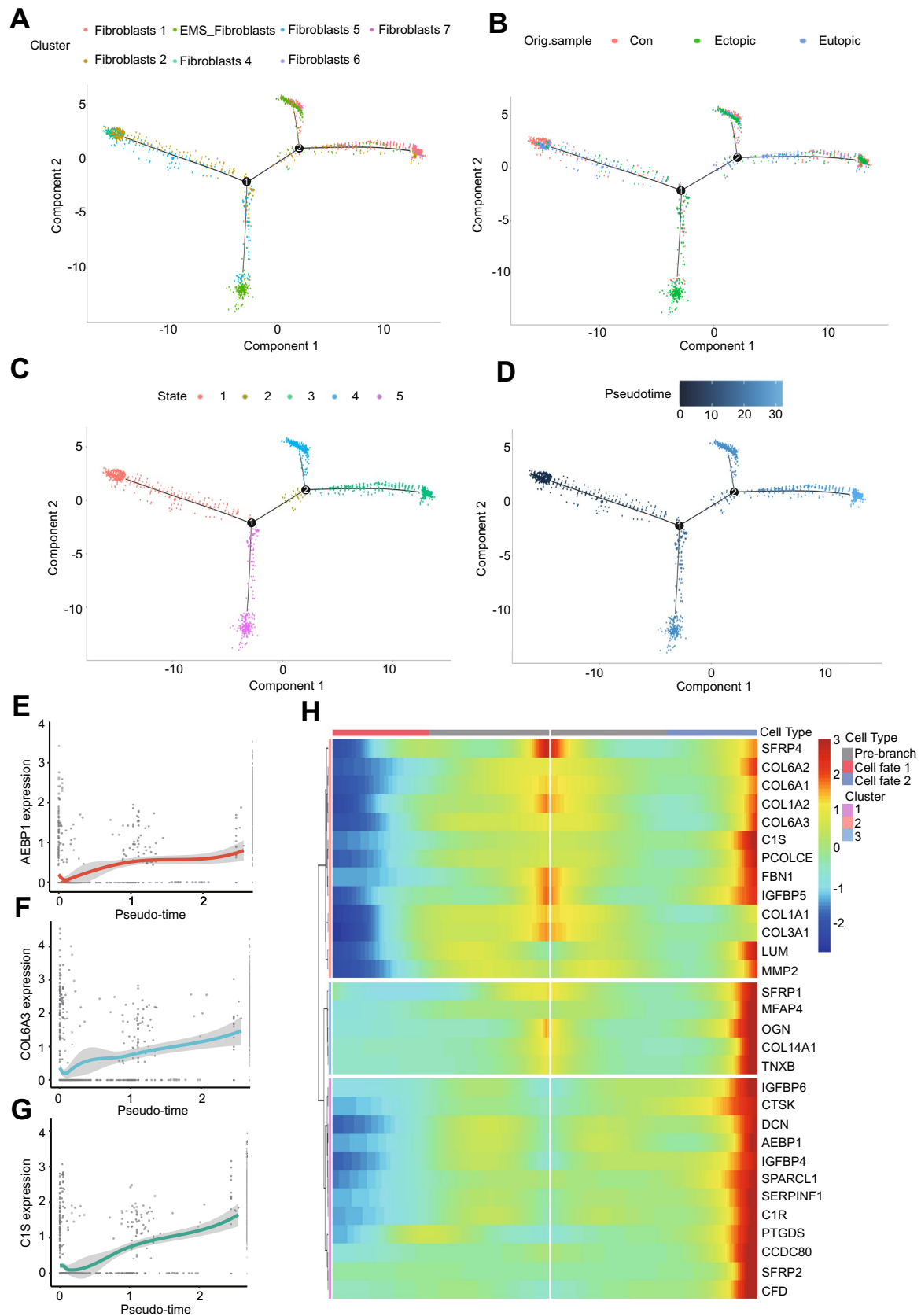


Fig. 5 Pseudotime analysis reveals a profibrotic differentiation trajectory of EMS-associated fibroblasts. **A** Monocle trajectory plot showing fibroblast differentiation states colored by fibroblast subtype. EMS_fibroblasts are preferentially distributed in later pseudotime regions. **B** Monocle trajectory plot colored by sample origin (control, ectopic, and eutopic), showing differential localization of fibroblasts from different tissue sources along the trajectory. **C** Monocle trajectory plot colored by cell state, showing multiple differentiation states during fibroblast progression. **D** Monocle trajectory plot colored by pseudotime, illustrating the transition from early to late differentiation states. **E–G** Dynamic expression of AEBP1, COL6A3, and C1S along pseudotime. Smoothed curves indicate the overall expression trend during fibroblast differentiation. **H** Branched heatmap showing genes dynamically regulated across branch-dependent cell fates. Genes related to extracellular matrix remodeling and fibrosis, including COL6A1, COL6A2, COL6A3, C1S, FBN1, IGFBP5, COL1A1, COL3A1, DCN, and AEBP1, are enriched in late-stage branches. EMS_fibroblasts, endometriosis-associated fibroblasts

Identified characteristic fibroblasts of endometriosis

Subsequently, we conducted the UMAP analysis again to hierarchically cluster the fibroblasts. Subclustering of fibroblasts revealed 8 different subtypes (Fig. 3A). We next quantified the distribution of the eight fibroblast subtypes across normal, ectopic, and eutopic samples. Subtype 4 was markedly enriched in ectopic lesions, whereas subtype 2 was significantly expanded in both ectopic and eutopic tissues compared with normal controls (Fig. 3B). Given their shared origin from endometriosis patients, we amalgamated these two fibroblast clusters and designated them as EMS-fibroblast (Fig. 3C).

To substantiate this classification, we systematically examined the differentially expressed genes (DEGs) across all fibroblast subtypes and highlighted the top 10 most significant shared DEGs between subtypes 2 and 4. These shared transcriptional features provide supportive evidence for defining EMS-associated fibroblasts. Notably, several key fibrosis-associated genes, including TGFB1, MMP2, and ACTA2, were prominently upregulated, implicating this population in fibrotic remodeling (Fig. 3D). Consistently, Hallmark pathway analysis revealed that EMS_Fibroblasts were significantly enriched in pathways related to cell cycle progression, TGF- β signaling, and estrogen response (Fig. 3E). Collectively, these data identify EMS_Fibroblasts as a distinct fibroblast population enriched in endometriosis, characterized by coordinated activation of proliferative and pro-fibrotic programs. This cell population may play a central role in driving lesion progression and fibrotic remodeling through the integration of TGF- β signaling, estrogen responsiveness, and cell cycle regulation.

The genes were subsequently analyzed by unsupervised clustering, leading to the emergence of unique gene groups. Furthermore, we categorized genes exhibiting analogous expression patterns, as indicated by the clustering outcomes.

Additionally, specific differential genes of seven fibroblast clusters were depicted in a heatmap (Fig. 3F). These subtype-specific molecular features may serve as functional hallmarks, facilitating a deeper understanding of the distinct roles of each fibroblast subpopulation in endometriosis development and fibrotic progression.

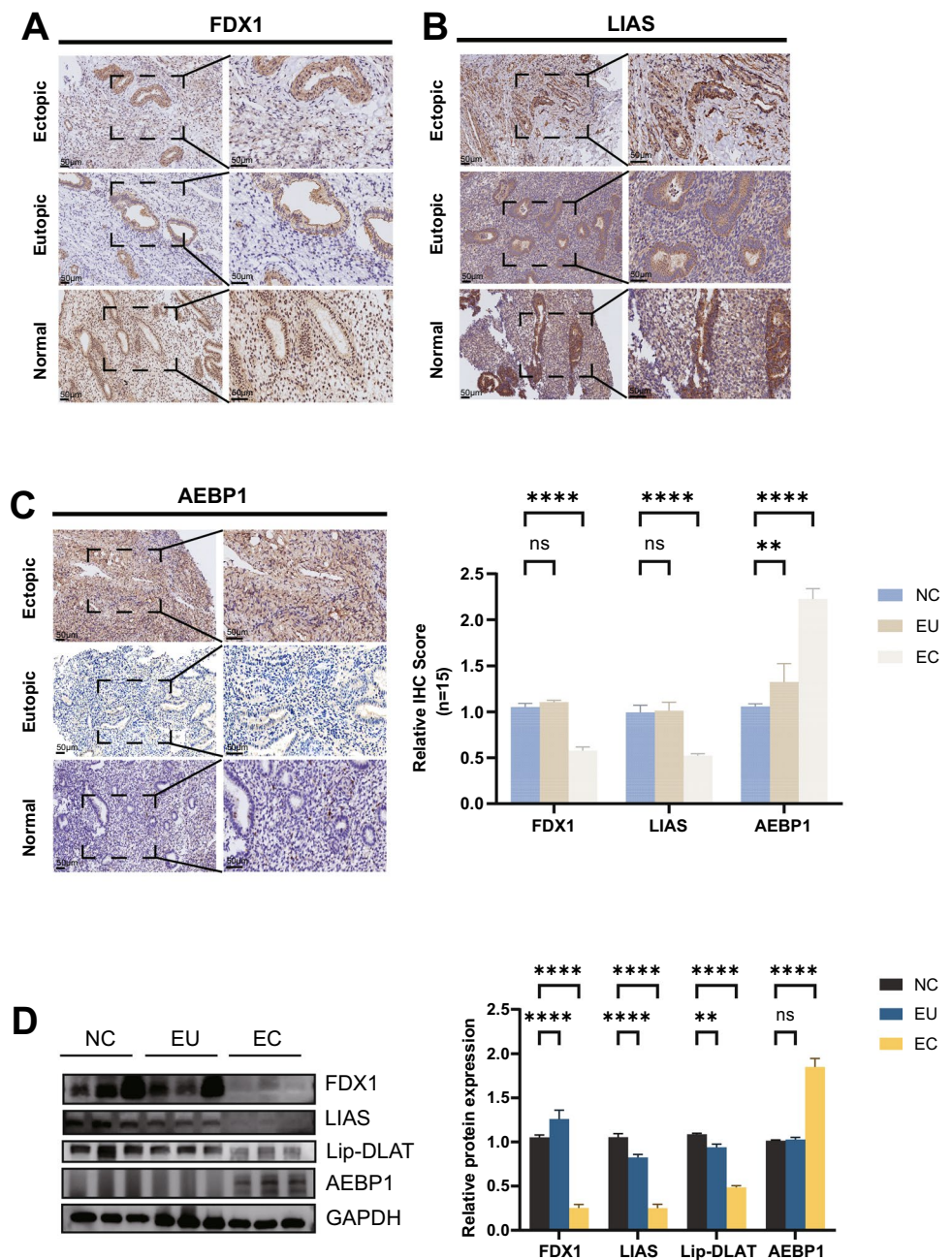
Identification of fibroblast-associated gene modules and hub genes using hdWGCNA and machine learning

We employed high-dimensional weighted gene co-expression network analysis (hdWGCNA) to identify the key molecular characteristics associated with fibroblasts in the context of endometriosis. The co-expression network construction revealed that a scale-free topology fitness index of 0.90 was achieved with a soft threshold power (β) of 5, which optimized the connectivity within the cell network (Fig. 4A). This analysis identified 13 co-expression modules (Fig. 4B), among which the blue module exhibited the strongest association with fibroblast activity (Fig. 4C). Within the blue module, highly connected genes were prioritized based on intramodular connectivity, and the top 120 genes were defined as key fibroblast-associated candidates. We further analyzed through protein–protein interaction (PPI) network analysis using the STRING database (Fig. 4D).

To identify robust hub genes, we integrated multiple machine learning approaches using bulk transcriptomic datasets (GSE7305 and GSE11691). We initially analyzed the combined dataset comprising 19 EMS tissue samples and 19 normal endometrial samples. The random forest approach revealed six genes with a gene relevance score exceeding 2 (Fig. 4E, F). The LASSO method revealed six genes of significant importance (Fig. 4G). The SVM-RFE algorithm found four genes of considerable significance (Fig. 4H). We derived the intersection of the genes identified by these three machine learning algorithms. Utilizing the LASSO regression technique, random forest algorithm, and SVM-RFE algorithm, we identified three pivotal genes, AEBP1, COL6A3, and C1S, that demonstrated correlation with endometriosis fibroblasts (Fig. 4I).

We next examined the expression patterns of these three genes across fibroblast subclusters. Heatmap analysis showed that AEBP1, COL6A3, and C1S were relatively enriched in EMS-associated fibroblasts, with AEBP1 showing the most prominent expression pattern (Fig. 4J). Feature plots further confirmed that these genes were preferentially expressed in fibroblast populations from ectopic and eutopic tissues, particularly within the EMS-associated fibroblast cluster (Fig. 4K). Based on its expression pattern and consistent identification across multiple analytical approaches,

Fig. 6 Clinical endometriosis specimens exhibit altered cuproptosis-related molecular signatures and increased AEBP1 expression. **A**, **B** Representative immunohistochemistry (IHC) staining of FDX1 and LIAS in ectopic, eutopic, and normal endometrial tissues. **C** Representative IHC staining of AEBP1 in ectopic, eutopic, and normal endometrial tissues, with quantification of relative IHC scores at right. **D** Representative western blots and densitometric quantification of FDX1, LIAS, Lip-DLAT, and AEBP1 in normal control (NC), eutopic (EU), and ectopic (EC) tissues. GAPDH was used as the loading control. Human tissue samples included NC (n = 15), EU (n = 15), and EC (n = 15). Data are presented as mean $\pm$ SD. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparisons test. ns, not significant; * $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$. Scale bars = 50 μ m. FDX1, ferrodoxin 1; LIAS, lipoic acid synthetase; Lip-DLAT, lipoylated dihydrolipoamide S-acetyltransferase; AEBP1, adipocyte enhancer-binding protein 1; IHC, immunohistochemistry

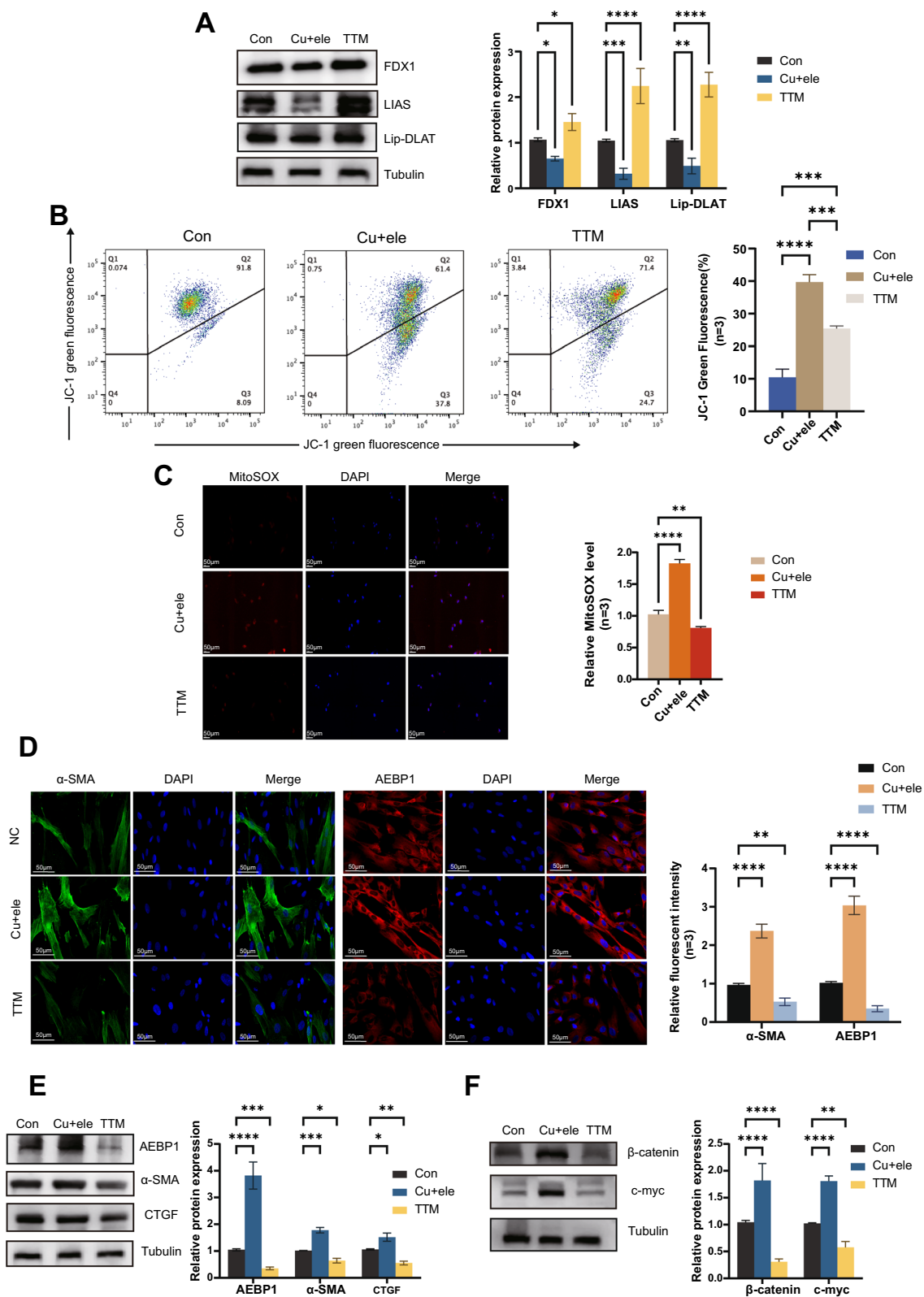


AEBP1 was prioritized for subsequent validation as a candidate fibrosis-associated marker in endometriosis.

Pseudotime analysis reveals a profibrotic differentiation trajectory of EMS-associated fibroblasts

Pseudotime analysis uncovered a continuous, branched differentiation trajectory for fibroblasts in endometriosis. Distinct fibroblast subclusters segregated along separate trajectory branches, with EMS-associated fibroblasts predominantly enriched in the relatively late-stage regions of

the trajectory—consistent with a disease-linked activated state (Fig. 5A). Fibroblasts isolated from control, eutopic, and ectopic tissue also exhibited distinct distribution patterns: ectopic and eutopic fibroblasts preferentially localized to specific trajectory branches and later pseudotime states, indicating altered differentiation dynamics in endometriosis-associated fibroblasts (Fig. 5B). In alignment with these findings, multiple distinct cell states were identified across different branches, supporting the occurrence of progressive, heterogeneous state transitions during fibroblast differentiation (Fig. 5C). Pseudotime-based color mapping



further illustrated a gradual progression from early to late cellular states toward distinct branch termini (Fig. 5D).

Notably, AEBP1, COL6A3, and C1S showed progressive upregulation along pseudotime, reflecting the gradual acquisition of profibrotic properties during fibroblast

Fig. 7 Copper and copper ionophore treatment is associated with cuproptosis-related molecular alterations, increased AEBP1 expression, and profibrotic activation in primary ectopic endometrial stromal cells. Primary ectopic endometrial stromal cells were treated with CuCl_2 (50 μM) plus elesclomol (10 nM) for 24 h, with or without tetrathiomolybdate (TTM), as indicated. **A** Representative western blots and densitometric quantification of FDX1, LIAS, and Lip-DLAT. Tubulin was used as the loading control. **B** Representative JC-1 flow cytometry plots and quantification of JC-1 green fluorescence. An increased proportion of green fluorescence indicates reduced mitochondrial membrane potential. **C** Representative MitoSOX staining images and quantification of mitochondrial reactive oxygen species (ROS). Nuclei were counterstained with DAPI. **D** Representative immunofluorescence staining of α -SMA and AEBP1, with quantification of relative fluorescence intensity. **E** Representative western blots and densitometric quantification of AEBP1, α -SMA, and CTGF. **F** Representative western blots and densitometric quantification of β -catenin and c-Myc. Western blot quantification was derived from three independent experiments. MitoSOX fluorescence was quantified from three independent microscopic fields per group. Data are presented as mean $\pm$ SD. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparisons test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. Scale bars = 50 μm . TTM, tetrathiomolybdate; α -SMA, alpha-smooth muscle actin; CTGF, connective tissue growth factor; DAPI, 4',6-diamidino-2-phenylindole; ROS, reactive oxygen species

differentiation (Fig. 5E–G). Consistent with this, branch-specific heatmap analysis revealed that a panel of extracellular matrix- and fibrosis-related genes—including COL6A1, COL6A2, COL6A3, C1S, FBN1, IGFBP5, COL1A1, COL3A1, DCN, and AEBP1—were enriched in late-stage trajectory branches, further corroborating the profibrotic transition of EMS-associated fibroblasts during disease progression (Fig. 5H).

Clinical endometriosis exhibits altered cuproptosis-related molecular signatures and elevated AEBP1 expression

The principal factors regulating cuproptosis are FDX1 and LIAS. FDX1 can convert divalent copper into the more hazardous monovalent copper and is also implicated in the regulation of lipoic acid modification of proteins. LIAS-encoded lipoic acid synthase (LIAS), an enzyme containing an iron-sulfur cluster, interacts with FDX1. Their connection facilitates the normal progression of protein acylation [21]. Consequently, LIAS and FDX1 are typically considered diagnostic markers for cuproptosis, with their expression levels diminishing during the process of cuproptosis. Lip-DLAT, the lipoylated form of DLAT, is one of the major lipoylated tricarboxylic acid (TCA) cycle proteins implicated in cuproptosis-related processes and can serve as an additional readout of cuproptosis-related molecular alterations. Therefore, we examined the expression of FDX1, LIAS, and Lip-DLAT to evaluate cuproptosis-related

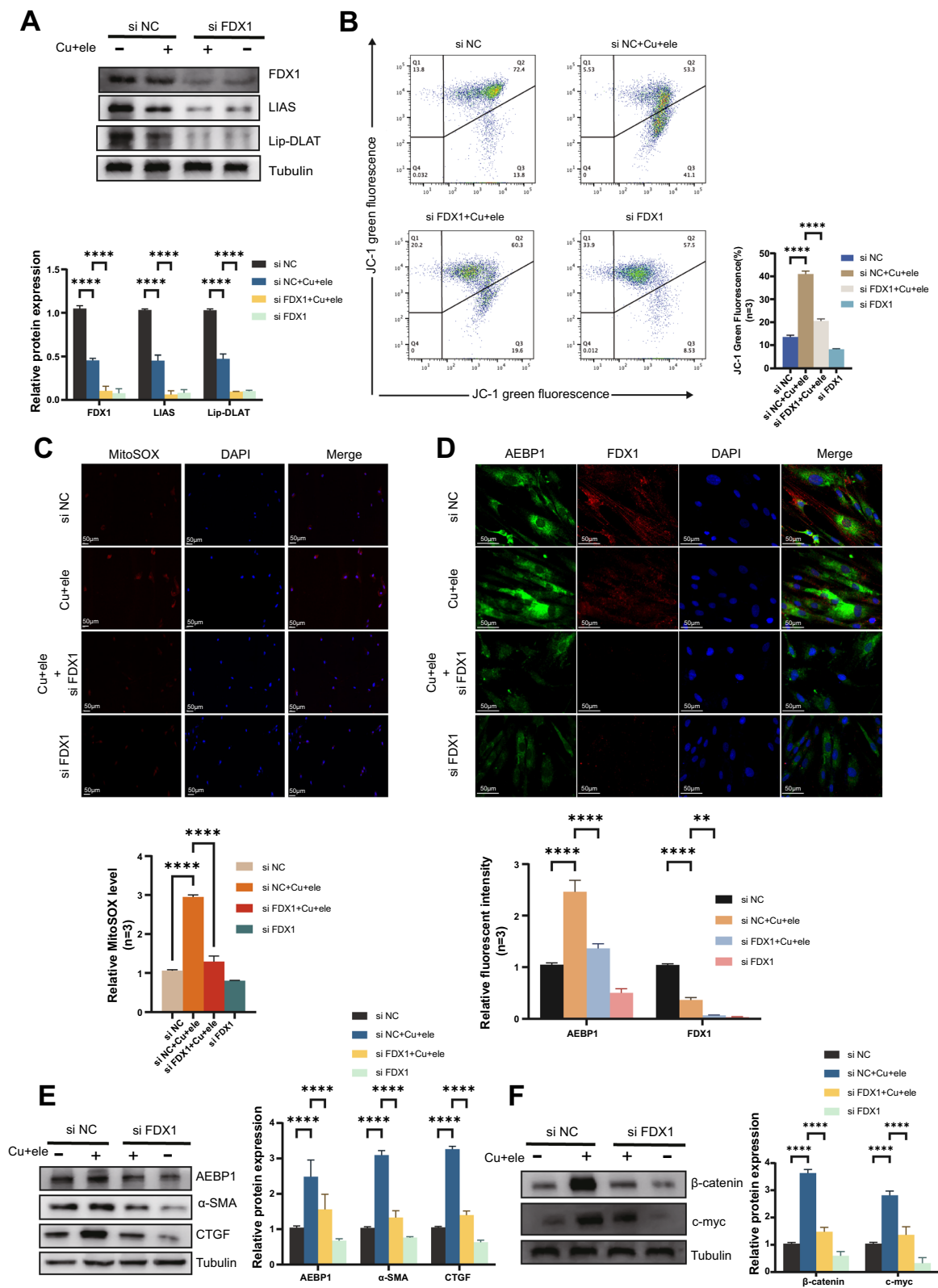
changes, together with AEBP1 as a fibrosis-associated marker.

To further characterize cuproptosis-associated molecular alterations in clinical endometriosis specimens, we assessed the expression levels of FDX1, LIAS and AEBP1 in normal control (NC), eutopic (EU) and ectopic (EC) endometrial tissues using immunohistochemistry (IHC) and Western blotting. IHC staining demonstrated that the expression of FDX1 and LIAS was notably downregulated in ectopic endometrial tissues, while AEBP1 expression was significantly upregulated, particularly within ectopic lesions (Fig. 6A–C). Western blot analysis further validated the decreased expression of FDX1, LIAS, Lip-DLAT as well as the increased abundance of AEBP1 in EC tissues (Fig. 6D). Additionally, altered Lip-DLAT expression was detected in endometriotic lesions, suggesting that copper-dependent mitochondrial alterations may be present in endometriosis. Collectively, these findings indicate that ectopic endometrial tissues display aberrant cuproptosis-related or copper-dependent mitochondrial stress signatures, accompanied by enhanced expression of the fibrosis-related marker AEBP1.

Cuproptosis-related changes are associated with increased AEBP1 expression and fibrotic activation in primary ectopic endometrial stromal cells

To further investigate the association between cuproptosis-related changes and profibrotic activation in endometrial stromal cells, cells were treated with CuCl_2 (50 μM) and elesclomol (10 nM) for 24 h, with or without the copper chelator TTM. Western blot analysis showed that CuCl_2 /elesclomol cotreatment reduced the expression of cuproptosis regulators FDX1, LIAS and Lip-DLAT, while TTM partially restored their expression levels (Fig. 7A). JC-1 staining revealed decreased mitochondrial membrane potential in the CuCl_2 plus elesclomol group, which was significantly attenuated by TTM (Fig. 7B). MitoSOX staining further confirmed increased mitochondrial ROS production following CuCl_2 /elesclomol treatment, an effect that was reversed by TTM (Fig. 7C).

We next evaluated whether cuproptosis-related changes were coupled with profibrotic activation. Immunofluorescence staining showed markedly increased expression of α -SMA and AEBP1 in the CuCl_2 plus elesclomol group, whereas TTM treatment significantly reduced their fluorescence intensity (Fig. 7D). Western blot analysis further verified that CuCl_2 /elesclomol cotreatment upregulated profibrotic proteins (AEBP1, α -SMA, CTGF) and activated β -catenin/c-Myc signaling, and these effects were notably suppressed by TTM (Fig. 7E, F). Taken together, these findings suggest that cuproptosis-related alterations are associated with increased AEBP1 expression and fibrotic marker



expression in endometrial stromal cells, accompanied by changes in β -catenin pathway-related proteins.

FDX1 knockdown attenuates cuproptosis-related alterations and profibrotic responses in endometrial stromal cells

Fig. 8 FDX1 knockdown attenuates copper ionophore-associated mitochondrial alterations and profibrotic responses in endometrial stromal cells. ThESCs were transfected with negative-control siRNA (siNC) or FDX1 siRNA (siFDX1), followed by treatment with CuCl₂ plus elesclomol (Cu+ele) or vehicle, as indicated. **A** Representative western blots and densitometric quantification of FDX1, LIAS, and Lip-DLAT. Tubulin was used as the loading control. **B** Representative JC-1 flow cytometry plots and quantification of JC-1 green fluorescence. **C** Representative MitoSOX staining images and quantification of mitochondrial ROS levels. Nuclei were counterstained with DAPI. **D** Representative immunofluorescence staining of AEBP1 and FDX1, with quantification of relative fluorescence intensity. **E** Representative western blots and densitometric quantification of AEBP1, α -SMA, and CTGF. **F** Representative western blots and densitometric quantification of β -catenin and c-Myc. Western blot quantification was derived from three independent experiments. MitoSOX fluorescence was quantified from three independent microscopic fields per group. Data are presented as mean $\pm$ SD. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparisons test. ** $P < 0.01$; **** $P < 0.0001$. Scale bars = 50 μ m. si NC, negative-control small interfering RNA; si FDX1, FDX1-specific small interfering RNA; Cu+ele, CuCl₂ plus elesclomol; α -SMA, alpha-smooth muscle actin; CTGF, connective tissue growth factor; ROS, reactive oxygen species

FDX1 is a well-established key regulatory factor in the process of cuproptosis. On one hand, during the occurrence of cuproptosis, FDX1 catalyzes the binding of toxic copper(I) to lipoylated proteins in the tricarboxylic acid (TCA) cycle, which leads to impairment of cellular respiration and subsequent induction of cuproptosis, manifested as a consumptive decrease in FDX1 expression. On the other hand, cuproptosis fails to occur following the knockdown of FDX1. Therefore, in this study, we induced cuproptosis in the human endometrial stromal cell line (ThESCs) while simultaneously performing FDX1 knockdown.

In the aforementioned bioinformatics analysis, we identified AEBP1 as a characteristic fibrosis marker of endometrial stromal cells. We observed that the expression of AEBP1 was downregulated after FDX1 knockdown, which indicates that the expression of AEBP1 is regulated by FDX1 to a certain extent. Simultaneously, after knocking down FDX1, the expression of FDX1, LIAS, and Lip-DLAT was markedly reduced (Fig. 8A). In parallel, JC-1 staining revealed that cotreatment with CuCl₂ and elesclomol enhanced green fluorescence intensity, indicative of reduced mitochondrial membrane potential. This effect was notably mitigated by FDX1 knockdown (Fig. 8B). Consistently, MitoSOX staining demonstrated that the elevation in mitochondrial ROS levels induced by CuCl₂/elesclomol cotreatment was partially reversed following FDX1 silencing (Fig. 8C).

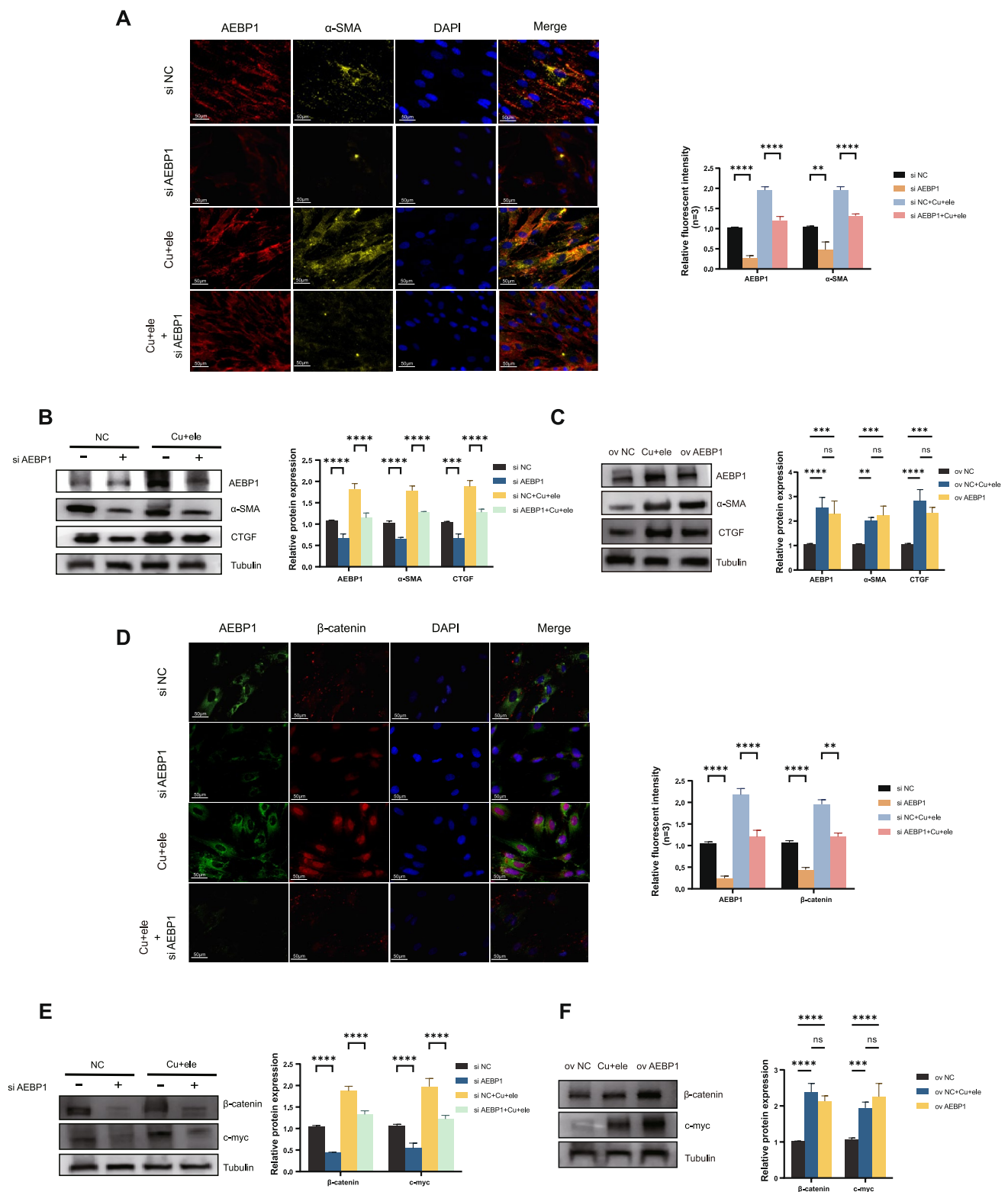
Next, we performed immunofluorescence analysis after FDX1 knockdown, confirming that AEBP1 expression levels significantly decreased when cuproptosis was induced concurrently with FDX1 knockdown (Fig. 8D). Western blot analysis further showed that the expression of the

fibrosis-related proteins AEBP1, α -SMA, and CTGF was elevated in the CuCl₂ plus elesclomol group but was attenuated following FDX1 knockdown (Fig. 8E). Similarly, the expression of β -catenin and its downstream target c-myc was also increased after CuCl₂ plus elesclomol treatment and was reduced by FDX1 silencing (Fig. 8F). Taken together, these findings indicate that FDX1 is closely involved in cuproptosis-associated molecular alterations and elevated AEBP1 expression, enhanced profibrotic marker levels, and modulated expression of β -catenin pathway-related proteins in endometrial stromal cells.

AEBP1 is associated with profibrotic responses and the β -catenin pathway-related protein expression in endometrial stromal cells

Prior research indicates that β -catenin pathway is involved in the progression of several fibrosis-associated disease and has also been implicated in endometriosis-related fibrosis [22–24]. The involvement of the β -catenin pathway in endometriosis has been substantiated by numerous prior investigations [25]. AEBP1, a fibrosis-associated factor, has been demonstrated in studies to have a regulatory role; particularly, the silencing of AEBP1 can mitigate β -catenin-mediated renal fibrosis [26]. Consequently, we silenced AEBP1 in ThESCs with AEBP1-specific small interfering RNA (siAEBP1). Immunofluorescence results indicated a reduction in the expression of α -SMA under CuCl₂ plus elesclomol treatment when AEBP1 was silenced (Fig. 9A). Consistently, Western blot analysis showed that the elevated expression of AEBP1, α -SMA, and CTGF induced by CuCl₂ plus elesclomol was attenuated following AEBP1 silencing (Fig. 9B). Conversely, AEBP1 overexpression was accompanied by increased expression of AEBP1, α -SMA, and CTGF, showing a pattern comparable to that observed after CuCl₂ plus elesclomol treatment (Fig. 9C).

We next examined β -catenin pathway-related changes after modulation of AEBP1. Immunofluorescence staining showed that CuCl₂ plus elesclomol increased AEBP1 and β -catenin signals, whereas AEBP1 knockdown reduced both signals (Fig. 9D). Western blot analysis further confirmed that the increased expression of β -catenin and its downstream target c-myc induced by CuCl₂ plus elesclomol was attenuated after AEBP1 silencing (Fig. 9E). In contrast, AEBP1 overexpression was accompanied by increased expression of β -catenin and c-myc, with levels similar to those observed in the CuCl₂ plus elesclomol group (Fig. 9F).



TTM attenuates fibrotic progression of ectopic lesions in vivo

We created an endometriosis animal model by implanting

mouse endometrial tissue fragments into the peritoneal cavity of C57 mice. To further examine whether copper-dependent alterations are associated with fibrotic progression in ectopic lesions in vivo, we created endometriosis

Fig. 9 AEBP1 modulates profibrotic marker expression and β -catenin pathway-related proteins in endometrial stromal cells. ThESCs were transfected with AEBP1-specific siRNA (siAEBP1) or AEBP1 overexpression plasmid (ovAEBP1), followed by treatment with CuCl_2 plus elesclomol (Cu+ele) or vehicle, as indicated. **A** Representative immunofluorescence staining of AEBP1 and α -SMA in siNC, siAEBP1, Cu+ele, and siAEBP1+Cu+ele groups, with quantification of relative fluorescence intensity. **B** Representative western blots and densitometric quantification of AEBP1, α -SMA, and CTGF in siNC, siAEBP1, Cu+ele, and siAEBP1+Cu+ele groups. **C** Representative western blots and densitometric quantification of AEBP1, α -SMA, and CTGF in ovNC, Cu+ele, and ovAEBP1 groups. **D** Representative immunofluorescence staining of AEBP1 and β -catenin in siNC, siAEBP1, Cu+ele, and siAEBP1+Cu+ele groups, with quantification of relative fluorescence intensity. **E** Representative western blots and densitometric quantification of β -catenin and c-Myc in siNC, siAEBP1, Cu+ele, and siAEBP1+Cu+ele groups. **F** Representative western blots and densitometric quantification of β -catenin and c-Myc in ovNC, Cu+ele, and ovAEBP1 groups. Tubulin was used as the loading control for all western blot analyses. Western blot quantification was derived from three independent experiments. Data are presented as mean $\pm$ SD. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparisons test. ns, not significant; ** P <0.01; *** P <0.001; **** P <0.0001. Scale bars=50 μm . si NC, negative-control small interfering RNA; si AEBP1, AEBP1-specific small interfering RNA; ov NC, empty vector control; ovAEBP1, AEBP1 overexpression plasmid; α -SMA, alpha-smooth muscle actin; CTGF, connective tissue growth factor

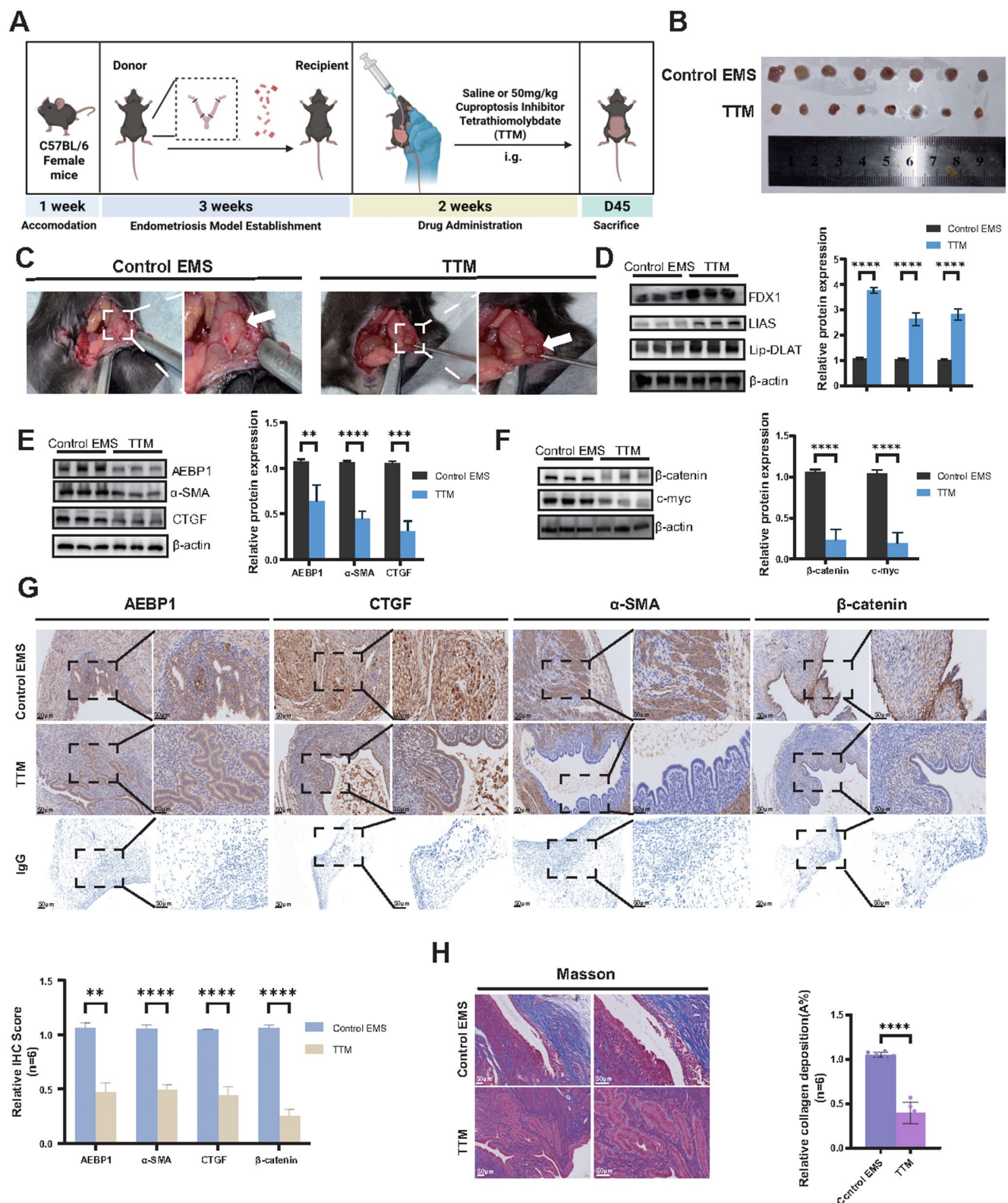
model mice and administered TTM (50 mg/kg), previously identified in studies as a cuproptosis inhibitor (Fig. 10A). Variations in average cyst sizes and lesion weights were noted between the two treatment groups (Fig. 10B). In comparison to the control EMS group, TTM treatment markedly suppressed the development of abdominal wall endometriotic lesions (Fig. 10C). We then identified cuproptosis indicators, fibrosis markers, and molecules associated with the β -catenin pathway in the ectopic lesions of the murine model. The results of Western blotting indicated that TTM treatment was associated with increased expression of FDX1, LIAS, and Lip-DLAT, suggesting a partial reversal of cuproptosis-related molecular alterations in ectopic lesions (Fig. 10D). Concurrently, the expression levels of the fibrosis markers α -SMA, CTGF, and AEBP1 were markedly reduced (Fig. 10E). The expression of β -catenin and c-myc was diminished subsequent to TTM therapy (Fig. 10F). Subsequently, we assessed the expression of four pivotal molecules (AEBP1, CTGF, α -SMA and β -catenin) using immunohistochemistry staining, and the findings were congruent with those derived from Western blotting (Fig. 10G). Masson staining demonstrated a considerable reduction in collagen fiber deposition following TTM treatment. A substantial area of blue-stained collagen fibers was noted in the ectopic lesions of the control group, while the proportion of blue-stained collagen fibers in the TTM group was markedly reduced (Fig. 10H). Taken together, these findings suggest that TTM treatment is associated with reduced fibrotic burden in ectopic lesions in vivo, accompanied by reversal

of cuproptosis-related molecular changes and decreased expression of β -catenin pathway-related and fibrosis-related proteins.

Discussion

Copper is an essential trace metal element in organisms, which acts as a cofactor or structural component of enzymes and participates in various life activities, including cellular free radical scavenging, connective tissue synthesis, pigment formation, immune regulation, and neurotransmitter synthesis [27, 28]. In the fibrotic process of multiple organs, tissue copper ion levels are consistently elevated. Copper iron overload induces the production of mitochondrial reactive oxygen species (ROS), thereby promoting the expression of fibrosis-related genes and the differentiation of myofibroblasts [29]. Specifically, copper accumulation in cardiomyocyte mitochondria triggers mitochondrial damage, cytochrome c release, and cell apoptosis—events that further contribute to cardiac injury and exacerbate cardiac fibrosis [30]. In patients with Wilson's disease, abnormal copper ion accumulation occurs within mitochondria. Notably, treatment with copper chelators leads to significant improvements in mitochondrial structure and function, accompanied by the alleviation of liver fibrosis [31, 32]. In patients with renal failure, plasma copper ion levels are markedly increased, indicating that copper ions accumulate to a certain extent in the body when renal function is impaired. Additionally, in a rat model of renal fibrosis induced by unilateral ureteral obstruction (UUO), administration of tetrathiomolybdate (TTM) significantly reduces copper ion concentrations in renal tissue and ameliorates fibrosis [27]. These observations provide a biological rationale for investigating whether copper-dependent stress responses are also involved in endometriosis-associated fibrosis.

Copper accumulation is a primary catalyst of cuproptosis. Excessive intracellular copper accumulation beyond homeostatic control induces cuproptosis through processes such as increasing the aggregation of lipoylated TCA cycle proteins, triggering overproduction of mitochondrial ROS, and interrupting cellular respiration [33, 34]. Our single-cell transcriptomic profiling identified that cells harboring elevated CRG scores were predominantly distributed in fibroblasts and smooth muscle cells. Specifically, fibroblasts with heightened CRG activity displayed marked enrichment of extracellular matrix and collagen-associated transcriptional programs. Given that fibroblast activation is a pivotal mediator of fibrotic remodeling in endometriosis, these results indicate that cuproptosis-related molecular signatures together with copper-dependent mitochondrial



stress may contribute to a profibrotic stromal phenotype. Importantly, however, the current data do not establish that canonical cuproptosis occurs in endometriotic tissues; rather, they support an association between CRG-related

transcriptional programs, copper-dependent mitochondrial stress, and fibrosis-relevant fibroblast phenotypes.

Although multi-omics approaches have substantially advanced the understanding of endometriosis heterogeneity,

Fig. 10 Tetrathiomolybdate attenuates lesion growth and fibrosis in a mouse model of endometriosis. **A** Schematic diagram of the mouse endometriosis model and treatment protocol. Uterine fragments from donor C57BL/6 female mice were implanted onto the abdominal wall of recipient mice. Three weeks after model establishment, recipient mice received saline or tetrathiomolybdate (TTM, 50 mg/kg, intragastric administration) for 2 weeks and were sacrificed on day 45. **B** Representative image of excised ectopic lesions from control EMS and TTM-treated mice. **C** Representative in situ images of abdominal wall endometriotic lesions in control EMS and TTM-treated mice. White arrows indicate lesion sites. **D** Representative western blots and densitometric quantification of FDX1, LIAS, and Lip-DLAT in ectopic lesions. **E** Representative western blots and densitometric quantification of AEBP1, α -SMA, and CTGF in ectopic lesions. **F** Representative western blots and densitometric quantification of β -catenin and c-Myc in ectopic lesions. β -actin was used as the loading control. **G** Representative immunohistochemistry staining of AEBP1, CTGF, α -SMA, and β -catenin in ectopic lesions from control EMS and TTM-treated mice; IgG staining served as the negative control. Quantification of relative IHC scores is shown below. **H** Representative Masson's trichrome staining of ectopic lesions and quantification of relative collagen deposition. For the animal study, recipient mice were assigned to control EMS ($n=15$) and TTM-treated EMS ($n=15$) groups; donor mice, $n=10$. IHC and Masson quantification were performed on six lesions per group ($n=6$). Data are presented as mean $\pm$ SD. Statistical analysis was performed using a two-tailed unpaired Student's *t*-test. $^{**}P<0.01$; $^{***}P<0.001$; $^{****}P<0.0001$. Scale bars = 50 μ m. EMS, endometriosis; TTM, tetrathiomolybdate; IHC, immunohistochemistry; α -SMA, alpha-smooth muscle actin; CTGF, connective tissue growth factor

the relationship between cuproptosis-related signaling and fibrosis-associated stromal states has remained poorly defined [17, 18, 35]. Prior research on cuproptosis in EMS have predominantly concentrated on conventional bulk transcriptome-based bioinformatics techniques. In this investigation, we initially conducted cuproptosis scoring for all cells at the single-cell level utilizing cuproptosis-related genes (CRGs). By integrating single-cell analysis with network-based and machine-learning approaches, our study extends previous transcriptome-based observations and identifies a fibroblast-associated CRG-high state linked to fibrotic remodeling.

Clinically, significant fibrosis is seen in endometriotic lesions, resulting in tissue or organ adhesions, anatomical disarray, and in extreme instances, compromised fertility. AEBP1 has been thoroughly investigated in various fibrotic diseases [15, 36–38]; specifically, endogenous AEBP1 expression is elevated in patients with cardiac hypertrophy and heart failure, and AEBP1 knockdown has been demonstrated to diminish the contractile ability of cardiac fibroblasts and the expression of the α -SMA gene, thereby underscoring AEBP1's pivotal role in cardiac fibrosis [14]. AEBP1 is significantly expressed in cancer-associated fibroblasts (CAFs) of patients with pancreatic ductal adenocarcinoma (PDAC), where it facilitates fibrosis in the tumor microenvironment (TME) and enhances the invasion and migration of PDAC cells; elevated AEBP1 expression correlates strongly with unfavorable prognosis in PDAC [13]. These studies collectively indicate that AEBP1 plays a

significant role in fibrogenesis. Our data extend these observations to endometriosis and support AEBP1 as a candidate fibrosis-associated mediator in EMS-associated fibroblasts. In subsequent experiments, copper and its ionophore treatment was accompanied by increased AEBP1 expression together with elevated profibrotic markers, increased mitochondrial ROS, and reduced mitochondrial membrane potential, whereas TTM or FDX1 knockdown attenuated these changes. These findings support the possibility that AEBP1 participates in copper-dependent profibrotic responses in endometrial stromal cells.

Cell–cell communication analysis further suggested that CRG-high stromal cells participate in stronger profibrotic signaling interactions, including TGF β and Wnt-related communication. This observation is notable because β -catenin signaling has previously been implicated in fibroblast activation and fibrosis in endometriosis and other organs. Together, these data raise the possibility that copper-dependent stress responses may influence not only stromal-cell intrinsic programs but also fibrosis-relevant intercellular signaling. Consistent with this mechanistic framework, manipulating copper-dependent stress in endometrial stromal cells coincided with modified expression of β -catenin and c-Myc proteins. As β -catenin signaling is known to regulate fibroblast activation and extracellular matrix production, these observations point to a plausible connection between stromal phenotypes with high CRG activity and profibrotic signaling driven by β -catenin.

Furthermore, considering our earlier identification of AEBP1 as a candidate fibrosis-associated marker of EMS fibroblasts, we noticed that AEBP1 knockdown attenuated the increases in profibrotic markers and β -catenin/c-Myc-related protein expression observed under copper ionophore treatment. These results support a potential association among cuproptosis-related signaling, AEBP1 expression, and β -catenin pathway activity. However, the present data do not yet define a direct linear mechanism or establish AEBP1 as the sole mediator linking these processes, and additional mechanistic studies will be required to clarify causality.

From a translational perspective, our findings indicate that AEBP1 merits further investigation as a fibrosis-associated biomarker candidate in endometriosis, and targeting copper metabolism may represent a promising antifibrotic therapeutic strategy. That said, our study did not assess circulating AEBP1 levels, its diagnostic performance, or stratification of fibrosis severity; thus, these translational implications should be regarded as preliminary. Additionally, while TTM mitigated fibrotic phenotypes in our *in vivo* models, its therapeutic effects should be attributed to the attenuation of copper-dependent molecular perturbations, rather than definitive blockade of core cuproptosis signaling. Future work should validate AEBP1 in larger and independent cohorts, define more precisely how AEBP1 relates to β -catenin pathway activity, and determine

whether modulation of copper metabolism has reproducible antifibrotic effects in more physiologically relevant preclinical models of endometriosis.

Several limitations of this study should be acknowledged. First, the single-cell analyses were based on a limited number of publicly available samples, although integration and quality-control procedures were applied to reduce technical bias. Second, the CRG score was derived from a predefined 16-gene signature and AUCell-based inference, which reflects pathway-related transcriptional activity rather than direct evidence of cell death. Third, although we assessed FDX1, LIAS, Lip-DLAT, mitochondrial ROS, and mitochondrial membrane potential, these measurements do not by themselves conclusively demonstrate canonical cuproptosis *in vivo*. Finally, while our perturbation experiments support a regulatory relationship between AEBP1 and β -catenin-related changes, additional studies, including rescue experiments and direct interrogation of pathway activity, will be needed to establish causality.

Collectively, our data support an interpretive framework linking cuproptosis-related molecular changes and copper-dependent mitochondrial stress to fibroblast activation and fibrotic remodeling in endometriosis. We further identify AEBP1 as a promising candidate mediator that warrants further in-depth investigation. These findings advance our understanding of stromal heterogeneity in endometriosis and lay the groundwork for exploring copper metabolism as a potential antifibrotic therapeutic target.

Conclusion

In conclusion, this study integrates single-cell transcriptomic analysis with experimental validation to investigate the association between cuproptosis-related molecular alterations and fibrotic progression in endometriosis. By leveraging single-cell RNA sequencing, pseudotime trajectory analysis, and hdWGCNA, we identified a fibroblast subpopulation associated with relatively high cuproptosis-related gene activity and profibrotic transcriptional features. Machine learning-based prioritization further highlighted AEBP1 as a candidate fibroblast-associated gene linked to cuproptosis-related signatures.

Functional experiments showed that copper ionophore treatment was accompanied by changes in fibrosis-related marker expression in endometrial stromal cells, together with altered β -catenin/c-Myc-related protein expression. *In vivo*, tetrathiomolybdate attenuated lesion fibrosis and reduced the expression of fibrosis-related markers, supporting the possibility that modulation of copper metabolism may have antifibrotic effects in endometriosis.

Collectively, our findings support an association between cuproptosis-related molecular alterations or copper-dependent mitochondrial stress and fibroblast-mediated fibrosis in

endometriosis, and identify AEBP1 as a candidate molecule for further investigation. These results also provide a rationale for further exploring copper metabolism as a potential antifibrotic target in this disease.

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Author contributions L Zhang and Y Liu proposed the idea and reviewed the manuscript, E Huang and J Li drafted and revised the initial manuscript. E Huang, D Zuo, and R Li performed the experiments. J Li, Q Wu, N Lin, J Zhao and H Wang analyzed the data. All authors read and approved the final manuscript.

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Data availability The public datasets used in this paper are available on the NCBI website (<https://www.ncbi.nlm.nih.gov/geo/>).

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval and consent to participate The collection of endometriosis samples from human subjects was approved by the Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology and followed the tenets of the Declaration of Helsinki. The patients provided their written informed consent to participate in this study.

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