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Epithelial cells with high TOP2A expression promote cervical cancer progression by regulating the transcription factor FOXM1

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Background: Cervical cancer (CC) remains a major malignancy threatening women's health, with high-grade squamous intraepithelial lesions playing a critical role in the progression toward CC. Exploring the molecular characteristics of epithelial cells (EPCs) as high-stage intraepithelial neoplasia evolves into CC is essential for the development of effective targeted drugs for cervical cancer. Single-cell RNA sequencing technology can fully understand the immune response at each molecular level, providing new ideas and directions for the precise treatment of CC.

Methods: Single-cell RNA sequencing was employed to comprehensively map EPCs characteristics. The differentiation trajectory of EPCs was inferred using Slingshot, while enrichment analysis highlighted the biological functions of EPCs. Cellchat visualized cell-cell interactions, and SCENIC was used to infer transcription factor regulatory networks in EPCs. CCK-8, colony formation, and EDU experiments were used to verify cell proliferation changes. Scratch assays and transwell assays were used to verify cell migration and invasion.

Results: A distinct EPCs subpopulation with high *TOP2A* expression was identified, predominantly originating from tumor tissues. This subpopulation exhibited disrupted mitosis and cell cycle regulation, along with features of high proliferation, high energy metabolism, and matrix plasticity. It played a key role in shaping the tumor microenvironment via the LAMC1-(ITGA3-ITGB1) signaling pathway. FOXM1, a key transcription factor in this cell subpopulation, significantly inhibited the proliferation and invasion of cervical cancer cells.

Conclusion: Through in-depth analysis of EPCs, this study provides promising insights and potential therapeutic targets for precision targeted treatment strategies for CC.

KEYWORDS

epithelial cells, cervical cancer, FOXM1, single-cell RNA sequencing, TOP2A

1 Introduction

Cervical cancer (CC) ranks among the most prevalent gynecological malignancies and is the fourth leading cause of cancer-related deaths in women, marked by high morbidity and mortality rates (1–3). Despite significant improvements in survival rates and quality of life for early-stage patients through a combined treatment approach of surgery, radiotherapy, and chemotherapy, some patients still experience drug resistance or disease progression. With the advancement of modern medicine, finding new targets for malignant tumors has become a hot issue in immunotherapy, precision therapy and reversing tumor immune resistance (4–7). Immunotherapy and targeted therapies offer promising alternatives for patients with recurrent or metastatic CC, with the potential to deliver more precise and effective treatment options (8).

In the progression of CC, cervical intraepithelial neoplasia (CIN) represents a critical turning point. While most low-grade CIN lesions can regress spontaneously, high-stage intraepithelial neoplasia (HSIL) poses a significantly higher risk of malignant transformation. Thus, investigating the core molecular mechanisms underlying the progression from HSIL to CC is essential for advancing early prevention strategies for cervical cancer (9–11).

The tumor microenvironment is a protective chamber that maintains the occurrence and development of tumors. Epithelial cells (EPCs), as the most common origin of tumor cells, play a vital role in tumor progression. As a type of stromal cells, EPCs are also able to support and maintain chronic inflammatory states and immune resistance in the tumor microenvironment. Therefore, treatment targeting EPCs may provide a promising approach for immune resistance in cervical cancer.

Multi-omics technologies technology has been widely used in cancer cell biology and molecular biology related research (12–20) especially in the field of immune resistance (8, 14, 20–27). This study used Single-cell RNA sequencing(scRNA-seq) to deeply explore the map characteristics of EPCs during CC progression, explained the biological characteristics of EPCs from the level of transcriptional regulatory network, and identified key pathogenic EPCs subpopulations. Our research provides potential cell vectors and molecular targets for the precise targeted treatment of CC, and provides a practical idea for the development of CC anti-tumor drugs.

2 Methods

2.1 Data source

Single-cell sequencing data were obtained by accessing the ArrayExpress (https://www.ebi.ac.uk/biostudies/arrayexpress) database with CC number E-MTAB-12305. The research data were derived from public databases, so no ethical approval was required.

2.2 Data quality control

The raw data were imported into R software (version 4.3.2) using Seurat R package for analysis. The DoubletFinder function (28–30) was used to perform strict quality control on the scRNA-seq data to filter low-quality cells and remove potential doublet cells. The quality control standards were as follows: 300<nFeature<6000; 500<nCount<50000; 0<pMT<25; 0<pHB<5.

The data were normalized using the NormalizeData function (31–35). The FindVariable Features function was used to find highly variable genes (36–40). The ScaleData R package was used for standardization processing of the data. Principal component analysis (PCA) (41–43) was performed on the data, and the top 30 principal components were selected. The harmony R package (44, 45) was used to remove batch effects.

2.3 Cell identification

The cells were clustered using the FindClusters function and the FindNeighbors function (28, 46), and the cells were annotated according to the CellMarker database (http://xteam.xbio.top/CellMarker/) and cell-typical markers. The FindAllMarkers function was used to find differentially expressed genes in different cell subpopulations.

2.4 InferCNV

Taking ECs as reference, the inferCNV algorithm was used to calculate the copy number variation of EPCs.

2.5 Enrichment analysis

Gene ontology (GO)enrichment analysi (47–52) of differentially expressed genes was performed using ClusterProfiler R package (51, 53, 54). Gene set enrichment analysis (GSEA)was performed by downloading GSEA software from the GSEA website (http://software.broadinstitute.org/gsea/msigdb).

2.6 Slingshot analysis

The getlineage function in the Slingshot R package is used to infer cell differentiation lineages (55–57). The getCurves function is used to calculate the cell expression levels of different lineages within the fitting time.

2.7 CellChat analysis

CellChat R package (58–60) was used to infer the interactions between cells. The identifyCommunicationPatterns algorithm is used to count the number of communication patterns, and the netVisual_diffInteraction algorithm is used to calculate the difference in the strength of communication between cells. Circle plots, violin plots, layer plots, and heat maps are used to visualize the communication between cells.

2.8 Scenic analysis

The activity of transcription factors in EPCs subsets was assessed using the SCENIC package in Python (version 3.7). The GRNBoost2 algorithm was used to infer co-expression modules between transcription factors and target genes. The RcisTarget algorithm was used to analyze the genes in each co-expression module to help identify enriched motifs. The AUCell score was used to evaluate the activity of transcription factors in EPCs.

2.9 Cell transfection

HeLa cell lines and SiHa cell lines were propagated in MEM medium. Cells were seeded at 50% density in 6-well plates and then transfected with FOXM1-specific knockdown constructs (si-*FOXM1*–1 and si-*FOXM1*-2) and negative control constructs (si-NC). Transfection was performed using Lipofectamine 3000RNAiMAX (Invitrogen, USA) according to the manufacturer's instructions. siRNA sequences: si-1: AAGAA GAAAUCCUGGUUAA; si-2: ACUAUCAACAAUAGCCUAU. qRT-PCR primers: F: AAACCTGCAGCTAGGGATGT; R: AGCCCAGTCCATCAGAACTC.

2.10 CCK-8 assay

After being plated in 96-well plates, the cells were cultured for a full day. After adding 10 μL of CCK-8 labeling reagent to each well, the wells were left in the dark for two hours. Using an enzyme marker, absorbance at 450 nm was used to measure cell viability.

2.11 Transwell assay

After the cells were starved for twenty-four hours, the cell suspension was combined with Matrigel and introduced onto the Costar plate's upper chamber. The lower chamber was filled with the serum-containing media. The cells were fixed with 4% paraformaldehyde and stained with crystal violet following a 48-hour incubation period.

2.12 Wound healing assay

The cell monolayer in each well of a six-well plate was consistently scratched using a sterile 200 μL pipette tip, and scratch photos were recorded after 0 and 48 hours of incubation. Scratch width was measured using Image-J software.

2.13 5-Ethynyl-2′-deoxyuridine proliferation assay

After adding the EdU working solution, the cells were incubated for two hours. Following a PBS wash, the cells were fixed using 4% paraformaldehyde solution, permeabilized and quenched using a solution containing 0.5% Tr and 2 mg/ml glycine, and stained using 1X Apollo solution and Hoechst staining reaction solution.

2.14 Statistical analysis

All data were processed using R or Python. $P< 0.05$ was considered statistically significant.

3 Results

3.1 Single-cell transcriptome profiles during CC progression

To explore the evolution of transcriptome features during CC development, we performed scRNA-seq analysis on 9 samples from normal cervix (NAT), HSIL and cervical cancer (Tumor), and finally identified 85,591 high-quality cells, as shown in Figure 1A. Using known cell type marker genes (Figure 1B), we finally identified 10 types of cells in these samples, including T cells and NK cells, ECs, fibroblasts, smooth muscle cells (SMCs), EPCs, B cells, plasma cells, mast cells (MCs), neutrophils and myeloid cells As shown in Figure 1C, EPCs are the main cell type present in tumor samples, accounting for 65.3%, and are mainly in the G2M and S phases of the cell cycle, with active cell replication and vigorous proliferation (Figure 1D). Figure 1E confirms this result. In addition, we also looked at the number of molecules and total number of genes detected in different cell types, and the results showed that EPCs had higher nCount-RNA and nFeature-RNA expression (Figure 1F). This suggests that EPCs have higher cell activity and rich gene expression diversity.

3.2 EPCs are heterogeneous during CC progression

As the precursor of tumor cells, the molecular characteristics of EPCs during cancer evolution are crucial to reveal the origin and development of tumors. Therefore, we conducted an in-depth exploration of the characteristics of EPCs during CC progression. First, we used inferCNV to analyze EPCs to view their copy number variations (Figure 2A). Next, we re-clustered EPCs and identified 6 EPCs subtypes: C0 *SPRR1B*+ EPCs, C1 *IGFBP7*+ EPCs, C2 *MUC5B* + EPCs, C3 *TOP2A*+ EPCs, C4 *CFAP126*+ EPCs, and C5 *PTPRC*+ EPCs (Figure 2B). Figure 2C shows the expression of the top 5 marker genes of the 6 EPCs subtypes and different groups. The expression levels of the named genes of the 6 EPCs subtypes are shown in bar graphs and UMAP graphs (Figures 2D, E). It is worth mentioning that we found that C3 *TOP2A*+ EPCs had the highest expression of G2M and S phase cell cycle phase scores (Figure 2F), which means that this EPCs subpopulation has active proliferation and may play an important role in the rapid progression of tumors. In-depth analysis found that C3 *TOP2A*+ EPCs tend to be distributed in tumor samples (Figure 2G), suggesting that this EPCs subpopulation may be tumor-related EPCs.

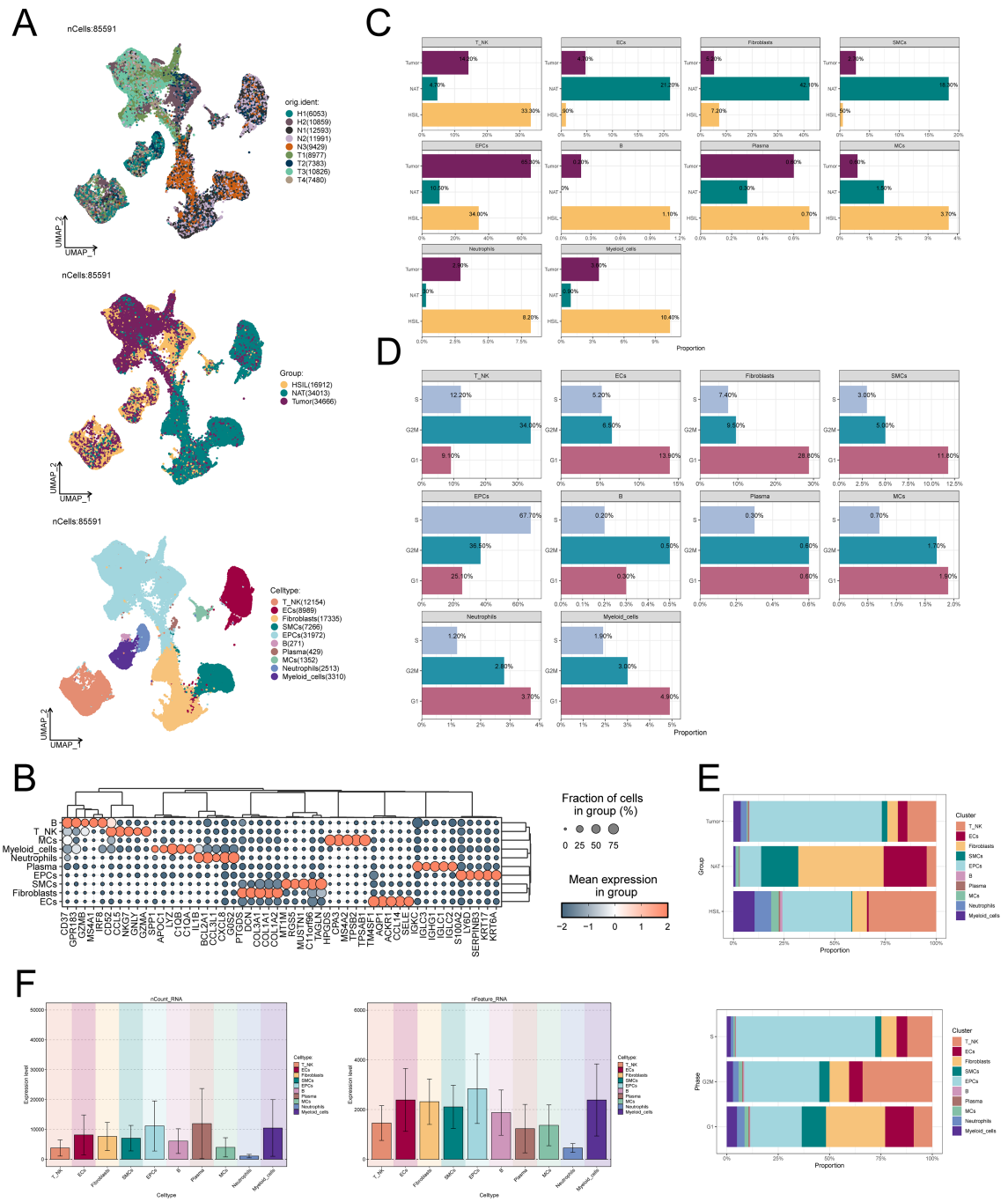

FIGURE 1
scRNA-seq characterizes the cellular landscape of CC. **(A)** Dimensionality reduction clustering diagram of 85,591 high-quality cells. From top to bottom, the sample sources, sample groups (HSIL, NAT and tumor) and cell types (T cells and NK cells, ECs, fibroblasts, SMCs, EPCs, B cells, plasma cells, MCs, neutrophils and myeloid cells) of all cells wereshown. **(B)** Top 5 marker genes of 10 cell types. The bubble size represents the Fraction of cells in group, and the bubble color represents the average expression level. **(C-E)** The proportion of 10 cell types in different samplegroups (HSIL, NAT and tumor) and different cell cycle phases (G1, G2M and S). **(F)** nCount-RNA and nFeature-RNA expression in 10 celltypes. nFeature_RNA: The number of different genes detected in each cell. nCount_RNA: The total number of all RNA molecules sequenced in each cell.

3.3 C3 TOP2A+ EPCs are the key EPCs subset for CC progression

To verify the above results, we used Slingshot to infer the differentiation trajectory of EPCs. As shown in Figure 3A, in the differentiation lineage 1 of EPCs, C3 *TOP2A*+ EPCs are located at the end of the differentiation trajectory, which is consistent with the results of Figure 2G, that is, the PCs subpopulation is tumor-associated EPCs and is of key significance in the evolution of CC. While in lineage 2, C4 *CFAP126*+ EPCs are located at the end.

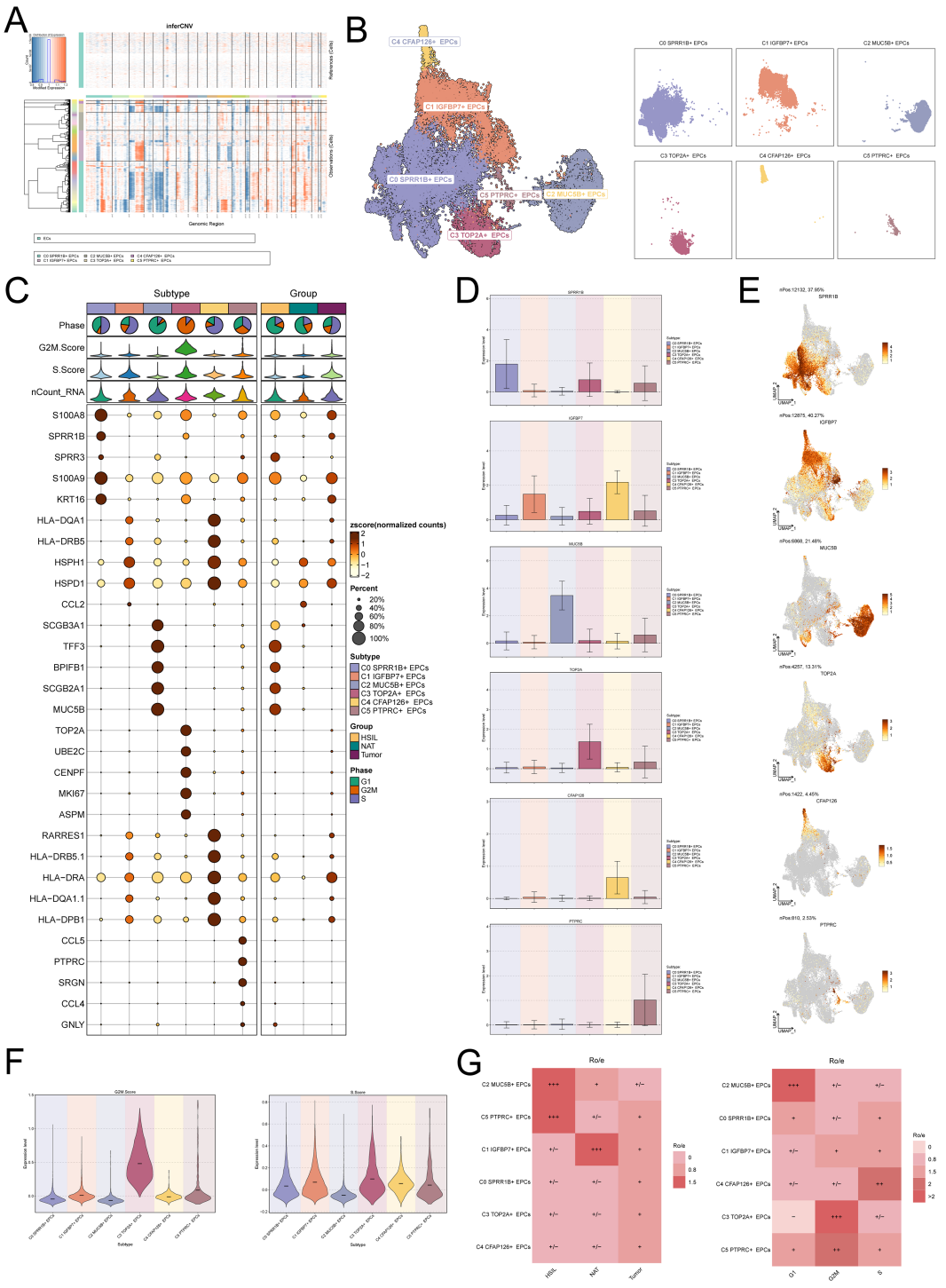

FIGURE 2
Heterogeneity profile of EPCs within CC. **(A)** inferCNV is used to infer the copy number variation level of EPCs. **(B)** Clustering and faceting diagrams of the six EPCs subtypes. **(C)** Expression levels of the top 5 markers of the six EPCs subtypes in different EPCs subtypes and sample source groups. The bubble size represents the expression percentage, and the bubble color represents the zscore. **(D, E)** The bar graph and umap graph show the expression levels of the named genes of the six EPCs subtypes. **(F)** G2M.score and S.score of the six EPCs subtypes. **(G)** Ro/e of the six EPCs subtypes in different sample groups (HSIL, NAT and tumor) and different cell cycle phases (G1, G2M and S).

Figures 3B, C show the differences in the expression levels of the named genes of the six EPCs subtypes over time, among which *TOP2A* is mainly expressed at the end of lineage 1.

3.4 C3 TOP2A+ EPC regulates mitosis and energy metabolism

To further explore the biological characteristics of C3 *TOP2A*+ EPCs, we performed differential gene analysis and enrichment analysis on different subtypes of EPCs (Figures 4A, B). As shown in the figure, C3 *TOP2A*+ EPCs are mainly enriched in chromosome segregation, mitotic nuclear division, nuclear chromosome segregation, mitotic sister chromatid segregation, sister chromatid segregation and nuclear division-related pathways, which are closely related to chromosome division and mitosis. In addition, GSEA further confirmed the above findings (Figure 4C). It is worth mentioning that with the progression of CC, the metabolic activity of EPCs gradually increased, especially in pathways such as riboflavin metabolism, pyruvate metabolism, oxidative phosphorylation, and glycolysis/gluconeogenesis. Consistent with this, the metabolic activity of these pathways was also significantly enhanced in C3 *TOP2A*+ EPCs (Figure 4D).

3.5 C3 TOP2A+ EPCs interact with other cells via the LAMC1-pathway

To reveal the crosstalk relationship between C3 *TOP2A*+ EPCs and other cells, we first analyzed the number and strength of interactions between EPCs and other cell types using cellular communication (Figure 5A). Further research found that when C3 *TOP2A*+ EPCs serve as signal senders, they have a strong interaction with immune cells, such as T cells and NK cells, B cells and myeloid cells, suggesting that they are closely related to the shaping of the tumor immune microenvironment (Figure 5B). When C3 *TOP2A*+ EPCs serve as signal receptors, they interact strongly with stromal cells, such as fibroblasts and SMCs, affecting the matrix shaping of the tumor microenvironment (Figure 5C). This suggests that C3 *TOP2A*+ EPCs have significant plasticity.

Analysis found that C3 *TOP2A*+ EPCs interacted with other cells mainly through the LAMININ signaling pathway, especially the LAMC1 - (ITGA3+ITGB1) ligand receptor pair (Figures 5D-F). In this pathway, C3 *TOP2A*+ EPCs mainly interact with immune cells such as T-NK cells and myeloid cells through paracrine effects. Finally, we used centrality scores to visualize the role of C3 *TOP2A*+ EPCs in this pathway (Figure 5G).

3.6 Transcription factor regulatory network of EPCs

Transcription factors can act directly on the genome, regulate gene transcription by binding to specific nucleotide sequences upstream of genes, and affect the biological function of cells. Therefore, we used the scenic algorithm to analyze the gene regulatory network of EPCs. Different transcription factors can jointly regulate the expression of certain genes. Therefore, we divided the transcription factors of EPCs into 5 modules based on the connection specificity index: M1, M2, M3, M4 and M5 (Figure 6A). As shown in Figures 6B, C, the transcription factors in the M4 and M5 modules may play a major regulatory role in the biological characteristics of C3 *TOP2A*+ EPCs. Further analysis of the top 5 transcription factors of different EPCs subtypes revealed that the transcription factors of C3 *TOP2A*+ EPCs were FOXM1, HOXA13, MYBL2, E2F8 and NFYB (Figures 6D, E). This is consistent with previous studies showing that E2F8 can maintain tumor cell proliferation and promote tumor cell migration (61, 62). Among them, FOXM1, as an important oxidative stress response regulator and proliferation-related factor, has a significant impact on the progression and outcome of tumors.

3.7 FOXM1 is a potential therapeutic target for CC

To verify the key role of *FOXM1* in cervical cancer, we knocked down *FOXM1* in CC cell lines (Figures 7A, B). CCK-8 showed that after *FOXM1* knockdown, the cell viability of cervical cancer cells decreased significantly (Figure 7C), and cell proliferation slowed down (Figure 7D). The EDU experimental results are consistent with this (Figure 7E). The results of the scratch experiment and transwell experiment showed that the reduced expression of *FOXM1* significantly inhibited the migration and invasion ability of CC cells (Figures 7F, G). Based on the above results, *FOXM1* is a key target for inhibiting the progression and invasion of CC and a potential target for future clinical drug development.

4 Discussion

Blocking the formation of the tumor microenvironment is of great significance for the early prevention and diagnosis and treatment of cancer (63–65). CIN is an important turning point in the development of CC (66, 67). We used scRNA-seq technology to comprehensively characterize the changes in the intracellular molecular profiles of normal tissues developing into HSIL and eventually evolving into CC. Tumor cells (especially epithelial malignant tumors) are usually derived from the malignant transformation of normal epithelial cells. Epithelial cells cover the body surface or form glands, and have polarity, tight arrangement, and reliance on intercellular connections under physiological conditions. Malignant epithelial cells (tumor cells) lose these characteristics and gain invasiveness and metastasis capabilities. Therefore, revealing the molecular biological characteristics of epithelial cells during cancer evolution is a core hotspot for studying tumor precision treatment strategies.

We identified a type of EPCs with high proliferation characteristics that mainly exist in tumor tissues, and further explored the cell interaction crosstalk and transcription factor regulatory network of

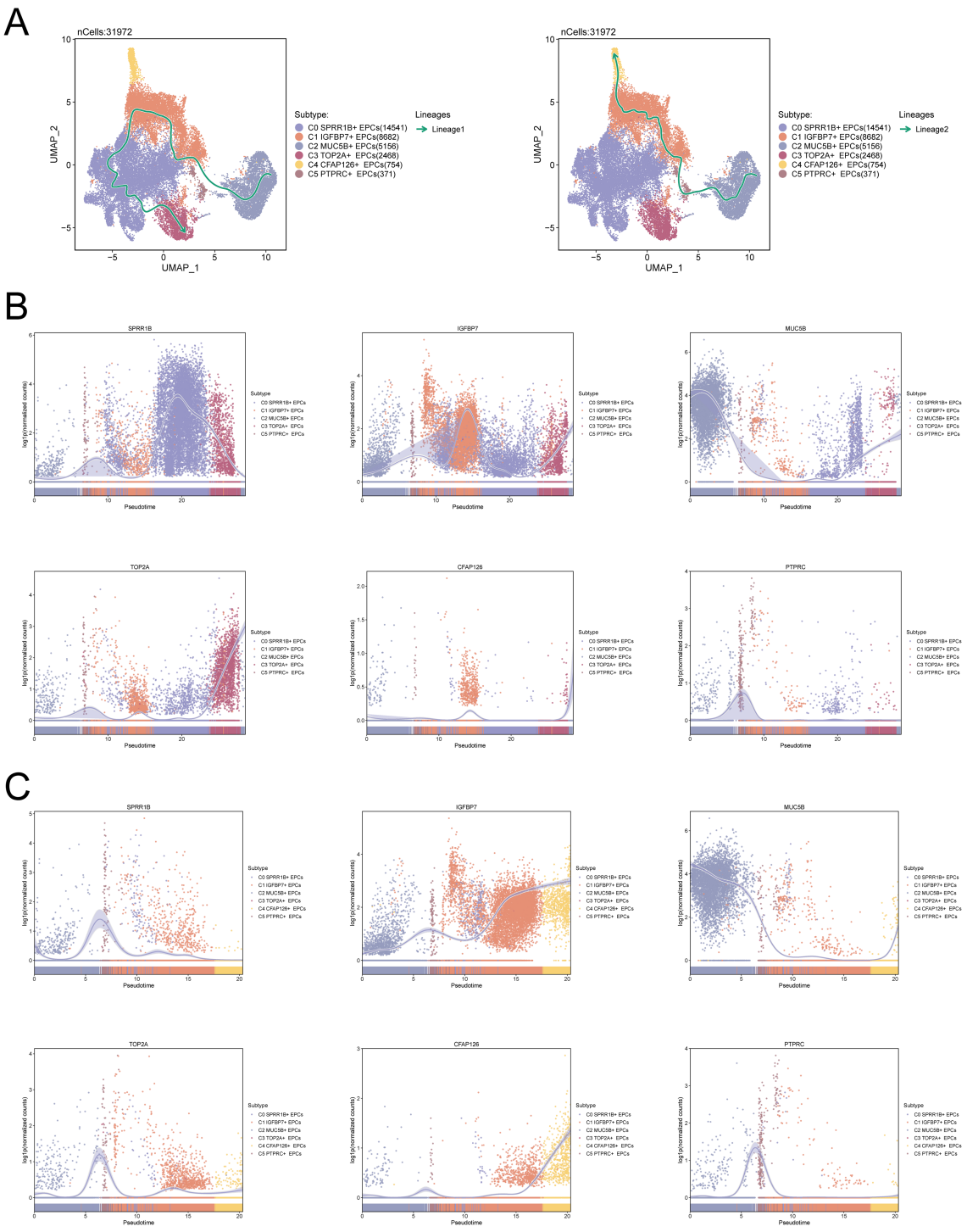

FIGURE 3
C3 *TOP2A*+ EPCs are located at the end of the EPCs differentiation trajectory. **(A)** Slingshot predicts two differentiation lineages of EPCs (left: lineage 1, right: lineage 2). The solid line represents the cell differentiation trajectory, and the arrow direction represents the transition of cell differentiation from immature to mature. **(B, C)** Dynamic trajectory diagram of the expression levels of the six EPCs subpopulation named genes predicted by Slingshot in the two differentiation lineages of EPCs over time (B: lineage 1, C: lineage 2).

this type of EPCs. Experiments confirmed that the transcription factor FOXM1 is a promising target for CC treatment. This study provides reference significance for the interception and treatment of CC, and also provides potential targets for the development of anti-tumor drugs.

TOP2A is a key subtype of DNA topoisomerase, and the DNA breaks mediated by it are of great significance for the carcinogenesis of normal cells (68). Studies have found that TOP2A can interact with RNA Pol II on mitotic chromosomes

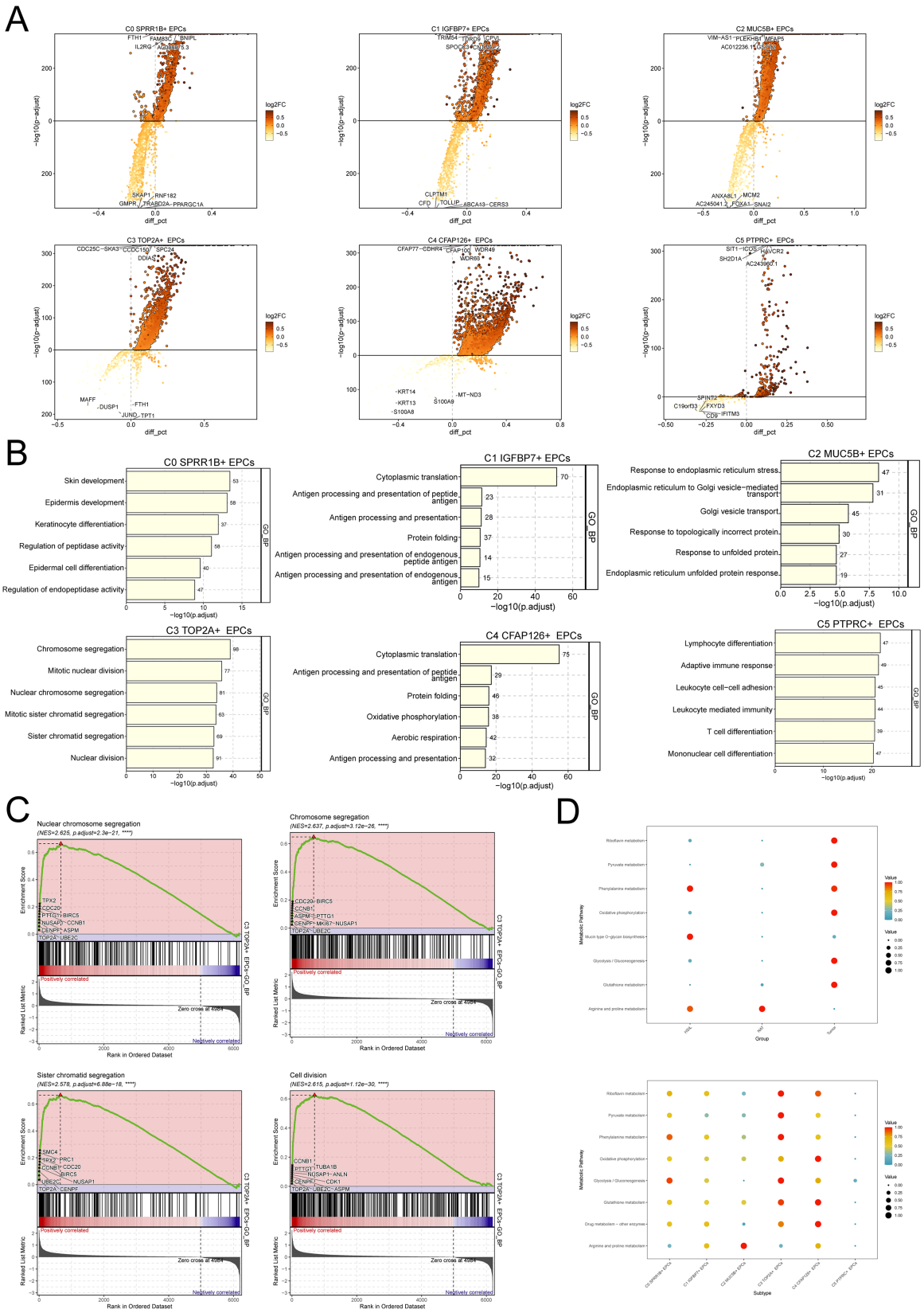

FIGURE 4
Heterogeneity of biological characteristics of different EPCs subgroups. **(A)** Differential gene analysis of 6 EPCs subtypes. The top 5 genes with upregulated differential expression are shown above the horizontal line, and the top 5 genes with downregulated differential expression are shown below the horizontal line. **(B)** GO-BP enrichment analysis of 6 EPCs subgroups. **(C)** GSEA results of C3 *TOP2A*+ EPCs. **(D)** Metabolic pathway enrichment analysis of EPCs.

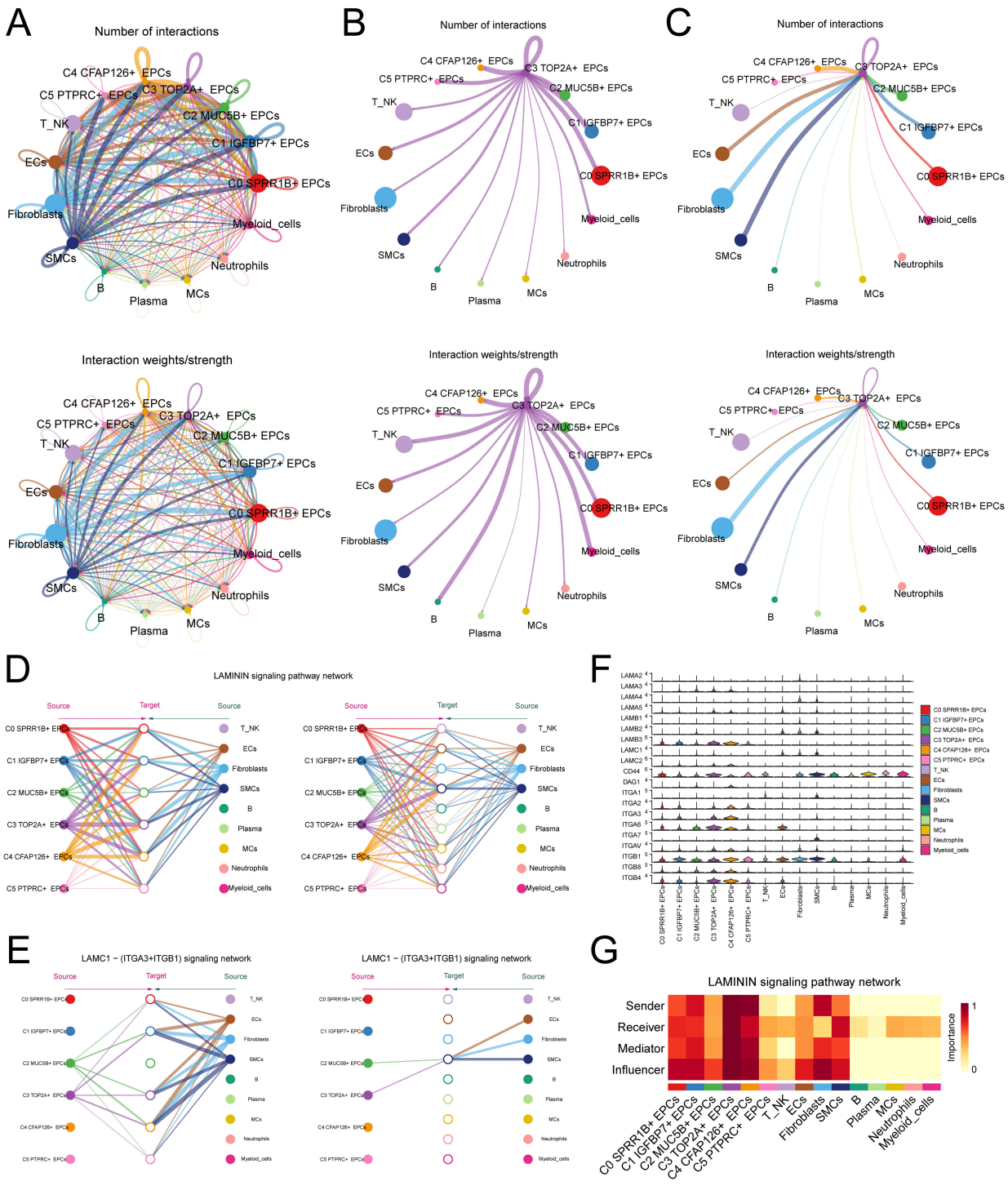

FIGURE 5
Cell interaction analysis of C3 *TOP2A*+ EPCs. **(A)** The number and weight of interactions between EPCs subtypes and other cell types. **(B)** Circle diagram of the number and weight of interactions with C3 *TOP2A*+ EPCs as signal senders and other cell types as signal receivers. **(C)** Circle diagram of the number and weight of interactions with C3 *TOP2A*+ EPCs as signal receivers and other cell types as signal senders. **(D)** A diagram of the communication hierarchy between cell types in the LAMININ signaling pathway. **(E, F)** Hierarchical diagram and violin plots of communication between cell types in the LAMC1 - (ITGA3+ITGB1) ligand receptor pathway. **(G)** Heat map of the centrality of communication between cells in the LAMININ signaling pathway.

to restart transcription, promote the mitotic process, and ensure cell proliferation and growth (69). In addition, this type of EPCs subtype highly expresses cell cycle-related genes such as *UBE2C*, *CENPF*, and *MKI67* (70, 71), explaining the gene characteristics behind the high proliferation function of C3 *TOP2A*+ EPCs. Slingshot analysis confirmed the finding that EPCs gradually evolved from normal cells to tumor-related EPCs, which is consistent with the sample composition in this study, that is, the

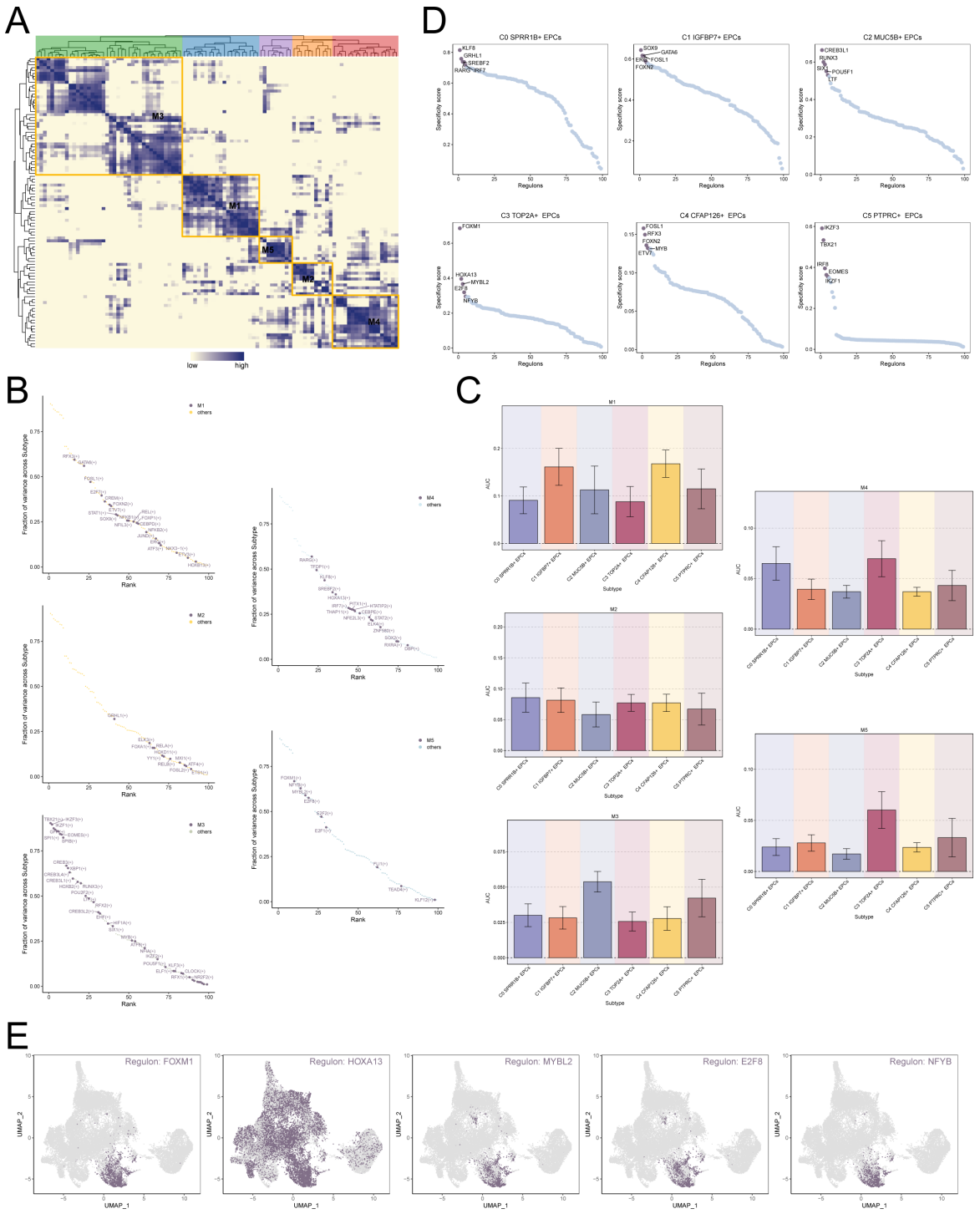

FIGURE 6
Transcription factor regulatory network of EPCs. **(A)** Based on the connection specificity index matrix, 5 regulatory modules of TAMs subtypes were identified. **(B)** In EPCs subtypes, the regulators in different regulatory modules were ranked based on variance scores. **(C)** Expression of different regulatory modules in EPCs subtypes. **(D)** Ranking of top 5 transcription factors in different EPCs subtypes based on regulator specificity score. **(E)** Expression of top 5 key regulators FOXM1, HOXA13, MYBL2, E2F8 and NFYB of C3 *TOP2A*+ EPCs in all EPCs.

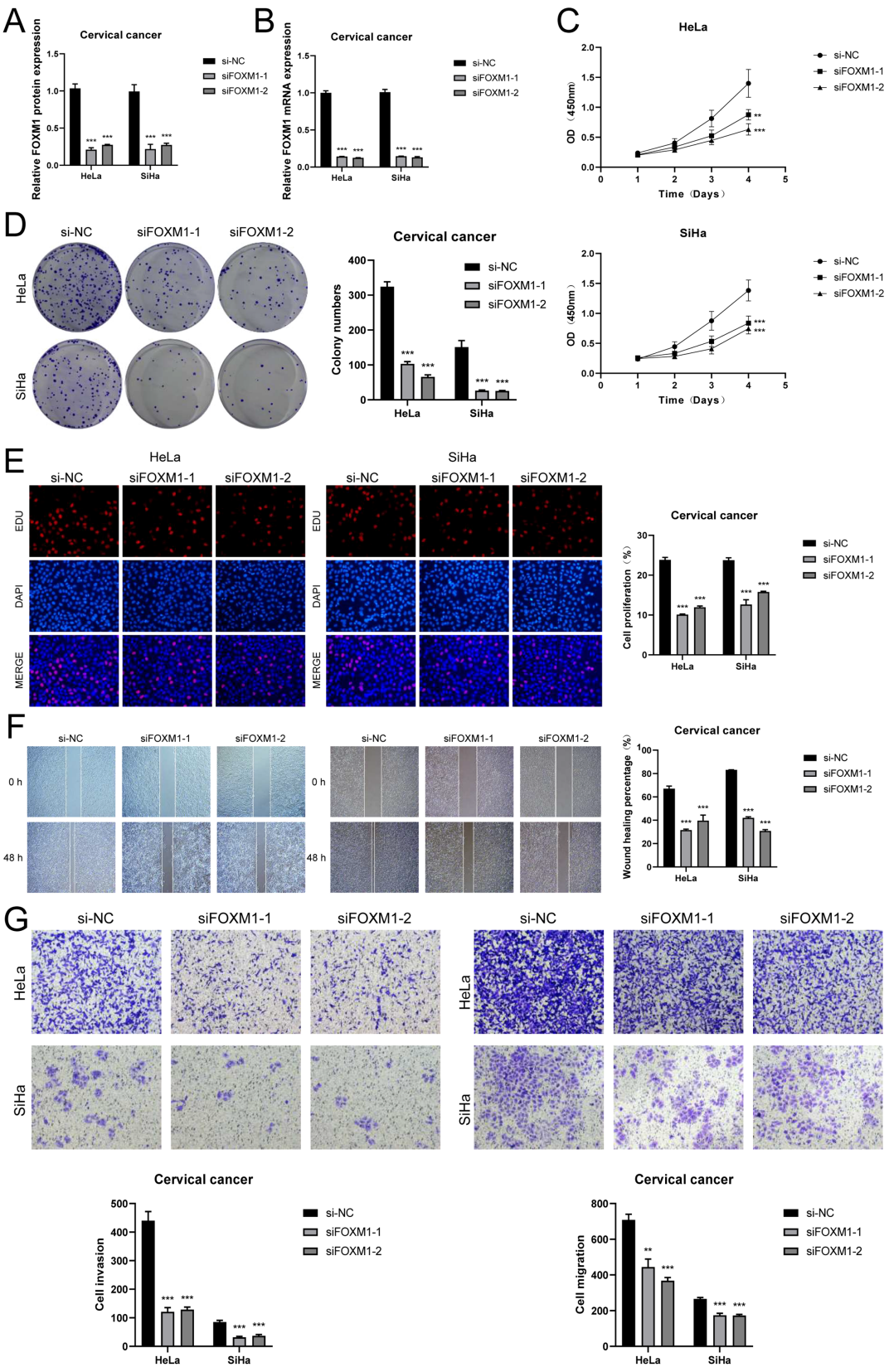

FIGURE 7
FOXM1 knockdown inhibits cervical cancer cell proliferation and invasion. **(A, B)** After *FOXM1* knockdown, its mRNA and protein expression abundance were significantly reduced. **(C)** CCK-8 showed changes in cervical cancer cell activity after *FOXM1* knockdown. **(D, E)** Colony formation and EDU showed that cell proliferation slowed down after *FOXM1* knockdown. **(F)** Scratch assay and quantitative analysis of FOXM1 knockdown. **(G)** Transwell assay and quantitative analysis of *FOXM1* knockdown. (**P < 0.01, ***P < 0.001.).

development of CC goes through three stages: normal tissue, HSIL, and tumor tissue. Combined with the results of enrichment analysis, the rapid proliferation of malignant EPCs in the tumor stage greatly accelerates the progression and invasion of CC.

Studies have found that increased glycolysis and enhanced oxidative stress response under hypoxic conditions can accelerate the proliferation of EPCs. This provides support for the high metabolic activity of C3 *TOP2A*+ EPCs and reveals the connection between EPCs and energy metabolism in CC.

The activation of laminin and integrin-related pathways creates favorable conditions for matrix shaping of the tumor microenvironment and immune escape of tumor cells. Previous studies have found that LAMC1, or laminin γ1, is a biomarker for CC prognosis (72, 73). Our study confirms this result and reveals its intrinsic molecular mechanism for promoting the progression of cervical cancer. LAMC1 binds to integrin (ITGA3-ITGB1) receptors, inducing the activation of the laminin signaling pathway, further leading to the formation of extracellular matrix, hindering the recruitment of immune effector cells into the TME to exert anti-cancer effects. Therefore, breaking the matrix niche and restoring normal immune response is the only way to avoid immune resistance and exert anti-tumor effects.

FOXM1, a forkhead box gene, plays an important role in cell proliferation and lifespan and has been considered as a potential target for CC treatment in recent years. FOXM1 is one of the main transcription factors that regulates PD-L1 expression and ICI immune response in tumors, and can increase the sensitivity of tumor cells to immunotherapy. Studies have found that FOXM1-mediated inactivation of inflammasome transcription can promote the immunosuppressive microenvironment of CC and accelerate the immune escape of cancer cells (74). Our study not only provides single-cell and experimental evidence for the treatment of cervical cancer with *FOXM1*, but also suggests that there may be a potential connection between *FOXM1* and *TOP2A*, but this hypothesis still needs to be verified experimentally (75). Previous studies have found that the co-expression of FOXM1 and TOP2A is significantly associated with poor prognosis in patients with colorectal cancer, bladder cancer, etc (76, 77). In addition, both can accelerate tumor immune escape and immunosuppression. At the same time, the close relationship between tumor proliferation and tumor cell extracellular matrix destruction still needs further exploration. Finally, the limited amount of sample data may lead to the lack of universality of our study.

5 Conclusions

In summary, our study provides a comprehensive and in-depth map of the molecular mechanism of EPCs in CC through scRNA-seq. EPCs with high expression of *TOP2A* are expected to become a key cell subpopulation for the treatment of CC in the future. On this basis, the development of anti-tumor drugs *FOXM1* inhibitors will also bring hope for the diagnosis and treatment of CC.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Author contributions

WS: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. LC: Conceptualization, Data curation, Visualization, Writing – review & editing. XF: Funding acquisition, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

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