

Original Research

Ketone Body Metabolism in Cancer-Associated Fibroblasts and *FSTL3* as Prognostic and Therapeutic Determinants of Chemoradiotherapy Response in Cervical Cancer

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Abstract

Background: Concurrent chemoradiotherapy (CCRT) is a cornerstone of treatment for locally advanced cervical cancer, although many patients show a poor response. The tumor microenvironment (TME) is a key determinant of therapeutic resistance and prognosis, with the metabolic reprogramming within specific cellular subsets playing a particularly important role. However, the cellular sources of metabolic heterogeneity in cervical cancer and their clinical implications have yet to be firmly established. **Methods:** An integrated analysis of single-cell RNA sequencing (scRNA-seq) datasets from eight patients cervical cancer, together with bulk RNA sequencing data retrieved from The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) datasets, was conducted. Cellular heterogeneity was characterized using the Seurat and Harmony, while metabolic activity was assessed by integrating five algorithms (AUCell, UCell, singscore, ssGSEA, and AddModuleScore). The Least Absolute Shrinkage and Selection Operator (LASSO)-Cox regression was used to construct a prognostic model, which was subsequently validated in external cohorts. The functional role of the key gene *FSTL3* was examined through both *in vitro* and *in vivo* experiments. **Results:** scRNA-seq analyses identified 10 major cell types within the cervical cancer TME. Among these, cancer-associated fibroblasts (CAFs) showed the greatest enrichment of ketone body metabolism-related gene signatures. CAFs with high enrichment of these signatures exhibited enhanced cell-cell communication and were associated with activation of the *AP-1* transcription factor network. Analysis of these CAFs led to the development of a nine-gene prognostic signature (*KLF4*, *SERPINF1*, *CD74*, *PRPS2*, *ERRF1*, *FSTL3*, *SOD2*, *JUN*, and *CXCL3*). A risk model based on these genes predicted survival in the TCGA training and validation sets and in independent GEO cohorts (disease-free survival in GSE44001 and overall survival in GSE52903). A nomogram combining the risk score with clinical variables showed improved predictive performance compared with either the risk score or clinical variables alone. Knockdown of *FSTL3* suppressed tumor growth and enhanced sensitivity to irradiation and cisplatin in both *in vitro* and *in vivo* models, supporting its functional role in cervical cancer progression and treatment response. **Conclusion:** These analyses identify a CAF population enriched in ketone body metabolism-related gene expression that may be associated with cervical cancer progression. In addition to establishing a prognostic model for further evaluation, this study identifies *FSTL3* as a potential therapeutic target that warrants further mechanistic and pharmacological investigation.

Keywords: cervical cancer; ketone body metabolism; cancer-associated fibroblasts; tumor microenvironment; *FSTL3*; prognostic model

1. Introduction

Cervical cancer is the most common gynecologic malignancy [1]. Cisplatin-based concurrent chemoradiotherapy (CCRT) is the standard treatment for locally advanced cervical cancer (LACC) [2]. However, the recurrence or metastasis rate in treated patients remains as high as 30%–50% [3]. Salvage radiotherapy or surgery carries significant risks and can significantly compromise quality of life, with 5-year survival rates below 20% [4]. Interpatient heterogeneity contributes to inconsistent treatment outcomes even

under identical radiotherapy conditions [5,6]. Developing high-quality predictive models for CCRT efficacy and prognosis in LACC is crucial to guide effective patient stratification and personalized treatment planning.

The tumor microenvironment (TME) plays a pivotal role in cancer initiation, progression, and therapeutic resistance, consisting of complex cellular communities and intercellular interactions [7,8]. A detailed understanding of the cellular composition and functional diversity of the TME in cervical cancer at single-cell resolution remains



lacking. Metabolic reprogramming has recently been identified as an emerging hallmark of cancer [9,10]. Within this context, the role of ketone body metabolism in tumor progression has gained increasing attention [11,12]; however, the cellular origins, regulatory mechanisms, and clinical significance within the cervical cancer TME are still unclear. Moreover, establishing reliable prognostic models derived from specific cellular subpopulations within the TME has the potential to markedly improve patient risk stratification [13].

In light of these current gaps in knowledge, this study was developed to create a detailed cellular atlas of the cervical cancer TME based on single-cell transcriptomic analysis and to explore ketone body metabolism activity within key cellular subsets in this context, with a particular focus on cancer-associated fibroblasts (CAFs). The overall aim of this approach was to create and validate a prognostic model based on TME metabolism-related multi-gene analysis, with a further focus on experimentally validating the functional role of the key gene *FSTL3* and identifying novel prognostic biomarkers, while also exploring its potential as a candidate for future targeted therapeutic studies.

2. Material and Methods

2.1 Data Acquisition and Preprocessing

Cervical cancer transcriptomic and single-cell RNA sequencing (scRNA-seq) profiles were retrieved from the Gene Expression Omnibus (GEO, <https://www.ncbi.nlm.nih.gov/geo/>), covering three independent cohorts, including GSE197461 (n = 8), GSE44001 (n = 300), and GSE52903 (n = 55). Multi-omics datasets from The Cancer Genome Atlas (TCGA)-Cervical Squamous Cell Carcinoma and Endocervical Adenocarcinoma (CESC) cohort (<https://portal.gdc.cancer.gov/projects/TCGA-CESC>), which included gene expression matrices, copy number variation information, and single nucleotide polymorphism profiles for 309 cervical cancer patients, were acquired from the Genomic Data Commons portal of TCGA. In total, this cohort consisted of 3 normal specimens and 306 tumor samples. For survival analyses, patients with both available gene expression data and complete survival follow-up information were included, yielding an analytic cohort of 291 patients. Quality control for the scRNA-seq data was performed using the Seurat package. Cells with phenotypic indicators more than three median absolute deviations from the median value were excluded in this step. DoubletFinder (v2.0.4, at the University of California, San Francisco, CA, USA) was utilized to screen and filter putative doublets in each sample.

2.2 scRNA-seq Quality Control, Clustering, and Cell Annotation

Following standard sequencing data preprocessing, the LogNormalize algorithm was applied to achieve global normalization of expression. Briefly, the total transcriptional expression of each cell was scaled to 10,000 through

proportional transformation, and logarithmic conversion was then conducted. The CellCycleScoring function was implemented to evaluate cell cycle status and calculate corresponding cell cycle scores, whereas the Find Variable Features function was utilized to screen for highly variable genes across cell populations.

The ScaleData function was employed to eliminate unwanted fluctuations in expression stemming from mitochondrial and ribosomal gene ratios, as well as intercellular cell cycle phase heterogeneity. Dimensionality reduction was accomplished using the RunPCA function, with qualified principal components reserved for subsequent analytical workflows. Harmony integration was performed to correct and minimize cross-sample batch biases. Uniform Manifold Approximation and Projection (UMAP) was further applied to achieve nonlinear dimensionality reduction for refined cell clustering.

Cell cluster annotation was accomplished by integrating previously published studies and the CellMarker database, enabling the identification of multiple cell subtypes closely correlated with cervical cancer pathogenesis. In addition, SingleR-based (version 2.15.2, developed by Aran et al. at the University of California, San Francisco, CA, USA; <https://github.com/SingleR-inc/SingleR>)-based automatic annotation was performed to accurately classify cell types and characterize specific marker genes associated with individual cell populations in the TME.

2.3 Candidate Gene Selection and Signature Establishment

A review of the relevant literature was used to establish a gene set related to the ketone body metabolism pathway. The ketone body metabolism gene set used for this study was established using the KEGG pathway (map00072) and prior pan-cancer metabolic signatures (Qian et al., 2021) [14]. In scRNA-seq data, pathway activity levels were evaluated using five algorithms: AUCell, UCell, singscore, ssGSEA, and AddModuleScore. The average scores derived from these five algorithms were used to compare ketone body metabolic activity among different cell types, leading to the identification of fibroblasts as the key cell population. To evaluate ketone-body metabolic activity, we applied five algorithms: AUCell, UCell, singscore, ssGSEA, and AddModuleScore. Scores from each algorithm were first Z-score standardized across all cells to ensure comparability of distributions and scales, and the averages of these standardized scores were then computed to serve as the composite metabolic activity score for each cell. Fibroblasts were categorized into high- and low-scoring groups according to pathway activity. Differential gene expression analysis was conducted using thresholds of $|\text{avg_log}_2\text{FC}| > 0.25$ and $p_val_adj < 0.05$. CellChat (version 2.2.0.9001, developed by Jin et al. at the University of California, Irvine, CA, USA; <https://github.com/jinworks/CellChat>) was applied to analyze ligand-receptor

interactions between these two groups. Regulon activity scores were visualized with heatmaps. To assess the robustness of our scoring approach, we performed sensitivity analyses using each of the five algorithms independently and also compared Z-score versus 0–1 range normalization. Both approaches yielded consistent results, with fibroblasts being consistently identified as the top-ranked cell type across all methods (**Supplementary Table 1**).

2.4 Patient-Aware Statistical Analyses Addressing Pseudoreplication

To account for the hierarchical structure of multi-patient scRNA-seq data stemming from the analysis of cells nested within patients, several complementary analyses were performed. First, pseudobulk analysis was conducted by aggregating raw counts of cells from the same cell type within each patient. Differential expression between high- and low-metabolic-activity CAF subgroups was then performed using DESeq2 (version 1.48.0, at the University of North Carolina at Chapel Hill, Chapel Hill, NC, USA; <https://github.com/mikelove/DESeq2>) on the pseudobulk count matrix. Linear mixed-effects models, incorporating patient identity as a random effect, were utilized to assess the relationship between fibroblast metabolic activity scores and cell-type identity, accounting for within-patient correlations. Third, the iDESC zero-inflated negative binomial mixed model was used as an additional method for assessing differential expression that explicitly models subject-level random effects and dropout events. Fourth, leave-one-patient-out (LOPO) sensitivity analysis was performed by iteratively omitting each patient, re-running the pseudobulk differential expression analysis on the remaining patients, and evaluating the stability of key findings.

2.5 Risk Model-Based Survival Analysis

A prognostic predictive model was developed based on signature genes screened from single-cell transcriptomic profiles. The LASSO Cox regression algorithm was used to filter genes closely associated with patient prognosis, and ten-fold cross-validation was performed to identify the optimal λ penalty coefficient for parameter tuning. All candidate genes with significant prognostic value screened in univariable Cox regression analyses were incorporated into the risk scoring system, with regression coefficients derived from LASSO analysis assigned as the weight of each gene. For model development, the TCGA-CESC cohort was randomly split into training and validation sets at a 1:1 ratio using a fixed random seed (set.seed(2024) in R) to ensure reproducibility. Patients were stratified into high-risk and low-risk subgroups using the optimal cutoff of the risk score determined by maximally selected rank statistics (surv_cutpoint function, survminer package). The cutoff was derived from the training cohort and then applied unchanged to the validation and external cohorts. The proportional hazards assumption was evaluated using Schoenfeld

residuals (cox.zph function in R), and no significant violation was detected (global $p > 0.05$). To correct for potential overfitting and optimism in the model's predictive performance, internal validation was performed using bootstrap resampling with 1000 iterations. The optimism-corrected C-index was calculated to provide a more conservative estimate of the model's discriminative ability. Survival data were compared between groups using Kaplan-Meier survival analyses combined with the log-rank test, and stratified analyses were used to further verify the stability and reliability of the prognostic performance of the developed risk signature. A rigorous multi-level validation framework was established for this study, including validation in an internal training dataset followed by external validation using multiple public datasets. For external validation, GSE44001 was analyzed for disease-free survival (DFS) and GSE52903 for overall survival (OS), according to the survival endpoints available in each dataset.

Receiver operating characteristic (ROC) curves, time-dependent ROC curves, and calibration curves were plotted to comprehensively quantify and validate the predictive accuracy of the prognostic model. Additionally, a visual nomogram model was constructed based on the individualized risk score. Decision curve analysis (DCA) and ROC verification were jointly implemented to confirm the excellent predictive efficiency and clinical application value of the nomogram.

2.6 Patient Cohort and Response Evaluation

Thirty cervical cancer patients undergoing CCRT treatment at the First Affiliated Hospital of Air Force Medical University were included to validate the developed nine-gene risk signature. Patients underwent pelvic external beam radiotherapy (50 Gy/25 fractions) alongside concurrent cisplatin administration (40 mg/m² weekly or 75 mg/m² tri-weekly). Pelvic magnetic resonance imaging (MRI) was used to assess tumor response at three months post-radiotherapy based on the RECIST v1.1 criteria. Patients with residual cervical primary lesions or lymphatic lesions on imaging were classified as exhibiting a poor response (PoR), while those with complete disappearance of all tumor lesions were defined as exhibiting a good response (GR). The good-response (GR) versus PoR grouping was a study-specific classification based on imaging findings and is not identical to the RECIST v1.1 response categories. Subsequent individualized treatment strategies were comprehensively determined by attending physicians according to each patient's clinical condition. Detailed clinical characteristics of all 30 patients are provided in **Supplementary Table 2**.

2.7 Multiplex Immunofluorescence (mIHC)

Paraffin-embedded cervical cancer tissue sections were first incubated at 56 °C for 1 h, followed by xylene-mediated deparaffinization and gradient rehydration with

serially diluted alcohol solutions ranging from 100% to 75%. Antigen retrieval was performed via microwave heating in 0.1 mM sodium citrate buffer (pH 6.0) for 15 min. To eliminate endogenous peroxidase activity, samples were treated with 3% H₂O₂, after which goat serum was applied for 20 min to block non-specific antigen binding sites. The following primary antibodies were used for multiplex immunofluorescence staining: anti-KLF4 (ab215036, Abcam, Cambridge, England, UK), anti-PEDF (ab307083, Abcam), anti-CD74 (ab108393, Abcam), anti-PRPS2 (ab96222, Abcam), anti-MIG-6 (ab227944, Abcam), anti-FSTL3 (ab232761, Abcam), anti-SOD2 (ab68155, Abcam), anti-JUN (ab40766, Abcam), and anti-CXCL3 (PA5-103136, Invitrogen, Waltham, MA, USA). All primary antibodies were used at a 1:200 dilution and incubated overnight at 4 °C. After a 1 h incubation with HRP-conjugated secondary antibodies (goat anti-rabbit IgG, HRP, cat. no. GB23303, Servicebio, Wuhan, Hubei, China; diluted 1:500) at room temperature, tyramide signal amplification (TSA) staining was implemented using fluorophore-labeled tyramide reagents (iF488-Tyramide, cat. no. G1231, Servicebio; iF555-Tyramide, cat. no. G1233, Servicebio; and iF647-Tyramide, cat. no. G1232, Servicebio; all diluted 1:500).

For sequential multiple staining, heat-induced antibody stripping was conducted with high-stringency antigen retrieval buffer after each staining round. Each tissue section underwent three independent staining cycles, using different primary antibodies for each cycle. After completing all staining procedures, DAPI (cat. no. G1012, Servicebio, Wuhan, Hubei, China) solution was applied for nuclear counterstaining, and anti-fade mounting medium was used for slide preservation. Individual fluorescence images from each optical channel were then captured. All experimental protocols were approved by the Medical Ethics Committee of the First Affiliated Hospital of Air Force Medical University. For quantitative analysis, three randomly selected non-overlapping high-power fields (HPFs) per tissue section were captured and analyzed using Aipathwell software (Version 2.2.4, Servicebio, Wuhan, Hubei, China). The primary quantitative metric used for all statistical comparisons was the percentage of positive cells for each target protein within the tumor region. Tumor regions were predefined for each section based on adjacent H&E-stained slides prior to mIHC analysis, and all image acquisition and quantitative analyses were performed blinded to patient clinical response status. Positive and negative staining controls were included in each staining run to ensure assay specificity and reproducibility. Quantitative data were derived from the mean values of three HPFs per case. For correlation analysis between protein and mRNA expression, Spearman's rank correlation coefficients were calculated between mIHC-derived protein expression (percentage of positive cells) and quantitative real-time PCR (qRT-PCR)-derived mRNA expression for each gene across all 30 patients. Statistical significance was defined as $p < 0.05$.

2.8 Cell Culture and Transfection

The human cervical cancer-derived HeLa cell line (Procell, Wuhan, Hubei, China; Cat. No. CL-0101) and SiHa cell line (Procell, Wuhan, Hubei, China; Cat. No. CL-0210), both of which were cryopreserved in our laboratory, were used for all experimental procedures in the present study. Cells were cultured in RPMI-1640 medium (Procell, Wuhan, Hubei, China) supplemented with 10% fetal bovine serum (FBS) at 37 °C under 5% CO₂. Mycoplasma contamination testing was routinely performed to ensure cell purity, and short tandem repeat (STR) profiling was conducted to verify cell line authenticity before experiments. All cell lines were validated by STR profiling and tested negative for mycoplasma. GenePharma (Shanghai, China) prepared shRNA lentiviral vectors targeting *FSTL3* to knock down *FSTL3* in HeLa cells and a corresponding negative control (NC) vector. The shRNA target sequence for *FSTL3* was [5'-AGGAAGAGAACTTCGTGTGAG-3'], and the NC shRNA sequence was [5'-TTCTCCGAACGTGTCACGT-3']. A single shRNA sequence targeting *FSTL3* was employed for gene silencing; while knockdown efficiency was validated in both HeLa and SiHa cells, the use of a single shRNA construct is acknowledged as a limitation addressed in the Limitations section. HeLa cells were infected during the exponential growth phase. After 24 hours, 4 µg/mL puromycin was added for selection and maintained until all uninfected cells were dead, thereby establishing the following stable cell lines: HeLa sh*FSTL3* and HeLaNC. To validate result generalizability, functional experiments were extended to SiHa cells (HPV16-positive squamous cervical carcinoma), which were cultured in RPMI-1640 medium. *FSTL3* knockdown in SiHa cells was achieved using the same shRNA lentiviral vector (sh*FSTL3*) as for HeLa cells. Knockdown efficiency was validated for both cell lines via qRT-PCR and Western blotting.

2.9 Western Blotting

Total protein was extracted from HeLa and SiHa cells using RIPA lysis buffer (Beyotime, Shanghai, China) supplemented with protease and phosphatase inhibitors. Protein concentrations were determined using a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts of protein (30 µg per lane) were separated by 10% SDS-PAGE and then transferred onto PVDF membranes (Millipore, Burlington, MA, USA) at 300 mA for 120 minutes. Membranes were blocked with 5% non-fat milk in TBST for 2 hours at room temperature, followed by overnight incubation at 4 °C with primary antibodies against *FSTL3* (1:1000, ab232761, Abcam, Cambridge, England, UK) and β -actin (1:1000, #4970, Cell Signaling Technology, Danvers, MA, USA). After washing, membranes were incubated with HRP-conjugated goat anti-rabbit secondary antibody (1:5000, #7074, Cell Signaling Technology, Danvers, MA, USA) for 2 hours at room temperature. Protein bands were visualized using

an enhanced chemiluminescence (ECL) substrate and imaged with a Bio-Rad ChemiDoc imaging system (Bio-Rad Laboratories, Hercules, CA, USA; acquisition software: Image Lab Touch; analysis software: Image Lab version 6.0.1). Densitometric analysis was performed using ImageJ (version 1.54r, Bethesda, MD, USA, and the Laboratory for Optical and Computational Instrumentation, University of Wisconsin-Madison, USA), with β -actin serving as the loading control for normalization. All experiments were repeated in three independent biological replicates. Full uncropped membrane images corresponding to the three independent replicate experiments are provided in **Supplementary Fig. 2** and **Supplementary Fig. 4**, and the corresponding densitometric measurements are included in **Supplementary Table 3**. For Western blot quantification, densitometric values are presented as mean $\pm$ standard deviation (SD) from three independent biological replicates. Statistical significance between groups was determined using unpaired two-tailed Student's *t*-test, with $p < 0.05$ considered statistically significant. Due to the close molecular weights of FSTL3 (~35 kDa) and β -actin (~42 kDa), parallel PVDF membranes were loaded with identical protein aliquots from the same lysates for independent antibody probing. Equivalent protein loading was ensured by (i) triplicate BCA quantification prior to electrophoresis; (ii) loading of equal protein amounts (30 μ g per lane); (iii) Ponceau S staining of membranes after transfer to visually verify consistent lane loading and uniform transfer efficiency, followed by destaining prior to antibody incubation; and (iv) simultaneous processing of all parallel membranes under identical transfer and incubation conditions. Densitometric analysis was performed using ImageJ software.

2.10 Quantitative Real-Time PCR (qRT-PCR)

Total RNA was separately isolated from clinical paraffin-embedded tissue specimens and cultured HeLa cells using matched commercial extraction kits. Specifically, the RNeasy Pure FFPE Kit was used for tissue RNA extraction, whereas the RNA Easy Fast Total RNA Extraction Kit (DP439 and DP451, TIANGEN, Beijing, China) was used for cellular samples. Reverse transcription for cDNA synthesis was then performed with the FastKing RT Kit (KR116, TIANGEN, Beijing, China). Quantitative real-time PCR reactions were prepared with SuperReal PreMix Plus (SYBR Green) (FP205, TIANGEN, Beijing, China). The relative expression levels of nine target genes, including *KLF4*, *SERPINF1*, *CD74*, *PRPS2*, *ERRF1*, *FSTL3*, *SOD2*, *JUN*, and *CXCL3*, were quantified using $2^{-\Delta\Delta C_t}$. β -Actin was used as a housekeeping reference gene to normalize expression. All primer sequences are presented in **Supplementary Table 4**.

2.11 Clonogenic Survival Assay

HeLa and SiHa cells stably transfected with NC and *shFSTL3* vectors were inoculated into 6-well plates at den-

sities 2000 cells per well and incubated overnight to allow for complete cell adherence. Cells were divided into four groups: untreated NC, untreated *shFSTL3*, NC/IR/DDP, and *shFSTL3*/IR/DDP. Cells were then exposed to X-ray irradiation at incremental doses of 0 Gy or 6 Gy alone or in combination with cisplatin (DDP) at a concentration of 10 μ M. After continuous incubation for 12 days, visible cell colonies were fixed with methanol and stained using 0.5% crystal violet. Colonies containing more than 50 cells were manually screened and counted. Cell survival fractions were calculated based on the relative colony formation rate compared with the non-irradiated control group. All functional assays in this study were performed with three independent biological replicates.

2.12 Cell Viability Assay

HeLa and SiHa cells were separated into four treatment groups: (1) NC, (2) *shFSTL3*, (3) NC with irradiation (IR) and cisplatin treatment (DDP), and (4) *shFSTL3* with IR and DDP. Cells were plated at 5×10^3 per well in 96-well plates and incubated overnight for adherence. In the combined treatment groups, cells were subjected to 6 Gy X-ray irradiation followed by exposure to 10 μ mol/L DDP, which remained in the culture medium for the entire incubation period. Cell Counting Kit-8 (CCK-8) reagent (10 μ L) was added to appropriate wells at 24 and 48 hours post-treatment. Analyses were independently repeated in triplicate, with three technical replicates per group. Absorbance at 450 nm in each well was then analyzed with a standard microplate reader.

2.13 Wound Healing Assay

Cell migration capacity was evaluated using a wound healing assay. HeLa and SiHa cells stably transfected with NC and *shFSTL3* vectors were seeded into 6-well plates at 5×10^5 cells per well. After cell adherence, cells were divided into four groups: NC, *shFSTL3*, NC/IR/DDP, and *shFSTL3*/IR/DDP. Only the NC/IR/DDP and *shFSTL3*/IR/DDP groups were subjected to 6 Gy X-ray irradiation and 10 μ mol/L DDP treatment, whereas the NC and *shFSTL3* groups received no irradiation or DDP treatment. A sterile pipette tip was used to generate a uniform straight scratch wound in the fully confluent cell monolayer. Floating, detached cells were washed away with PBS, and serum-free culture medium was then added into each well.

Cells were continuously cultured under conventional incubation conditions, and microscopic images of the scratch wound area were captured at 0 h and 48 h. The degree of wound healing in each group was quantitatively analyzed using the ImageJ software. Specifically, five random visual fields were selected per group, and all experiments were repeated three times as independent biological replicates to ensure the reliability of the results. Representative images of the five randomly selected visual fields per well for the wound healing assay at 0 h and 48 h are provided in **Supplementary Fig. 5**.

2.14 Transwell Assay

HeLa and SiHa cells stably transfected with NC and *shFSTL3* vectors were inoculated at 2×10^4 cells/well into the upper chambers of Matrigel-precoated Transwell inserts. After cell adherence, cells were divided into four groups: NC, *shFSTL3*, NC/IR/DDP, and *shFSTL3*/IR/DDP. Only the NC/IR/DDP and *shFSTL3*/IR/DDP groups were subjected to 6 Gy X-ray irradiation and 10 $\mu\text{mol/L}$ DDP treatment, whereas the NC and *shFSTL3* groups received no irradiation or DDP treatment. The upper chamber was filled with serum-free medium, while the lower chamber was filled with serum-containing complete medium to induce cell invasion.

After 48 hours of culture, residual non-invasive cells attached to the upper membrane surface were thoroughly removed via PBS rinsing. The invasive cells that successfully penetrated the Matrigel matrix were fixed with 4% paraformaldehyde for 15 minutes and stained with a 0.1% crystal violet solution. For quantitative analysis, five randomly selected microscopic fields from each Transwell insert were captured and analyzed using ImageJ, with all assays conducted in three independent biological replicates. Representative images of the five randomly selected fields per Transwell insert are provided in **Supplementary Fig. 6**.

2.15 In Vivo Xenograft Tumor Model

Six-week-old female BALB/c nude mice (BALB/c-nu) were obtained from the Animal Experimental Center of Xi'an Jiaotong University (Xi'an, Shaanxi, China). All experimental mice were raised in a specific pathogen-free (SPF) barrier system with standardized environmental parameters, including a 12-hour light-dark cycle, a constant ambient temperature of $22 \pm 2^\circ\text{C}$, and a relative humidity of 50% to 60%. All mice were provided with ad libitum access to standard laboratory feed and sterile drinking water. Prior to formal experimental manipulation, all animals were adaptively raised for one week to acclimatize to the breeding environment.

No experimental animals were excluded from any experiments. The exact sample size in each group is indicated in the corresponding Figure Legends and experimental descriptions. Inclusion and exclusion criteria were defined prior to the commencement of experiments. Experimental animals were randomly assigned to groups using random number sequences generated in GraphPad Prism. Animal cage positions were rotated daily, and sample testing order was randomized to eliminate confounding factors such as housing location or measurement sequence.

The mice were randomly assigned to four groups ($n = 5/\text{group}$): (a) HeLa/NC, (b) HeLa/*shFSTL3*, (c) HeLa/NC with IR and DDP, and (d) HeLa/*shFSTL3* with IR and DDP. Treatment administration (irradiation and cisplatin injection) was performed according to this randomization scheme by personnel separate from those conducting tumor

measurements and data analyses, who remained blinded to group assignments. Each mouse received a subcutaneous injection of 5×10^6 HeLa-NC or HeLa-*shFSTL3* cells in 100 μL PBS in the right flank. After tumors had grown to a volume of approximately 500 mm^3 (~14 days), groups (c) and (d) were subjected to weekly irradiation (6 Gy) and 2 mg/kg injections of DDP.

Tumors were measured at three-day intervals throughout the experimental period, and tumor volume was calculated using the standard formula: $(\text{length} \times \text{width}^2) / 2$. Following three weeks of continuous treatment, all experimental mice were sacrificed via cervical dislocation. Subcutaneous tumor tissues were subsequently dissected, weighed, and photographed for phenotypic observation. All animal experimental protocols implemented in this study were formally approved by the Biomedical Ethics Committee of Health Science Center of Xi'an Jiaotong University. All *in vivo* xenograft experiments adhered to the ARRIVE 2.0 guidelines and institutional animal care ethics standards.

2.16 Statistical Analysis

All statistical analyses were performed using R software (version 4.4.2, R Foundation for Statistical Computing, Vienna, Austria) and GraphPad Prism (version 9.0, GraphPad Software, San Diego, CA, USA). Normality of continuous variables was assessed using the Shapiro-Wilk test, and homogeneity of variances was evaluated using the F-test. All data are presented as mean $\pm$ SD from at least three independent biological replicates unless otherwise specified. Effect sizes with 95% confidence intervals were calculated for intergroup comparisons, and a two-sided $p < 0.05$ was considered statistically significant.

2.16.1 For In Vitro Functional Assays

CCK-8 proliferation data were analyzed using two-way analysis of variance (ANOVA) with Tukey's post-hoc test to evaluate the effects of treatment, time, and their interaction. Comparisons of multiple groups in Transwell invasion, wound-healing migration, and clonogenic survival assays were performed using one-way ANOVA followed by Tukey's post-hoc test. Pairwise comparisons between two independent groups were conducted using unpaired two-tailed Student's *t*-test.

2.16.2 For In Vivo Xenograft Studies

Longitudinal tumor volume data over time were analyzed using linear mixed-effects models, with group, time, and their interaction as fixed effects and mouse identity as a random intercept. Pairwise post-hoc comparisons were adjusted using Tukey's method. Endpoint tumor weights among the four groups were compared using one-way ANOVA with Tukey's post-hoc test.

2.16.3 For Prognostic Model Development and Validation

Gene selection for the risk signature was performed using LASSO-Cox regression with ten-fold cross-validation. The proportional hazards assumption was verified using Schoenfeld residual tests (global $p > 0.05$). Internal validation was conducted via bootstrap resampling (1000 iterations) to derive optimism-corrected C-indexes. Survival differences between risk groups (OS in TCGA and GSE52903; DFS in GSE44001) were assessed using Kaplan–Meier curves with the log rank test. Time-dependent ROC curves, calibration plots, and DCA were employed to evaluate model discrimination, calibration, and clinical net benefit.

2.16.4 For Clinical Sample Validation

Correlations between protein expression (mIHC) and mRNA expression (qRT-PCR) for the nine signature genes were evaluated using Spearman's rank correlation coefficients.

3. Results

3.1 Cervical Cancer Cell Subtyping and Marker Gene Annotation

To investigate molecular markers associated with cervical cancer development and prognosis, we began by analyzing the GSE197461 scRNA-seq dataset from the GEO database, which included eight tumor tissue samples from distinct patients. After processing gene expression matrices using the Seurat package and performing rigorous quality control across multiple samples, we excluded low-quality cells and cells with fewer than 200 detected genes. Doublets were further removed using the DoubletFinder package, yielding a final set of 21,518 high-quality cells for subsequent analysis. Cell filtering results were then visualized using violin and scatter plots (**Supplementary Fig. 1A,B**), and the top 10 genes exhibiting the highest standard deviation values are presented in **Supplementary Fig. 1C**.

Using the ElbowPlot method, 20 was identified as the optimal number of principal components (PCs) (**Supplementary Fig. 1D**). PC analysis identified batch effects among samples (**Supplementary Fig. 1E**), prompting the use of the Harmony algorithm for data integration and correction (**Supplementary Fig. 1F**). Dimensionality reduction and clustering performed via a UMAP approach revealed 11 distinct cell subpopulations (Fig. 1A). These subpopulations were systematically annotated and categorized into 10 major cell types based on classic cell marker gene expression patterns: fibroblasts, pericytes, macrophages, CD4⁺ T cells, CD8⁺ T cells, B cells, plasma cells, epithelial cells, mast cells, and endothelial cells (Fig. 1B). To validate cell type annotation reliability, a bubble plot was generated to visualize expression levels and proportions of cells positive for canonical marker genes (e.g., *CD3D*, *CD4*, *CD8A*, *MS4A1*, *S100A9*, *ACTA2*, *VWF*, etc.) across different cell types (Fig. 1C). *CD3D* and *CD4* served as markers for

CD4⁺ T cells, *CD8A* for CD8⁺ T cells, *MS4A1* and *CD79A* for B cells, *S100A9* and *CIQB* for macrophages, *ACTA2* for fibroblasts, *RGS5* for pericytes, and *VWF* and *PECAM1* for endothelial cells. Fig. 1D illustrates the proportional distribution of different cell types across sample groups, revealing notable differences in the abundances of certain immune and stromal cell populations between the Adenocarcinoma (ADC) and Squamous Cell Carcinoma (SCC) groups. This suggests that the histological background may influence the cellular composition of the TME. In summary, systematic scRNA-seq analysis was herein used to successfully establish a cellular atlas for the cervical cancer-associated TME, identifying 11 cell subpopulations belonging to 10 major cell types. This atlas provides a sound foundation for further efforts to elucidate the heterogeneity that characterizes cervical cancer and the underlying molecular mechanisms.

3.2 Ketone Body Metabolism is Specifically Enriched in Cancer-Associated Fibroblasts

To systematically investigate the potential role of ketone body metabolism in the cervical cancer-associated TME, we first compiled a gene set related to this metabolic pathway through a search of the relevant literature [14]. We then integrated five computational algorithms (AUCell, UCell, singscore, ssGSEA, and AddModuleScore) to comprehensively evaluate the activity of this metabolic program across different cell types at single-cell resolution. By aggregating normalized scores from these algorithms, we found that fibroblasts exhibited the highest levels of ketone body metabolic activity, thus identifying them as the key cellular subset for further investigation. Notably, sensitivity analyses using each of the five algorithms independently confirmed that fibroblasts consistently exhibited the highest ketone-body metabolic activity across all scoring methods (**Supplementary Table 1**), supporting the robustness of our metabolic activity classification. Based on median ketone body metabolism scores, we stratified fibroblasts into high-activity (Hexp_Fibroblasts) and low-activity (Lexp_Fibroblasts) subgroups. Differential expression analysis identified 109 significantly differentially expressed genes between these two subgroups (Fig. 2A), which were retained as a candidate gene set for subsequent analyses. We utilized the CellChat package to analyze ligand-receptor interactions to assess functional differences in cell-cell communication between these two subgroups. This approach revealed that Hexp_Fibroblasts exhibited markedly enhanced communication capabilities, with both outgoing and incoming interaction strengths substantially higher than those of Lexp_Fibroblasts (Fig. 2B), indicating that these cells play a more active role within the TME signaling network. Further visualization revealed distinct ligand-receptor expression patterns in key signaling pathways between the two subgroups (Fig. 2C). To further elucidate the transcriptional regulatory mechanisms underlying these metabolic differences, we subjected the fibrob-

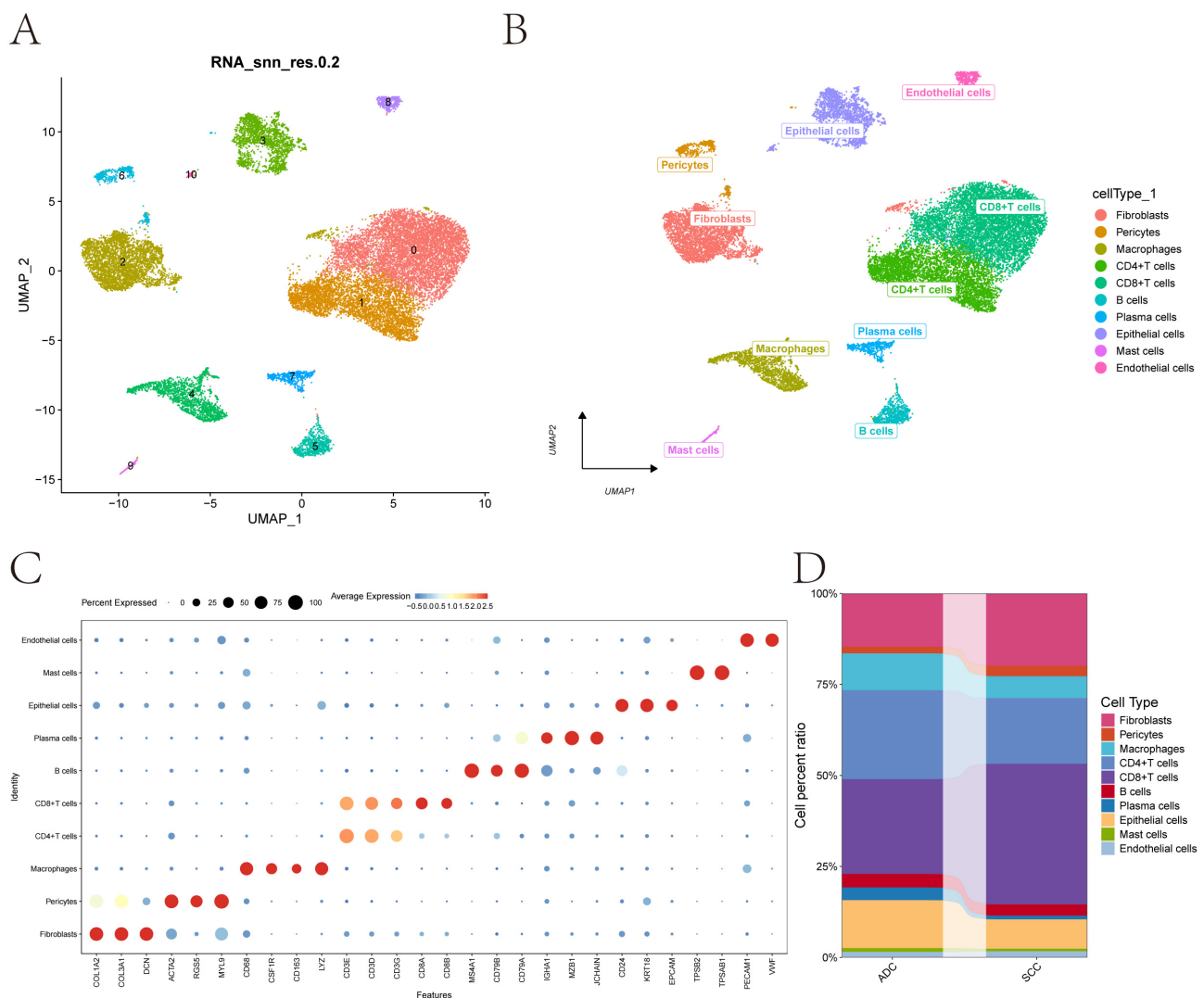


Fig. 1. Clustering and annotation of single-cell RNA sequencing data from cervical cancer patients. Uniform Manifold Approximation and Projection (UMAP) clustering visualizations depict: (A) The cell type landscape; (B) Cell annotations; (C) Expression patterns of lineage-specific marker genes, with darker red corresponding to elevated gene expression levels, and larger circle sizes indicating a greater number of gene-expressing cells; and (D) cell type distributions across sample groups.

last population to SCENIC analysis, revealing pronounced differences in transcription factor regulatory networks between Hexp_Fibroblasts and Lexp_Fibroblasts (Fig. 2D). A substantial number of regulons exhibited elevated activity levels in Hexp_Fibroblasts, indicating a more active transcriptional network. Through a Regulon Specificity Score (RSS) analysis, we identified several transcription factors—including *JUNB*, *FOS*, *EGR1*, *ATF3*, and *FOSB*—that were specifically enriched in Hexp_Fibroblasts (Fig. 2E). Notably, these factors are primarily members of the *AP-1* family or are associated with stress response and metabolic regulation, suggesting their potential role in mediating ketone body metabolism-related signaling and functional specialization in fibroblasts. Taken together, our findings indicate that fibroblasts represent the primary cell type with the highest enrichment of ketone-body-

metabolism-related transcriptional signatures in the cervical cancer TME. Those fibroblasts with high levels of metabolic activity exhibit an enhanced capacity for communication together with a unique transcriptional regulatory landscape, providing new insights into how metabolic heterogeneity influences the TME.

3.3 Establishment of a Prognostic Gene Signature Related to Ketone Body Metabolism in Cancer-Associated Fibroblasts

We next examined the link between transcription factors and ketone body metabolism in CAFs and sought to identify prognostic molecular markers by integrating clinical data from cervical cancer patients in the TCGA database, focusing on 109 previously identified candidate genes. Univariate Cox regression analysis identified 22

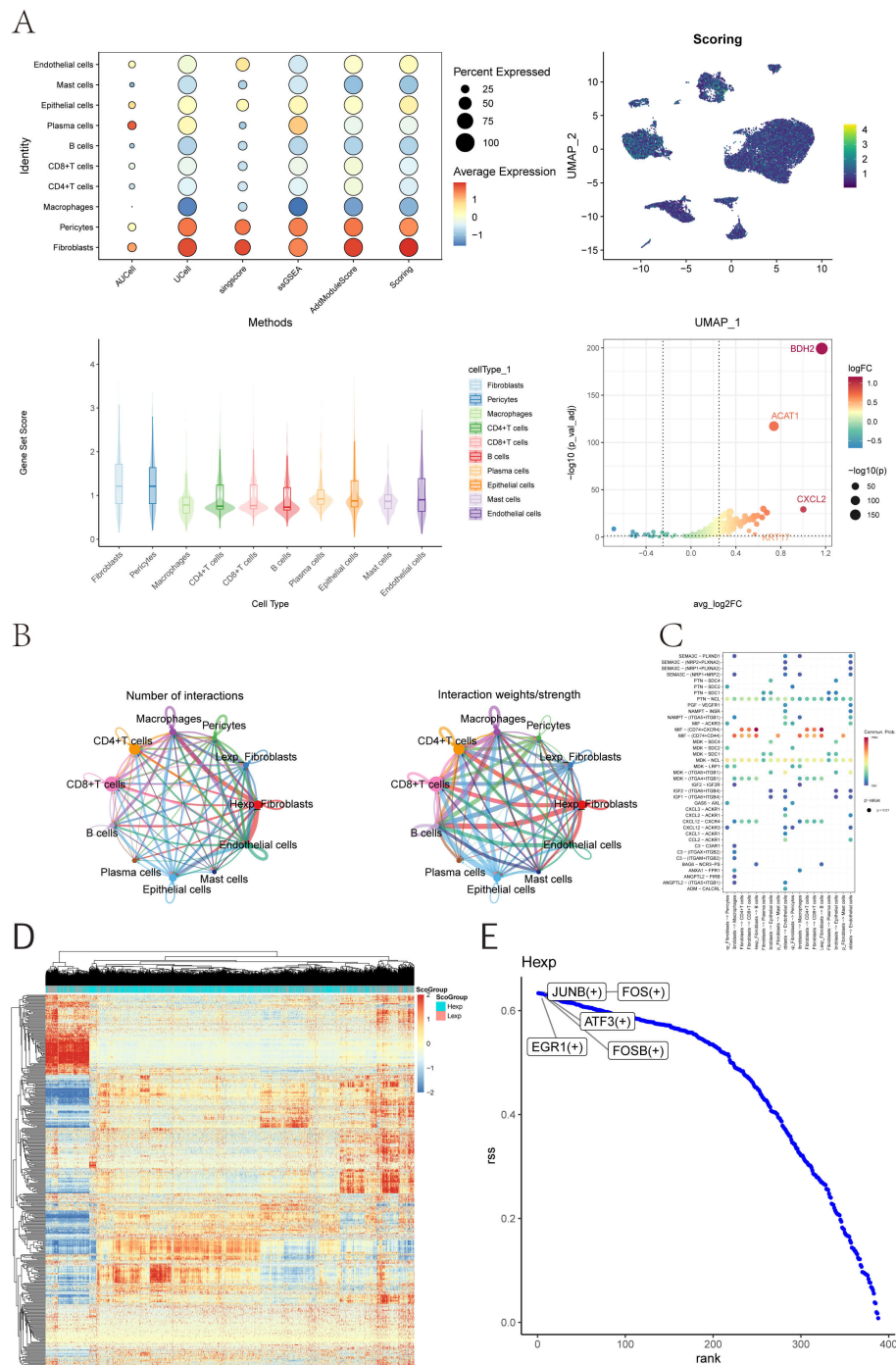


Fig. 2. Analysis of ketone body metabolism-related activity in cancer-associated fibroblasts (CAFs). (A) Differential expression analysis plot: Fibroblasts were divided into high- and low-activity subgroups based on ketone body metabolism activity; 109 significantly differentially expressed genes were screened as a candidate gene set for subsequent analysis. (B) CellChat analysis results showing that the bidirectional interaction strength of high-activity fibroblasts was markedly higher than that for the low-activity subgroup, consistent with a more active role in tumor microenvironment (TME) signaling network. (C) Ligand-receptor expression pattern plot of key signaling pathways, demonstrating clear differences in expression between the two fibroblast subgroups in core pathways. (D) SCENIC analysis plot presenting pronounced differences in transcription factor regulatory networks between the two fibroblast subgroups, with elevated activity of numerous regulons in the high-activity subgroup. (E) Regulon Specificity Score (RSS) analysis plot identifying transcription factors specifically enriched in high-activity fibroblasts.

genes significantly associated with overall survival ($p < 0.05$; Fig. 3A). We then utilized a LASSO regression approach for feature selection to build a robust prognostic model. The TCGA cervical cancer dataset was split into training and validation sets in equal proportions. Following LASSO regression analysis (Fig. 3B–D), a nine-gene risk score model was established with the following formula:

Risk Score = $KLF4 \times (-0.423) + SERPINF1 \times (-0.255) + CD74 \times (-0.248) + PRPS2 \times (-0.082) + ERRFI1 \times (0.073) + FSTL3 \times (0.163) + SOD2 \times (0.231) + JUN \times (0.294) + CXCL3 \times (0.331)$. The training cohort consisted of 145 patients with 31 (21.4%) death events, and the validation cohort comprised 146 patients with 40 (27.4%) death events. Schoenfeld residual analysis confirmed that the proportional hazards assumption was met (global $p = 0.40$). Bootstrap internal validation with 1000 resamples yielded an optimism-corrected C-index of 0.73 (95% CI: 0.66–0.79), suggesting that the model maintained reasonable discriminative ability without substantial overfitting. Patients were stratified into high- and low-risk groups using the optimal cutoff determined by maximally selected rank statistics (`surv_cutpoint` function, `survminer` package); the cutoff was derived from the training cohort and then applied to the validation cohort. Kaplan-Meier survival curves revealed that patients assigned to the high-risk subgroup possessed remarkably shorter overall survival than their low-risk counterparts within both training and validation cohorts (Fig. 3E,F). Moreover, ROC curve analysis demonstrated consistent predictive accuracy of the model in both cohorts (Fig. 3G,H). These findings suggest that the risk score model, derived from genes linked to ketone body metabolism in TME-associated fibroblasts, may offer potential as a prognostic biomarker for cervical cancer patients. This model also provides a valuable reference for clinical risk stratification that may inform the formulation of individualized treatment strategies.

3.4 External Validation of Prognostic Model Performance

To further validate the accuracy of the established prognostic model, we downloaded processed cervical cancer patient data with available survival information from the GEO database (GSE44001, GSE52903). Patients in the GEO datasets were categorized into risk groups using the model, and Kaplan-Meier analysis was used to compare survival differences between these groups. In both external validation datasets, the high-risk group consistently exhibited significantly worse survival outcomes than the low-risk group (disease-free survival in GSE44001 and overall survival in GSE52903; Fig. 4A,B). ROC curve analysis confirmed the model's modest prognostic performance in these cohorts (Fig. 4C,D). Based on these results, nine model genes were retained for subsequent analysis, including *KLF4*, *SERPINF1*, *CD74*, *PRPS2*, *ERRFI1*, *FSTL3*, *SOD2*, *JUN*, and *CXCL3*.

3.5 Development and Validation of a Predictive Nomogram for Cervical Cancer

To assess the clinical utility of the constructed risk model for prognostic evaluation in cervical cancer patients, we combined the risk score with key clinicopathological features, including age, grade, and TNM stage, to develop a nomogram for OS prediction (Fig. 5A). Age, grade, T stage, N stage, and risk score were included as independent predictors in the final model, with each having a unique contribution weight, highlighting the complementary and additive value of integrating clinical variables with molecular characteristics for prognostic prediction. Calibration curves were then used to evaluate the predictive accuracy of this nomogram when assessing 3- and 5-year OS. The results demonstrated strong alignment between predicted and observed survival probabilities, confirming the model's excellent calibration and clinical applicability (Fig. 5B). Time-dependent ROC curves were generated to assess the model's discriminative performance, confirming stable predictive performance based on respective 1-, 3-, and 5-year OS AUC values of 0.7482, 0.7926, and 0.7891 (Fig. 5C).

Model utility in the context of clinical decision-making was next evaluated through a DCA approach, which revealed that the combination model incorporating the risk score outperformed individual clinical variables and the 'treat-all' or 'treat-none' strategies in terms of net benefit across various threshold probabilities (Fig. 5D), suggesting its potential utility as a means of aiding risk stratification and treatment decision-making for cervical cancer patients.

3.6 Expression Patterns and Functional Activity of Model Genes at Single-Cell Resolution

We next conducted a comprehensive analysis of single-cell transcriptomic data from cervical cancer to characterize the expression patterns of the nine prognostic model genes identified above (*KLF4*, *SERPINF1*, *CD74*, *PRPS2*, *ERRFI1*, *FSTL3*, *SOD2*, *JUN*, and *CXCL3*). This approach revealed unique expression profiles for these model genes across 10 primary cell types, including fibroblasts, pericytes, macrophages, CD4+ T cells, CD8+ T cells, B cells, plasma cells, epithelial cells, mast cells, and endothelial cells (Fig. 6A,B). We additionally applied the AUCell algorithm to quantify the activity of Hallmark pathway gene sets and visualized the activity of key genes across different pathways using a heatmap. This analysis revealed notable differences in pathway activity among the model genes. *CXCL3*, *ERRFI1*, *FSTL3*, *KLF4*, *PRPS2*, *SERPINF1*, and *SOD2* exhibited increased activity in epithelial-mesenchymal transition (EMT) pathways, while Jun proto-oncogene (*JUN*) was particularly active in the Tumor Necrosis Factor-alpha (TNF- α) signaling via Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF- κ B) pathway, and *CD74* showed heightened activity in pathways related to allograft rejection (Fig. 6C). This suggests that these candidate genes may jointly influence

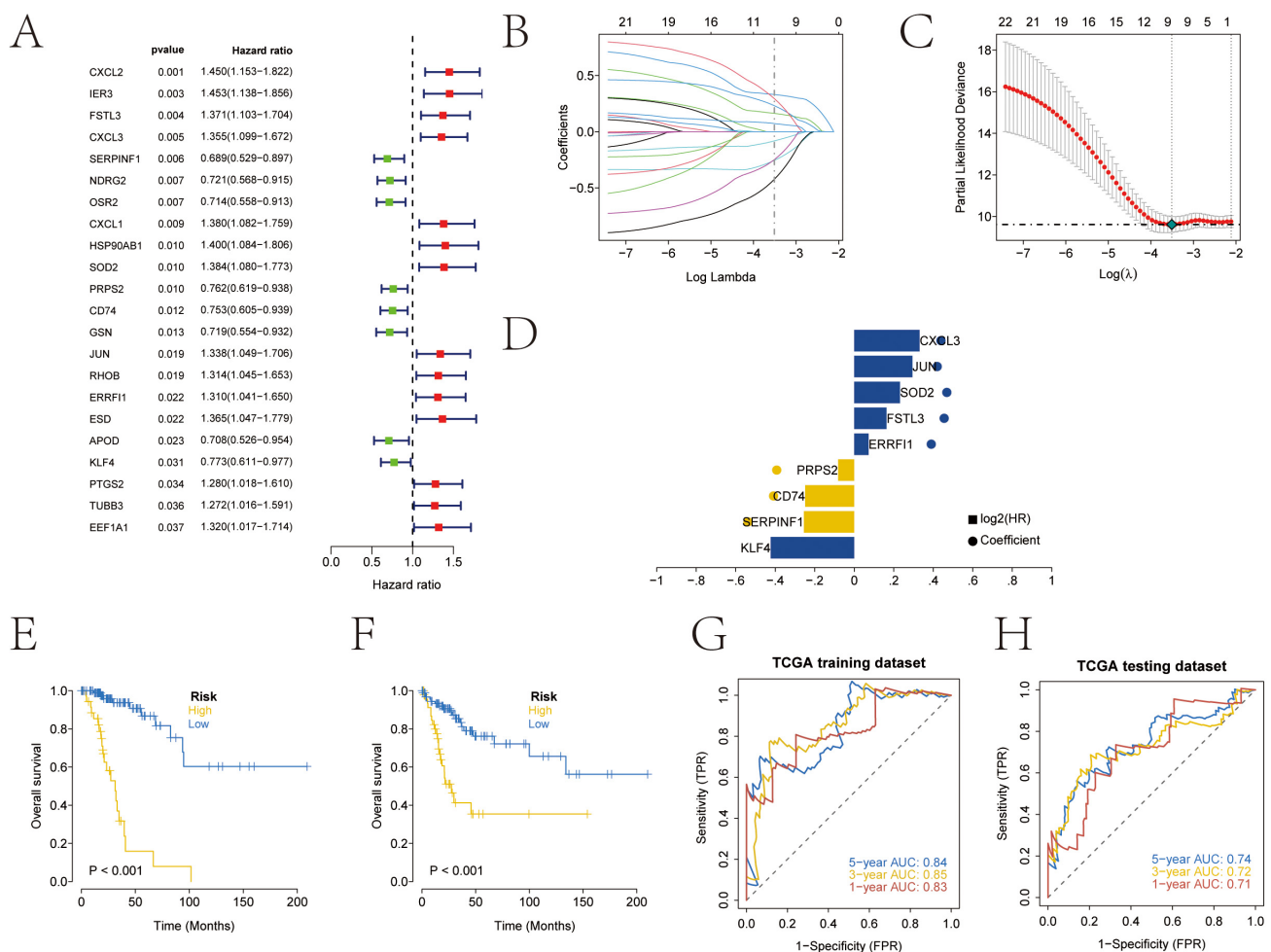


Fig. 3. Establishment of prognostic gene signature related to ketone body metabolism in cancer-associated fibroblasts. A range of methods was used to assess associations between gene expression and overall survival (OS) in The Cancer Genome Atlas (TCGA) cervical cancer dataset. (A) Univariate Cox regression analyses identified a total of 22 genes that were significantly correlated with patient OS ($p < 0.05$). (B–D) LASSO regression was employed for feature selection in the training and validation cohorts (split 1:1), yielding a 9-gene risk score model. (E) Kaplan-Meier curves showing overall survival of high- versus low-risk groups in the training cohort ($n = 145$). Median survival time: high-risk group ($n = 39$, events = 21), 31.3 months (95% CI: 19.9–39.7); low-risk group ($n = 106$, events = 10), not reached. HR = 4.296 (95% CI: 2.139–8.628); log-rank $p < 0.001$. (F) Kaplan-Meier curves showing overall survival of high- versus low-risk groups in the validation cohort ($n = 146$). Median survival time: high-risk group ($n = 51$, events = 22), 27.4 months (95% CI: 17.1–NA); low-risk group ($n = 95$, events = 18), not reached. HR = 3.568 (95% CI: 1.903–6.689); log-rank $p < 0.001$. (G,H) Receiver operating characteristic (ROC) curve analysis confirming the stable predictive performance of the model across cohorts, highlighting its potential as a prognostic biomarker for cervical cancer. In (E,F), survival differences between risk groups were assessed using the log-rank test. In (G,H), time-dependent ROC curves were generated to evaluate predictive accuracy.

cervical cancer progression and prognosis by coordinating metabolic-immune interactions among various cell types within the TME.

3.7 Validation of Candidate Gene Expression in Clinical Tissue Specimens

To confirm the association between the 9-gene signature and prognosis in patients undergoing CCRT, we analyzed paraffin-embedded tissue sections from 30 individuals, evaluating gene expression via mIHC. Quantitative analysis of the percentage of positive cells revealed that

patients who exhibited a PoR to treatment exhibited significantly higher expression levels of SOD2 ($p < 0.001$), CXCL3 ($p < 0.001$), JUN ($p < 0.001$), CD74 ($p < 0.001$) and FSTL3 ($p < 0.001$), along with significantly lower expression of KLF4 ($p < 0.001$), compared with the GR group. In contrast, patients with a GR presented with low levels of SOD2, CXCL3, JUN, CD74, and FSTL3, together with high KLF4 expression. No significant differences were detected for SERPINF1 ($p = 0.81$), PRPS2 ($p = 0.07$), or ERRFI1 ($p = 0.62$) between the prognostic groups (Fig. 7A,B and Supplementary Fig. 7). The complete quan-

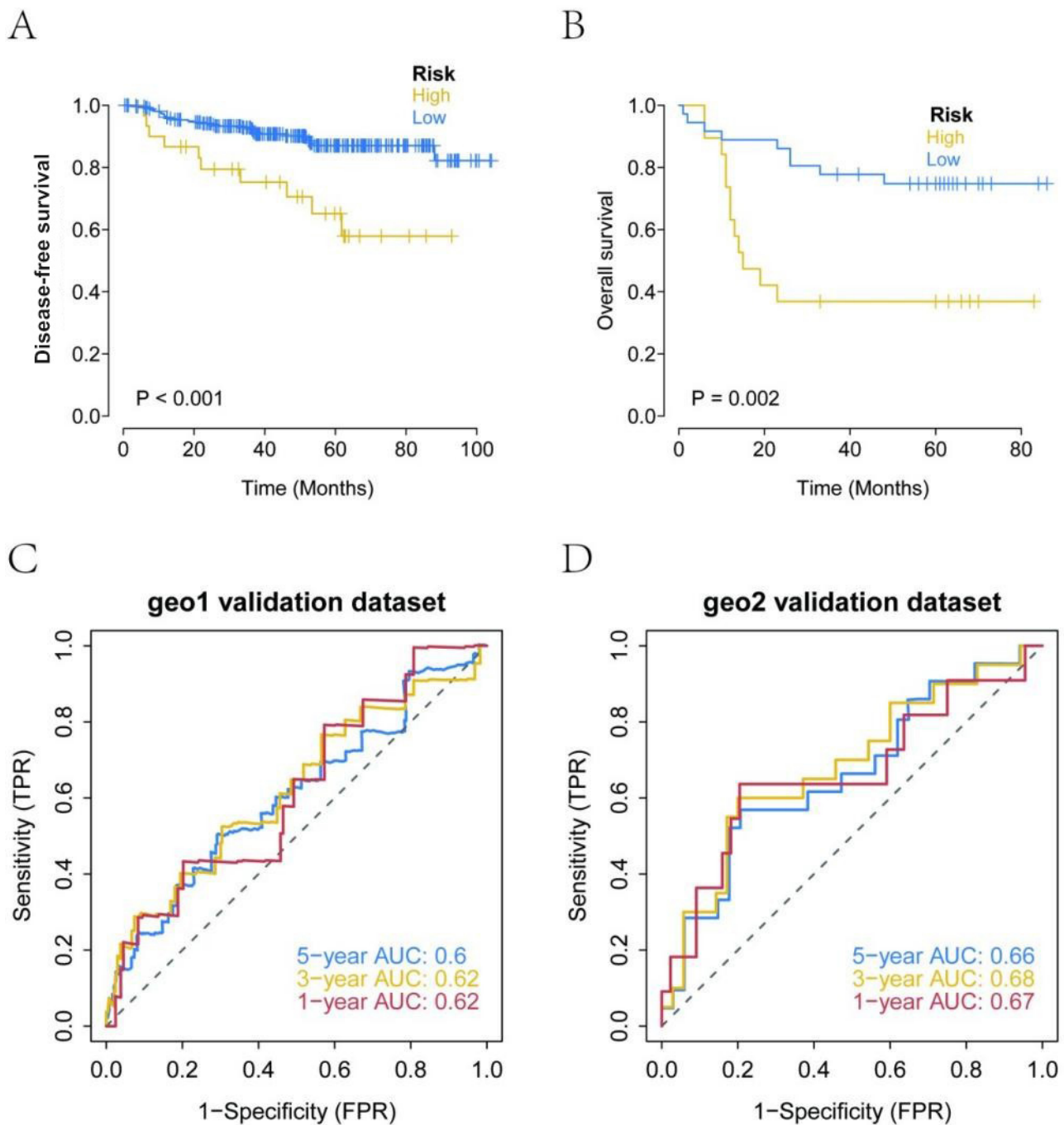


Fig. 4. External validation of the cervical cancer prognostic model and identification of model genes. (A) Kaplan-Meier curves showing disease-free survival (DFS) of high- versus low-risk groups in the GSE44001 cohort ($n = 300$). Median DFS was not reached in either group (high-risk: $n = 37$, events = 11; low-risk: $n = 263$, events = 27). HR = 3.33 (95% CI: 1.65–6.71); log-rank $p < 0.001$. (B) Kaplan-Meier curves showing overall survival (OS) of high- versus low-risk groups in the GSE52903 cohort ($n = 55$). Median survival time: high-risk group ($n = 19$, events = 12), 15.0 months (95% CI: 12.0–NA); low-risk group ($n = 36$, events = 9), not reached. HR = 3.744 (95% CI: 1.584–8.849); log-rank $p = 0.002$. (C,D) ROC curve analysis demonstrating the model's modest prognostic performance and accuracy.

titative data for each patient, including the percentage of positive cells for all nine markers across the 30 tissue samples, are provided in **Supplementary Table 5**. Generally concordant trends were obtained through qRT-PCR analy-

sis of the same set of paraffin-embedded tissues (Fig. 7C and **Supplementary Table 6**). Specifically, qRT-PCR confirmed that *SOD2* ($p < 0.001$), *CXCL3* ($p < 0.001$), *JUN* ($p < 0.001$), *CD74* ($p < 0.001$), and *FSTL3* ($p < 0.001$) were

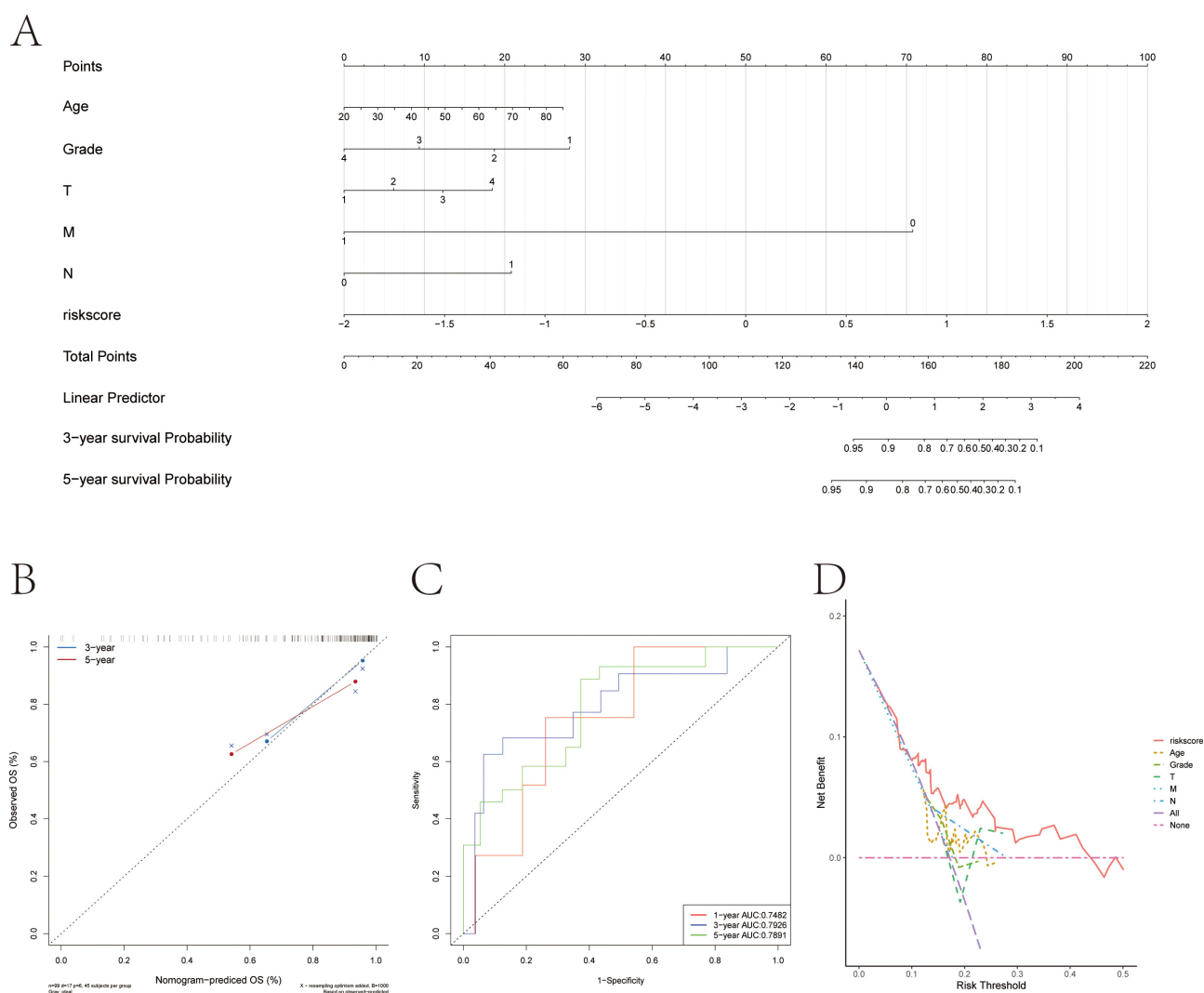


Fig. 5. Development and validation of a prognostic nomogram for cervical cancer. (A) A prognostic nomogram was developed that combines risk scores with clinicopathological features including age, grade, and TNM stage. (B) Calibration curves were generated, revealing strong agreement between predicted and actual 3-year and 5-year overall survival (OS) probabilities. (C) Time-dependent ROC curves with corresponding AUC values of 0.7482, 0.7926, and 0.7891 for 1-year, 3-year, and 5-year OS, respectively. (D) Decision curve analysis (DCA) revealing that the combination model offers greater net benefit compared to individual clinical variables and the ‘treat-all’ and ‘treat-none’ strategies.

significantly upregulated in the PoR group compared with the GR group, while *KLF4* ($p < 0.001$) was significantly downregulated. No significant differences were detected for *SERPINF1* ($p = 0.577$), *PRPS2* ($p = 0.650$), or *ERRFII* ($p = 0.868$) at the mRNA level. These results indicate that elevated *SOD2*, *CXCL3*, *JUN*, *CD74*, and *FSTL3* expression, combined with reduced expression of *KLF4*, is associated with poor therapeutic response and prognosis in cervical cancer patients undergoing CCRT treatment.

3.8 *FSTL3* Facilitates Cervical Cancer Development and Progression

Previous studies have reported that high levels of *SOD2* [15], *CXCL3* [16], *JUN* [17], and *CD74* [18] expres-

sion, as well as low *KLF4* [19] expression, are associated with cervical cancer therapeutic responses. Based on our earlier screening results, which also linked *FSTL3* to poor treatment outcomes in cervical cancer, we further investigated its specific functional role.

First, we knocked down *FSTL3* expression in HeLa using a lentivirus-mediated approach (Fig. 8A,B; **Supplementary Fig. 2**) and evaluated cell proliferation. In HeLa cells, two-way ANOVA revealed significant effects of treatment ($p < 0.001$), time ($p < 0.001$), and their interaction ($p = 0.035$) on cell viability. *FSTL3* knockdown significantly reduced cell proliferation compared with the NC group at both 24 h ($p = 0.037$) and 48 h ($p = 0.0004$). In the combined treatment setting, sh*FSTL3*/IR/DDP further enhanced the

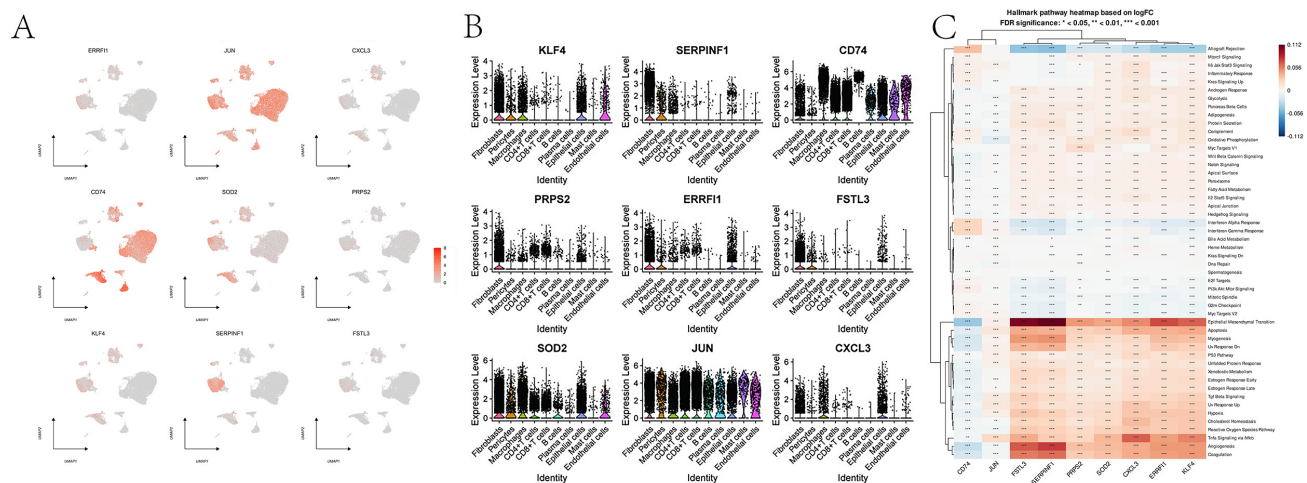


Fig. 6. Expression patterns and functional activity of model genes at single-cell resolution. (A) UMAP plots showing the expression distribution of the nine model genes across single cells. (B) Violin plots showing the expression levels of the nine model genes across 10 primary cell types in cervical cancer single-cell transcriptomic data. (C) A heatmap illustrating Hallmark pathway activity (based on logFC, with FDR significance) of the nine model genes, analyzed using the AUCell algorithm.

anti-proliferative effect compared with NC/IR/DDP at 24 h ($p = 0.039$) and 48 h ($p = 0.008$) (Fig. 8C). Furthermore, Transwell invasion assays revealed that *FSTL3* knockdown markedly reduced the invasive capability of these cells (Fig. 8D; **Supplementary Fig. 6**). One-way ANOVA revealed a significant difference in invasive cell numbers among the four groups ($p < 0.001$). *FSTL3* knockdown significantly reduced invasion compared with the NC group ($p = 0.0001$). In the combined treatment setting, sh*FSTL3*/IR/DDP further reduced invasion compared with sh*FSTL3* alone ($p = 0.0003$). In addition, wound-healing assays demonstrated that *FSTL3* knockdown suppressed cell migration (Fig. 8E; **Supplementary Fig. 5**). One-way ANOVA revealed a significant difference in wound closure among the four groups ($p < 0.001$). *FSTL3* knockdown significantly reduced cell migration compared with the NC group ($p = 0.007$). In the combined treatment setting, sh*FSTL3*/IR/DDP further reduced migration compared with sh*FSTL3* alone ($p < 0.001$) and compared with NC/IR/DDP alone ($p = 0.001$). These results suggest that *FSTL3* enhances cervical cancer cell proliferation, migration, and invasion, potentially affecting treatment outcomes.

To exclude any cell line-specific phenotypic bias and to validate the general biological function of *FSTL3*, additional functional validation experiments were performed in SiHa cells. Both qRT-PCR and Western blotting confirmed the efficient knockdown of *FSTL3* in these cells (**Supplementary Fig. 3A,B**; **Supplementary Fig. 4**). Consistent with the phenotypic alterations observed in HeLa cells, *FSTL3* knockdown substantially suppressed the proliferative, migratory, and invasive abilities of SiHa cells (**Supplementary Fig. 3C–E**; **Supplementary Fig. 5** and **Supplementary Fig. 6**). In SiHa cells, two-way ANOVA revealed significant effects of treatment ($p <$

0.001), time ($p < 0.001$), and their interaction ($p < 0.001$) on cell viability. *FSTL3* knockdown significantly reduced cell proliferation compared with the NC group at 24 h ($p = 0.0003$) and 48 h ($p < 0.001$). In the combined treatment setting, sh*FSTL3*/IR/DDP further enhanced the anti-proliferative effect compared with NC/IR/DDP at 24 h ($p < 0.001$) and 48 h ($p < 0.001$) (**Supplementary Fig. 3C**). Furthermore, Transwell invasion assays revealed that *FSTL3* knockdown significantly reduced the invasive capability of SiHa cells (**Supplementary Fig. 3D**). One-way ANOVA revealed a significant difference in invasive cell numbers among the four groups ($p < 0.001$). *FSTL3* knockdown significantly reduced invasion compared with the NC group ($p = 0.015$). In the combined treatment setting, sh*FSTL3*/IR/DDP further reduced invasion compared with sh*FSTL3* alone ($p = 0.039$). (**Supplementary Fig. 3D**). In addition, wound-healing assays demonstrated that *FSTL3* knockdown significantly suppressed cell migration in SiHa cells (**Supplementary Fig. 3E**). One-way ANOVA revealed a significant difference in wound closure among the four groups ($p < 0.001$). *FSTL3* knockdown significantly reduced cell migration compared with the NC group ($p = 0.002$). In the combined treatment setting, sh*FSTL3*/IR/DDP further reduced migration compared with sh*FSTL3* alone ($p < 0.001$) and compared with NC/IR/DDP alone ($p < 0.001$) (**Supplementary Fig. 3E**). These findings thus provide validation for the phenotypic specificity of *FSTL3* knockdown.

In addition, clonogenic survival assay results indicated that *FSTL3* deficiency significantly impaired colony formation efficiency for HeLa cell lines. One-way ANOVA revealed a significant difference in colony numbers among the four groups ($p < 0.001$). *FSTL3* knockdown significantly reduced colony formation compared with the

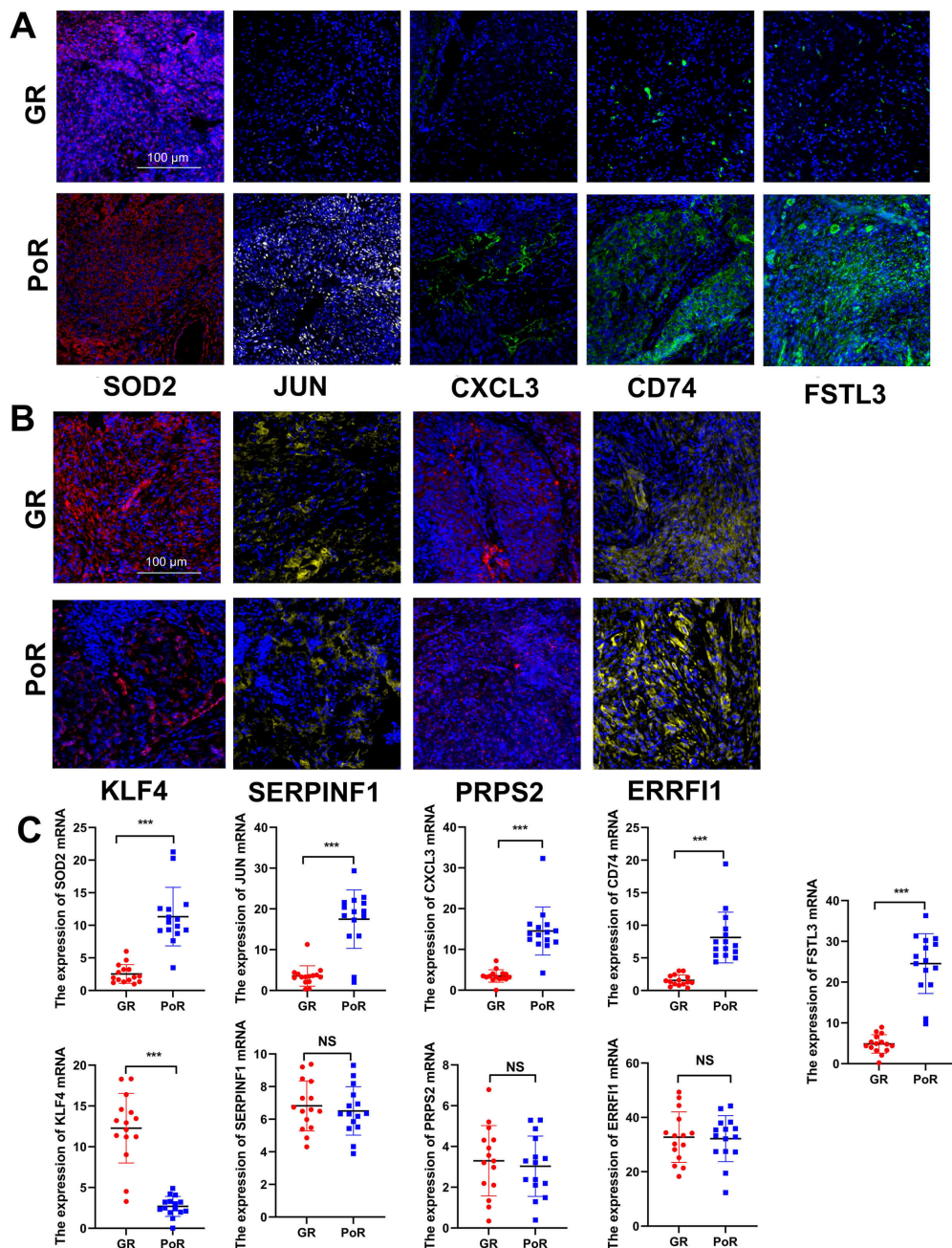


Fig. 7. Validation of the nine candidate signature genes in human cervical cancer clinical tissue samples. (A) Representative multiplex immunofluorescence (mIHC) staining images for SOD2, JUN, CXCL3, CD74, and FSTL3 in tumor tissues from patients who achieved good response (GR) and poor response (PoR) following cisplatin-based concurrent chemoradiotherapy (CCRT) treatment; scale bar = 100 μ m. (B) Representative mIHC staining images for KLF4, SERPINF1, PRPS2, and ERRF1 in GR and PoR clinical samples; scale bar = 100 μ m. (C) Quantification of mRNA levels for nine signature genes in GR and PoR tissue samples via quantitative real-time PCR (qRT-PCR) revealed that *SOD2*, *JUN*, *CXCL3*, *CD74*, and *FSTL3* were significantly upregulated, while *KLF4* was markedly downregulated in the PoR group relative to the GR group. No significant intergroup differences were detected for *SERPINF1*, *PRPS2*, or *ERRF1*, in line with mIHC staining results. For mIHC (A,B), representative images from three patients per group are shown; quantitative analysis was performed on all 30 patients ($n = 15$ per group) and is presented in **Supplementary Table 5**. For qRT-PCR (C), data are presented as mean $\pm$ standard deviation (SD) ($n = 15$ per group). Statistical comparisons between GR and PoR groups were performed using the Mann-Whitney U test. GR, good therapeutic response group; PoR, poor therapeutic response group; NS, no significant difference; *** $p < 0.001$. Scale bar = 100 μ m.

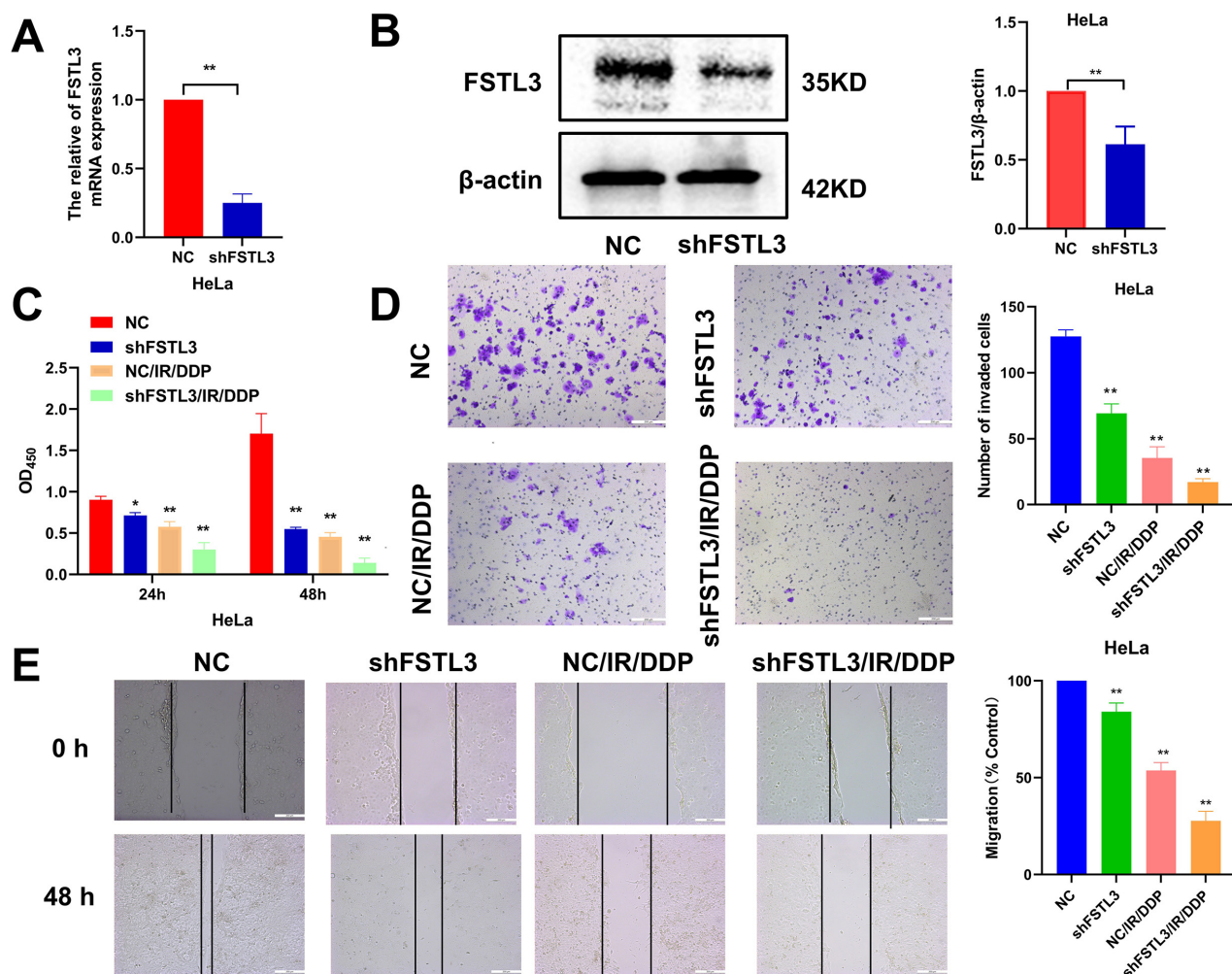


Fig. 8. *In vitro* functional exploration of *FSTL3* in cervical cancer cells. (A) qRT-PCR analysis of relative *FSTL3* mRNA levels in HeLa cells transfected with either negative control shRNA (negative control (NC)) or *FSTL3* knockdown shRNA (*shFSTL3*) constructs. (B) Representative Western blotting images and corresponding quantification of *FSTL3* protein expression, with β-actin serving as the internal reference. (C) CCK-8 cell proliferation assay demonstrating cell viability at 24 h and 48 h across four experimental groups (NC, *shFSTL3*, NC/IR/DDP, *shFSTL3*/IR/DDP). (D) Representative Transwell invasion staining images and corresponding quantification of invasive cell numbers in each group. (E) Wound healing assay micrographs and corresponding quantification of cell migratory capacity, normalized to the NC group. For (A), data are presented as mean ± SD from three independent biological replicates (n = 3 per group). For (C–E), data are presented as mean ± SD from three independent biological replicates (n = 3 per group). Statistical significance in (A) was determined by unpaired two-tailed Student's *t*-test; in (C) by two-way analysis of variance (ANOVA) with Tukey's post-hoc test; and in (D,E) by one-way ANOVA with Tukey's post-hoc test. Representative images in (B,D,E) are from one experiment representative of three independent replicates. NC, negative control shRNA; IR, irradiation; DDP, cisplatin treatment. In (A,B), significance markers indicate comparisons between *shFSTL3* and NC; in (C–E), significance markers above each bar indicate comparisons versus the NC group. **p* < 0.05, ***p* < 0.01. Scale bar = 200 μm.

NC group (*p* = 0.001). In the combined treatment setting, *shFSTL3*/IR/DDP further reduced colony formation compared with *shFSTL3* alone (*p* < 0.001) and compared with NC/IR/DDP alone (*p* = 0.017) (**Supplementary Fig. 8A**). Similarly, in SiHa cells, one-way ANOVA revealed a significant difference in colony numbers among the four groups (*p* < 0.001). *FSTL3* knockdown significantly reduced colony formation compared with the NC group (*p* = 0.001). In the combined treatment setting,

shFSTL3/IR/DDP further reduced colony formation compared with *shFSTL3* alone (*p* < 0.001) and compared with NC/IR/DDP alone (*p* = 0.017) (**Supplementary Fig. 8B**). These inhibitory effects were more prominent under X-ray irradiation, emphasizing the regulatory role of *FSTL3* as a mediator of cervical cancer radioresistance.

3.9 In Vivo Validation of the Role of *FSTL3* in Cervical Cancer

To validate the function of *FSTL3* at the *in vivo* level, we established a subcutaneous xenograft model in nude mice, which were assigned to four experimental groups: (a) HeLa/NC, (b) HeLa with *FSTL3* knockdown (HeLa/*shFSTL3*), (c) HeLa/NC with irradiation/DDP treatment (HeLa/NC/IR/DDP), and (d) HeLa/*shFSTL3* with irradiation/DDP treatment (HeLa/*shFSTL3*/IR/DDP). After 21 days of treatment, mice were euthanized, and tumor tissues were collected for imaging and analysis (Fig. 9A). Tumor growth curves were plotted using serial tumor volume measurements, and tumor weight was assessed following resection (Fig. 9B,C). Linear mixed-effects model analysis of tumor volume revealed significant main effects of group ($p < 0.001$), time ($p < 0.001$), and their interaction ($p = 0.0002$), indicating that the tumor growth patterns differed significantly among the four groups. *FSTL3* knockdown significantly reduced tumor volume compared with the NC group at the endpoint ($p = 0.007$). Moreover, the combination of *FSTL3* knockdown with IR/DDP further reduced tumor volume compared with NC/IR/DDP alone ($p = 0.002$), supporting an enhanced anti-tumor efficacy of the combined regimen. One-way ANOVA revealed a significant difference in tumor weight among the four groups ($p < 0.001$). *FSTL3* knockdown significantly reduced tumor weight compared with the NC group ($p < 0.001$). In the combined treatment setting, *shFSTL3*/IR/DDP further reduced tumor weight compared with *shFSTL3* alone ($p = 0.0097$). These *in vivo* findings provide functional evidence that *FSTL3* knockdown suppresses cervical tumor growth and may enhance responsiveness to irradiation and cisplatin, although further studies with larger sample sizes are needed to confirm these observations.

4. Discussion

Cervical cancer remains one of the most prevalent malignant tumors globally [20], and is characterized by a complex [21], multi-step pathogenesis that presents considerable challenges for treatment and prognostic assessment [22]. Despite continuous medical advancements and the progressive optimization of comprehensive treatment strategies, the lack of overt symptoms in the early stages of disease results in many cervical cancer patients only being diagnosed when the disease is already at an advanced stage. There is thus a pressing need to identify prognostic biomarkers and develop accurate prognostic stratification strategies for this cancer type.

In recent years, the pivotal importance of the TME in the pathogenesis, progression, and prognosis of cervical cancer has been increasingly established [23]. The composition of this intricate ecosystem, consisting of tumor cells, immune cells, CAFs, and additional elements, is strongly linked to patient clinical outcomes. Specifically, activated CAFs drive angiogenesis and matrix remodeling by se-

creting factors such as Vascular Endothelial Growth Factor (VEGF) and Transforming Growth Factor-beta (TGF- β) [24], thereby promoting tumor invasion and metastasis. Clinical evidence has confirmed that stromal enrichment of CAFs is an independent risk factor predicting shorter OS and disease-free survival in cervical cancer patients [25,26]. In this study, we systematically unraveled the cellular heterogeneity of the TME in cervical cancer, identified the crucial role of ketone body metabolism in CAFs, and constructed a prognostic model with potential clinical utility. Our scRNA-seq analysis effectively mapped the cervical cancer-associated TME, identifying 10 major cell types and highlighting significant compositional differences in immune and stromal cells between histological subtypes, including ADC and SCC. This underscores the close relationship between TME cellular composition and tumor pathological background, suggesting its potential importance in driving tumor heterogeneity. Subsequently, through an integrated multi-algorithm analysis of single cells, we determined that the ketone body metabolism pathway is specifically enriched in fibroblasts. By further categorizing fibroblasts into high- and low-metabolic activity subgroups, we found that the high-activity subgroup exhibited stronger cell-cell communication capabilities as well as a unique transcriptional regulatory network dominated by AP-1 family transcription factors such as JUNB and FOS. These results suggest that metabolically active CAFs may represent a functionally specialized subpopulation within the TME, potentially contributing to an immunosuppressive microenvironment or promoting tumor progression through active signaling and specific transcriptional programs. However, due to the small sample size of the single-cell cohort, caution is warranted when interpreting these results, and further large-scale studies are necessary to confirm the generalizability of these CAF subgroups.

Based on these findings, we developed and validated a nine-gene prognostic risk model based on differentially expressed genes associated with ketone body metabolism in CAFs. This model demonstrated consistent prognostic predictive ability across the internal TCGA cohort and multiple external GEO datasets. The integration of model genes with clinicopathological parameters resulted in a nomogram that was able to accurately predict 1-, 3-, and 5-year survival rates, demonstrating its potential as a useful clinical tool. Single-cell expression and pathway activity analysis revealed the importance of these genes in essential biological processes, including epithelial-mesenchymal transition, NF- κ B signaling [27], and allograft rejection. This suggests that the prognostic signature could affect patient outcomes by mediating metabolic-immune interactions within the TME. Finally, we investigated the role of *FSTL3* in cancer development and progression, confirming its function through both *in vitro* and *in vivo* experiments [28,29,30]. Knockdown of *FSTL3* effectively inhibited the proliferation, migration, and invasion of cer-

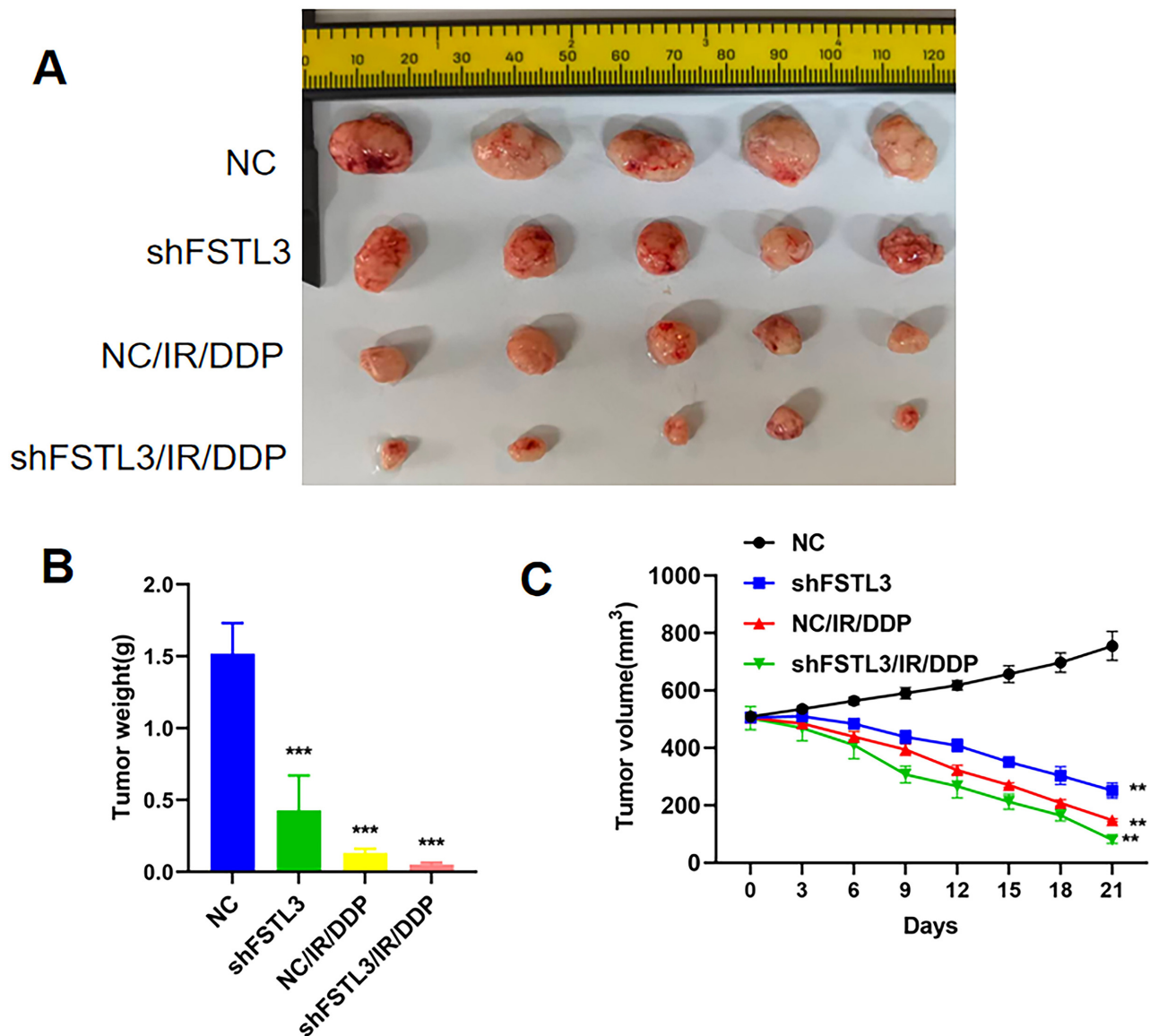


Fig. 9. In vivo functional validation of the role of *FSTL3* in cervical cancer xenograft models. (A) Representative macroscopic images of dissected xenograft tumors harvested from the four indicated experimental groups. (B) Quantification of final tumor weights, revealing a significant reduction in the *shFSTL3* group compared with the NC group; the combined treatment of irradiation plus cisplatin (IR/DDP) further reduced tumor weight upon *FSTL3* knockdown. *** $p < 0.001$. (C) Tumor volume growth curve over the 21-day study period, revealing that the *shFSTL3* group exhibited slower tumor progression relative to the NC group; simultaneous *FSTL3* knockdown and IR/DDP treatment enhanced anti-tumor activity, resulting in the smallest tumor mean volume at the endpoint. For (A), representative images from each group ($n = 5$ per group) are shown. For (B), data are presented as mean $\pm$ SD ($n = 5$ per group); statistical significance was determined by one-way ANOVA with Tukey's post-hoc test. For (C), data are presented as mean $\pm$ SD ($n = 5$ per group); tumor volume curves were analyzed using linear mixed-effects models with group, time, and their interaction as fixed effects and mouse identity as a random intercept. NC, negative control shRNA group; *shFSTL3*, *FSTL3* stable knockdown group; IR, radiotherapy irradiation; DDP, cisplatin chemotherapy. In (B), significance markers (***) indicate comparisons versus the NC group. In (C), significance markers (**) indicate comparisons versus the NC group at the endpoint (day 21). ** $p < 0.01$, *** $p < 0.001$.

vical cancer cells. In mouse models, it inhibited tumor growth and improved the effectiveness of both irradiation and chemotherapy. This not only validates the reliability of the bioinformatic predictions conducted herein but also suggests that *FSTL3* may represent a candidate ther-

apeutic target; however, further studies—including rescue experiments, pharmacological blockade, and mechanistic dissection—are required to establish its direct targetability and translational potential. Cervical cancer radioresistance is co-regulated by multiple factors including stromal

metabolism, oxidative stress, and microenvironmental disturbance, which cannot be fully explained by *FSTL3* alone. Our multi-gene signature integrated with eight additional CAF metabolism-related genes allows for the more comprehensive characterization of TME abnormalities, avoiding the limitations of single gene-based predictive assessment and thereby potentially improving prognostic stratification and clinical applicability.

Our study systematically highlights the cellular and metabolic diversity that exists within the cervical cancer TME, underscoring the importance of ketone body metabolism in CAFs. The multi-gene prognostic signature we established, together with the functional validation of *FSTL3*, provides useful insights and a foundation for improving prognostic stratification and developing new combination treatment strategies for cervical cancer patients.

Several limitations of this study should be acknowledged and are discussed in detail in the Limitations section, including the lack of rescue experiments, incomplete mechanistic characterization, limited cell models, and the need for further clinical validation.

5. Limitations

This study has several inherent limitations. First, the TCGA, GSE44001, and GSE52903 datasets contain cases with diverse treatment regimens, limiting their applicability for research focused specifically on concurrent CCRT. Nevertheless, the conserved molecular signatures that regulate chemoradiosensitivity in cervical cancer tissues may still reflect the intrinsic CCRT responsiveness of tumors. Notably, our cellular and animal experiments partially compensate for the incomplete clinical information of public datasets. In addition, the nine-gene signature constructed in this study represents a transcriptome-derived biomarker for predicting CCRT sensitivity in cervical cancer, and its predictive efficacy requires further validation in prospective CCRT cohorts. Second, all experimental validation in this study was performed in cervical cancer cells and subcutaneous xenograft tumor mouse models, without supplementary verification using patient-derived xenograft (PDX) models or organoid models. While our *in vivo* xenograft experiments provide supportive functional evidence for the role of *FSTL3* in tumor growth and treatment sensitivity, these findings should be interpreted with caution due to the limited sample size ($n = 5$ per group) and the inherent limitations of subcutaneous xenograft models, which do not fully recapitulate the human tumor microenvironment. Third, rescue experiments using *FSTL3* overexpression to verify the specificity of the knockdown phenotype, as well as pharmacological inhibition of *FSTL3*, were not performed in the current study. Additionally, only a single shRNA construct was used for gene silencing, which may raise concerns regarding potential off-target effects. While our loss-of-function experiments in both HeLa and SiHa cells consistently demonstrated the tumor-promoting role

of *FSTL3*, we acknowledge that complementary gain-of-function or rescue assays, the use of multiple independent shRNA sequences, and pharmacological blockade studies would provide additional evidence to confirm the specificity of the observed phenotypes, exclude off-target effects of shRNA and evaluate therapeutic translatability. This represents a limitation of our current experimental validation, and we plan to address this in future investigations. Fourth, while our *in vitro* and *in vivo* experiments demonstrate that *FSTL3* knockdown affects tumor cell proliferation, migration, invasion, and sensitivity to irradiation and cisplatin, the downstream molecular pathways—such as apoptosis signaling, DNA damage repair, or cell-cycle regulation—underlying these effects remain unexplored. Therefore, our conclusions are presented as functional associations rather than mechanistic definitions, and further mechanistic investigations are required to elucidate the precise regulatory network. Fifth, multivariable analyses adjusting for clinical covariates were not performed due to the limited sample size ($n = 30$) of the clinical validation cohort, which precluded meaningful adjustment for multiple confounders. Larger prospective cohorts are required to confirm the independent predictive value of the identified markers. Sixth, the CAF ketone-body metabolism–*FSTL3* axis evaluated in this study is currently supported by correlative transcriptomic evidence. Functional validation through experiments involving CAF-derived soluble factors, CAF-specific *FSTL3* manipulation, and direct ketone-body measurements will be necessary to establish causality. These experiments are beyond the scope of this study but are crucial for future research. Finally, the prognostic model developed herein yielded modest AUC values of 0.60–0.68 during external validation, with limited discriminatory power. Sample heterogeneity stemming from inconsistent therapeutic regimens across datasets is likely the major factor restricting the broader generalizability of this model.

6. Conclusion

In summary, this study employed a multi-level analytical approach to establish a cellular atlas of the cervical cancer TME based on scRNA-seq data, revealing the cellular heterogeneity that characterizes this tumor type. We identified enriched ketone body metabolism in CAFs, and further identified enhanced communication capacity and a unique *AP-1* transcriptional regulatory network in this cellular subpopulation. We developed and validated a nine-gene prognostic model associated with ketone body metabolism and determined that integrating this model with clinicopathological features in a nomogram afforded promising predictive performance and clinical utility. *In vitro* and *in vivo* experiments provided functional evidence supporting the tumor-promoting role of the key gene *FSTL3* and highlighted its potential as a candidate for further therapeutic exploration, though additional validation is needed to con-

firm its suitability as a directly targetable molecule. These findings provide novel biomarkers and a theoretical foundation for prognostic stratification and individualized therapy in cervical cancer.

Availability of Data and Materials

The scRNA-seq datasets generated and analyzed during the current study are available in the GEO repository (GSE197461). The bulk RNA-seq datasets are available from the TCGA database (<https://portal.gdc.cancer.gov>) and GEO database (GSE44001, GSE52903). All other data supporting the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions

HY, RM, and LM designed the research study. HY drafted the initial manuscript. HY, RM, and WL performed the research. LM and DH performed the bioinformatics analysis and interpreted the results. JL and YZ provided clinical samples and patient data. LW analyzed the data. ZL contributed to the design of the work, and to the analysis and interpretation of data. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The animal study was reviewed and approved by the Biomedical Ethics Committee of Health Science Center of Xi'an Jiaotong University (Approval No. XJTUAE2026-2028). The animal experiments were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and the study adhered to the ARRIVE 2.0 guidelines. All procedures performed in this study were approved by the Medical Ethics Committee of the First Affiliated Hospital of the Air Force Medical University (KY20233336-1). The study was carried out in accordance with the guidelines of the Declaration of Helsinki. Written informed consent has been obtained from the patients to publish this paper.

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Conflicts of Interest

The authors declare no conflicts of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.31083/FBL54670>.

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