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Estrogen receptor α suppresses hepatocellular carcinoma by restricting M2 macrophage infiltration through the YAP-CCL2 axis

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Abstract

Purpose Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide, with significant differences in incidence and outcomes between men and women. Estrogen receptor alpha (ERα) expression is associated with sex-based differences and poor prognostic outcomes in HCC. However, the detailed function of ERα in the tumor microenvironment of HCC remains unclear.

Methods Bioinformatics analysis of differentially expressed genes in HCC samples was performed from publicly available databases, and ERα was selected. The function of ERα was examined in the cell experiments. A co-culture system was built to study function of ERα-treated liver cells on macrophages in vitro. The precise mechanism was determined using quantitative real-time PCR, western blotting, immunohistochemistry, mass spectrometry, co-immunoprecipitation, and dual-luciferase reporter assay.

Results ERα played an important role in the pathogenesis of sexual dimorphism in HCC. ERα mainly acted on macrophages in the tumor microenvironment (TME) of HCC and reduced M2 macrophage infiltration through CCL2. By acting on NF2 and 14-3-3theta, ERα enhanced YAP phosphorylation and attenuated the nuclear translocation of YAP, thereby suppressing CCL2 expression. It also acted as a transcription factor that regulated CCL2 expression at the transcriptional level.

Conclusion ERα/YAP/CCL2 signaling reduced M2 macrophages infiltration to inhibit HCC progression, revealing the effect of ERα in cancer cells on immune cells in HCC microenvironment.

Highlight

- ERα mainly acted on macrophages in the tumor microenvironment of HCC.
- ERα mainly reduced M2 macrophage infiltration through CCL2.
- ERα promoted the activation of Hippo pathway through NF2 and enhanced p-YAP through 14-3-3theta, preventing YAP nuclear translocation and reducing CCL2 expression.
- ERα played an important role in the pathogenesis of sexual dimorphism in HCC, and has a good value in the prognosis and treatment of HCC.

Keywords HCC, Estrogen receptor α, Macrophage, CCL2, YAP

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Introduction

HCC is one of the most common malignant tumors worldwide. Owing to its high recurrence rate, high metastasis rate and poor prognosis, the 5-year survival rate of HCC is less than 20% [1]. The liver is the organ with the most obvious sex dimorphism besides the reproductive organ [2]. Males experience a 2–3 times higher incidence of HCC than females. Additionally, the overall survival rate of male patients is considerably lower than that of female patients, indicating the potential role of sex hormones in the pathophysiology of HCC [3].

We discovered that ESR1 is crucial for providing protection against the development of HCC, following differential genes analysis and screening through several public databases on HCC. ERα, encoded by ESR1,is the most predominant estrogen receptor in the liver [4]. Hepatocarcinogenesis is significantly promoted when females lack ERα [5]. ERα can suppress HCC occurrence and development by regulating genes such as PTPRO, P53, TNF and inhibiting Wnt/β-catenin and activating Hippo signaling [6, 7]. However, the detailed functions of ERα in the HCC tumor microenvironment remain ambiguous.

Macrophages(Mϕ) are the highest proportion of immune cell population in the liver, which are of great significance in maintaining liver homeostasis. Mϕ in the tumor microenvironment have two functionally distinct types of polarization, namely M1 (immune-promoting) and M2 (immune-suppressive) [8]. ERα polarized macrophages differently in different tumors. In lung cancer and endometrial cancer, ERα mainly promoted the M2 polarization of macrophages [9, 10], while in prostate cancer, ERα in cancer associated fibroblasts inhibited the M2 polarization of macrophages to suppress tumors [11]. However, the role of ERα on macrophages in HCC remains unclear.

Hippo signaling is a critical regulatory pathway for normal liver development and HCC occurrence owing to its vital role in maintaining liver size and function [12, 13]. Hippo signaling is vital in the early liver cancer development [14, 15]. Hippo signaling pathway regulates target genes and mediates the generation of inflammatory cytokines, thus facilitating a variety of malignancies [16]. CCL2 is a very important macrophage chemokine and has been shown to be a target gene of YAP [17, 18]. In HCC, YAP was also shown to promote CCL2 expression, thereby inducing macrophage migration [19, 20]. We focused on how ERα regulates the cytokine CCL2 through Hippo signaling.

In this study, we investigated the mechanism by which ERα acted as a tumor suppressor gene to restrict macrophage infiltration in HCC. ERα inhibited the YAP-CCL2 axis by acting on NF2 and 14-3-3theta. ERα also downregulated CCL2 at the transcriptional level. This study aimed to reveal the regulatory mechanisms of ERα-mediated macrophages in suppressing HCC through the Hippo pathway, which may be helpful for ERα-based prognostic prediction and development of anticancer therapeutic strategies.

Materials and methods

Transcriptome data analysis

The Cancer Genome Atlas (TCGA) website (https://portal.gdc.cancer.gov/) and clinical information of all patients with HCC were downloaded for subsequent analyses. We obtained the GSE55092, GSE76427, and GSE121248 datasets from the gene expression omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/). The "limma" package(version 3.44.3) was used for differential analysis. For each dataset, data correction was performed using the normalizeBetweenArrays in the limma package. Each dataset was independently processed. Genes with$|\log_2$fold change (FC)$|>0.4$ and a p value <0.05, were considered differentially expressed genes. Cox univariate analysis used the 'survival' R package, and least absolute shrinkage and selection operator (LASSO) analysis used the "glmnet" package. SsGSEA method was used to analyze the correlation between ESR1 gene expression and 23 kinds of immune cells. Gene set expression analysis (GSEA) was performed with the "GSEABase" package, "ClusterProfler" package, and "org.Hs.eg.db" package. The Gene Ontology (GO) database was used for the GSEA (http://geneontology.org/). The pathway was deemed to be significantly enriched if $P<0.05$. The "ggplot2" and "ggpubr" packages were utilized for the visualization.

Tissue specimens

Twenty-two HCC tissue samples were obtained from the Fifth Hospital of Shijiazhuang between 2017 and 2022. These samples included tumor and adjacent paracancerous tissues. Detailed clinical data of the 22 patients including gender, age, stage of liver cancer, Edmondson grades, cirrhosis grades, etiology, and viral replication are shown in Supplementary Table 1. The study was approved by the Medical Ethics Committee of The Fifth Hospital of Shijiazhuang(ethical approval No.202316-1).

Cell lines and reagents

Human HCC cell lines HepG2, HuH7, SMMC-7721, Hep3B, and hepatic stellate.

LX2 cells were provided by the Fifth Hospital of Shijiazhuang and grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, Thermo Fisher Scientific, USA) and minimum essential medium (MEM, Gibco, Thermo Fisher Scientific, USA). The human acute monocytic leukemia cell line THP-1 was purchased from Shanghai Zhong Qiao Xin Zhou Biotechnology Co., Ltd. (China) and grown in Roswell Park Memorial Institute 1640

medium (RPMI 1640, Gibco, Thermo Fisher Scientific, USA). The medium was supplemented with 10% fetal bovine serum (VivaCell, Shanghai, China), 10 KU/Ml penicillin, and 10 mg/Ml streptomycin (Ruipate Biotech, Shijiazhuang, China) at 37 °C in a humidified atmosphere with 5% CO_2. THP-1 cells required phorbol 12-myristate 13-acetate (PMA) (100 ng/mL, Ruipate Biotech) to differentiate into macrophages (Mϕ) in PRIM 1640 for 48 h.

Cell transfection

The ERα overexpression plasmid was supplied by Hunan YouBio Co., Ltd. and the pcDNA3.1-NC plasmid served as the control. Small interfering RNA (siRNA; GenePharma, Suzhou, China) were designed to knock down ERα. Si-NC was used as a control. SiRNA and Si-NC sequences are shown in Supplementary Table 2. Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific, USA) was used to transfect siRNAs and plasmids into cells according to the manufacturer's instructions.

Table 1 List of PCR primers used in this study

Name		Sequence, 5′ → 3′
ESR1	Sense	ATGGTCAGTGCCTTGTTGGATGC
	Antisense	GTCTGCCAGGTTGGTCAGTAAGC
CCL2	Sense	CAGCCAGATGCAATCAATGCC
	Antisense	TGGAATCCTGAACCCACTTCT
CCL5	Sense	CCAGCAGTCGTCTTTGTCAC
	Antisense	CTCTGGGTTGGCACACACACTT
MMP9	Sense	CGGAGCACGGAGACGGGTAT
	Antisense	TGCAGGCGGAGTAGGATTGG
TGF-β	Sense	CATCAACGGGTTCACTACC
	Antisense	CTCCGTGGAGCTGAAGCA
IFN-γ	Sense	TCGGTAACTGACTTGAATGTCCA
	Antisense	TCGCTTCCCTGTTTTAGCTGC
TNF-α	Sense	CCTCTCTCTAATCAGCCCTCTG
	Antisense	GAGGACCTGGGAGTAGATGAG
IL-4	Sense	CCGTAACAGACATCTTTGCTGCC
	Antisense	GAGTGTCCTTCTCATGGTGGCT
CSF-1	Sense	TGAGACACCTCTCCAGTTGCTG
	Antisense	GCAATCAGGCTTGGTCACCACA
CCL15	Sense	TGATGTCAAAGCTTCCACTGGAAA
	Antisense	GAGTGAACACGGGATGCTTTGTG
CCL18	Sense	GTTGACTATTCTGAAACCAGCCC
	Antisense	GTCGCTGATGTATTTCTGGACCC
CX3CL1	Sense	ACAGCACCACGGTGTGACGAAA
	Antisense	AACAGCCTGTGCTGTCTCGTCT
CXCL8	Sense	GAGAGTGATTGAGAGTGGACCAC
	Antisense	CACAACCCTCTGCACCCAGTTT
CXCL12	Sense	CTCAACACTCCAAACTGTGCCC
	Antisense	CTCCAGGTACTCCTGAATCCAC
CD163	Sense	CCAGAAGGAACTTGTAGCCACAG
	Antisense	CAGGCACCAAGCGTTTTGAGCT
CD68	Sense	CGAGCATCATTCTTTCACCAGCT
	Antisense	ATGAGAGGCAGCAAGATGGACC
IL-6	Sense	AGACAGCCACTCACCTCTTCAG
	Antisense	TTCTGCCAGTGCCTCTTTGCTG
IL-10	Sense	TCTCCGAGATGCCTTCAGCAGA
	Antisense	TCAGACAAGGCTTGGCAACCCA
YAP	Sense	TACTGATGCAGGTACTGCGG
	Antisense	TCAGGGATCTCAAAGGAGGAC

Co-culture of HCC cells and macrophages

The co-culture of HCC cells and Mϕ was performed using a Transwell system of six-well plates (Jet Bio-Filtration, Guangzhou, China). In this system, 1×10^6 Mϕ cells were cultured in the upper chamber, HCC cells with ERα knockdown or overexpression were seeded in the six-well plates for 48 h before harvest, and the co-culture medium with Mϕ and HCC cells was separately used to detect cytokines.

Quantitative real-time PCR (qPCR) assay and western blotting

TRIzol reagent (Solarbio, Beijing, China) was used for the RNA extraction. A PrimeScript RT Reagent Kit (Perfect Real Time, Takara, Japan) was used for cDNA synthesis. Hieff qPCR SYBY® Green Master Mix (Yeasen, Shanghai, China) was used to perform qPCR. All the experimental steps were performed in accordance with the manufacturer's instructions. Relative mRNA expression was normalized to that of the endogenous control GAPDH using the $2^{-\triangle\triangle Ct}$ method. The primer sequences used are listed in Table 1.

For western blotting, cells were lysed using a phosphatase inhibitor cocktail (Ruipate Biotech, Shijiazhuang, China) and radioimmunoprecipitation assay (RIPA) buffer (Solarbio, Beijing, China) containing phenylmethylsulfonyl fluoride (PMSF). A Bicinchoninic acid (BCA) Protein Quantification Kit (Beyotime, Shanghai, China) was used for protein quantification. Detailed procedures for the western blot experiments are presented in the reference article [21]. Primary antibodies used are listed in Table 2.

Cell proliferation assays

Cell proliferation was detected using the MTS assay. Transfected cells (2×10^3 cells/well) were seeded in 96-well plates. Twenty uL (500 mg/mL) of MTS reagent was added to each well and incubated in a CO_2 incubator for 2 h. The absorbance was measured at 492 nm using a ReadMax 1200 (Flash) instrument after 0, 24, 48, and 72 h of cell culture, respectively.

Transwell migration and invasion assays

A Transwell chamber (Corning, NY, USA) with an 8 μm pore membrane was used for the migration tests. HCC cells with ERα knockdown or overexpression were placed in the lower chamber, whereas 1×10^5 Mϕ were seeded in the upper chamber. After a 36-hour incubation period,

Table 2 List of antibodies used in this study

Experiment	Name	LOT	Manufacturer
WB	ERα	D8H8	CST
	CCL2	#RA1084	ReportBio
	yap	#RA1104	ReportBio
	p-YAP(s127)	Et1611-69	ReportBio
	MST	HA500031	HuaAn
	p-MST	AF2367	Affinity
	LATS	AF7669	Affinity
	p-LATS(Thr1079)	AF7169	Affinity
	14-3-3theta	A8936	ABclonal
	GAPDH	60004-1-Ig	proteintech
	Lamin-B1	ET1606-27	HuaAn
	NF2	HA721320	HuaAn
IHC	ERα	D8H8	CST
	yap	#RA1104	ReportBio
Co-IP	ERα	D8H8	CST
	14-3-3theta	A8936	ABclonal

Mϕ cells were fixed with paraformaldehyde, stained with crystal violet, and examined using a Leica microscope (DM12000M, Germany). A Transwell chamber with 50 μL of Matrigel (Corning) was used in the upper chamber for the invasion assay. A total of 700 μL of culture medium was added to the lower compartment, and the upper chamber was seeded with 1×10^5 HCC cells with either ERα knockdown or overexpression, and incubated for 40 h. The remaining steps were performed in the same way as described previously.

Flow cytometry analysis

Annexin V-FITC/PI double-staining assays were performed for analysis of apoptosis. Transfected cells were cultured for 48 h and harvested at a density of 1×10^6 cells/ml. Propidium iodide (5 μL) and Annexin V-FITC (10 μL) were added to the cells, followed by incubation in the dark. The single-staining reagent PI (4 A Biotech, Beijing, China) was used for the cell cycle experiments. The increase in CD11b expression was quantified using flow cytometry and PE-labeled anti-CD11b antibody (ICRF44; Zenbio, Chengdu, China).

Immunohistochemical (IHC) staining and immunofluorescence (IF)

Primary and secondary antibodies were used to immunostain sections of tumor and paracancerous tissues embedded in paraffin to measure protein expression. Simultaneously, two seasoned pathologists examined each portion and performed immunological scoring without being aware of clinical information. Pathologists performed histology scoring: tissue sections were scored according to the degree of staining (0–3 divided into negative staining, light yellow, light brown, dark brown) and positive range (1–4 divided into 0 ~ 25%, 26 ~ 50%, 51 ~ 75%, 76 ~ 100%). The scores were added at the end of the experiment, and the results were compared.

In IF experiments, HepG2 cells were plated on glass coverslips, transfected with siRNA or overexpression plasmid containing ERα, and incubated for 48 h. ERα was stained red, phosphorylated YAP (p-YAP) in green, and 4′,6-diamidino-2-phenylindole (DAPI, blue) was used as a nuclear counterstain. A Leica confocal laser scanning microscope was used to obtain images of the specimens.

ELISA

Macrophage-related cytokines were tested using ELISA. Mϕ cells were co-cultured for 48 h with HepG2 cells having knockdown or overexpression of ERα. Thereafter, the co-culture medium was collected to measure the levels of IL-6, IL-4, CCL-2, IFN-γ, TNF-α, IL-10, and CCL-5 using a human ELISA assay kit (Jonln, Shanghai, China) according to the manufacturer's protocol.

Nuclear and cytoplasmic extraction

The Minute^{TM} Cytoplasmic and Nuclear Extraction Kit for cells (Invent, Beijing, China) was used to extract the nuclear and cytoplasmic fractions of HepG2 and HuH7 cells (5×10^6) to shed light on the cellular localization of YAP according to the manufacturer's guidelines. The cytoplasmic and nuclear localization controls were GAPDH and Lamin B1, respectively.

Co-immunoprecipitation (Co-IP) and mass spectrometry (MS) analysis

HuH7 cells were lysed in RIPA buffer containing phenylmethylsulfonyl fluoride (PMSF), and the extracts were centrifuged at 12,000 rpm at 4 °C for 10 min. Protein A/G magnetic beads (MedChemExpress, Shanghai, China) were washed three with PBST, incubated with PBST containing ERα or 14-3-3θ antibodies, and shaken for 30 min at 20 °C. After washing with PBST, the cell lysate extract was added to the protein A/G magnetic beads and shaken for 30 min at 20 °C. After another PBST wash, the protein A/G magnetic beads were suspended in 1 × sodium dodecyl sulfate-polyacrylamide gel electrophoresis loading buffer and boiled for 10 min.

The binding proteins of ERα and IgG in HepG2 and HuH7 cells were identified by MS analysis (Shanghai, Aptbiotech, Co., Ltd) and verified by Co-IP.

Dual-luciferase reporter assay

Full-length CCL2 promoter plasmids, CCL2 promoter plasmids containing estrogen response element 3(ERE3) /estrogen response element 4, and CCL2 promoter plasmids with a mutant ERE3 area were constructed by Sangon Biotech Co., Ltd. HepG2 cells were co-transfected with empty or ERα-overexpressing plasmids, CCL2 promoter-reporter gene plasmids, or Renilla plasmids

for 48 h. A Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) was used to evaluate luciferase activity.

Statistical analysis

Statistical analyses were performed using the R software (version 4.1.2) or GraphPad Prism software (v 8.0.0, GraphPad Inc., CA). Two-tailed unpaired Student's t test or paired sample t-test was used for comparison between two groups, and one-way ANOVA tests were used for comparison among the three or more groups. *P*-values less significance was set at $P < 0.05$.

Results

ESR1 was screened and correlated with better HCC prognosis

To investigate the key genes influencing sexual dimorphism in HCC, we first analyzed publicly available data from GSE55092, GSE76427, and GSE121248 from NCBI and TCGA_LIHC. The intersection of the DEGs in these four databases was used to obtain 608 candidate target genes, and Venn diagrams were generated (Fig. 1A) (Figure S1). We subsequently applied univariate Cox regression analysis to screen the prognostic roles of these differentially expressed genes based on TCGA_LIHC cohort, and 264 genes were identified (Fig. 1B). Genes

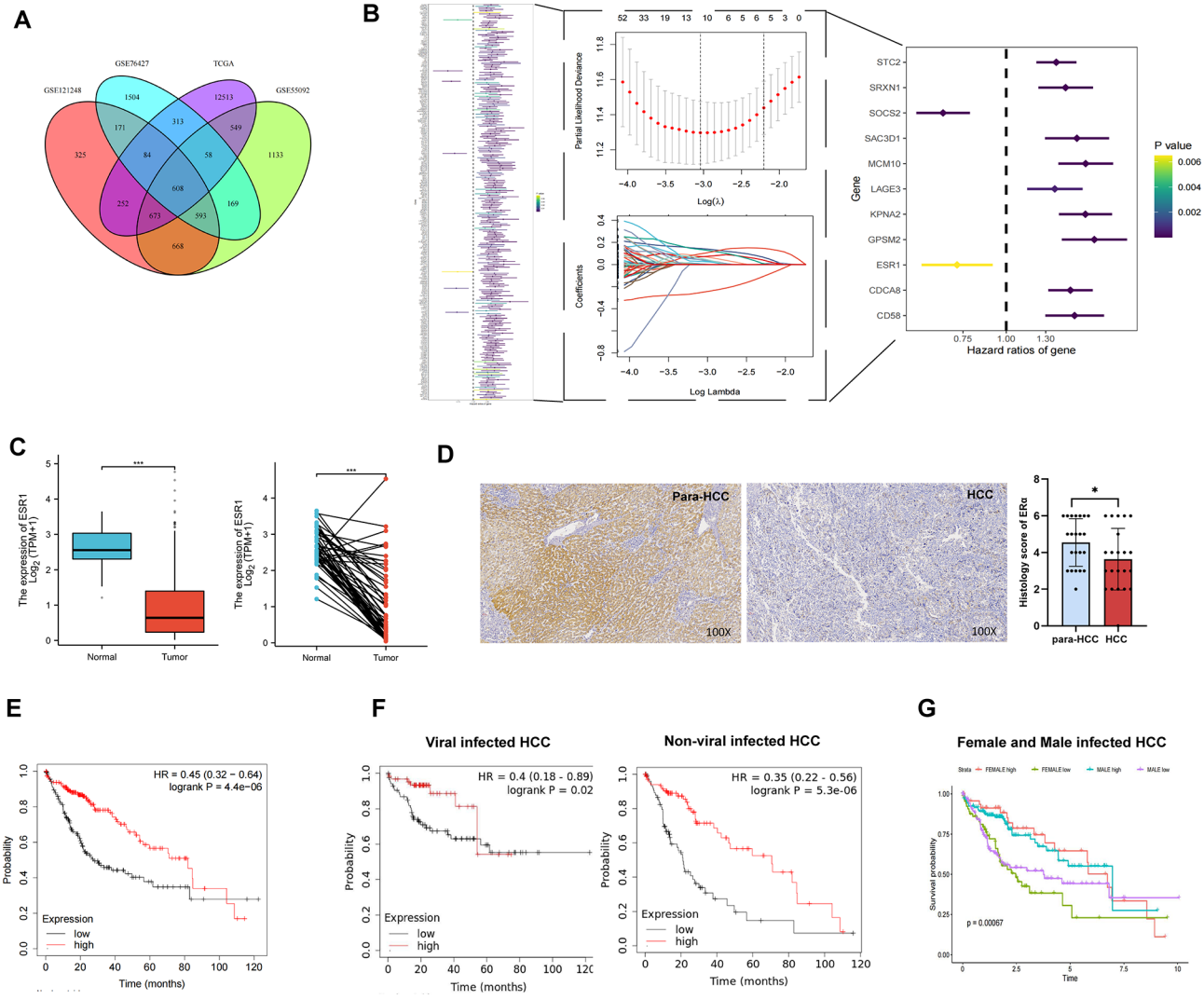

Fig. 1 ESR1 is screened and is correlated with better prognosis in HCC patients. **(A)** Venn diagram showed the intersection of the differentially expressed genes from GSE55092, GSE76427 and GSE121248 and TCGA_LIHC datasets. **(B)** Univariate Cox regression analysis and LASSO analysis were used to screen for DEGs in TCGA_LIHC. **(C)** ERα expression in normal and tumor tissues (left) and paired normal and tumor tissues (right). **(D)** Images of ERα IHC staining of para-cancerous and HCC tissues. **(E)** Survival curve based on TCGA database. **(F)** Survival curve of viral infected HCC and non-viral infected HCC based on TCGA database. **(G)** Survival curve of different gender in HCC based on TCGA database. The Student's t-test was used to compare the means between groups. * $P < 0.05$

obtained by Cox regression analysis were analyzed using LASSO regression to further screen for core genes. Finally, we identified 11 key genes closely associated with HCC prognosis (Fig. 1B). Among these 11 genes, *ESR1* was the optimal hub gene related to sexual dimorphism in HCC and acted as a protective factor.

Comparison between paired tumorous tissues (HCC) and adjacent normal tissues, as well as between unpaired tumorous tissues and adjacent normal tissues in the TCGA-LIHC database, showed that ERα expression in HCC was significantly lower than that in the normal tissue group (Fig. 1C). We also analyzed the IHC staining of HCC pathological tissue sections from 22 clinical samples. The histology score of ERα in HCC was 3.64 ± 1.68, which was much lower than that of the corresponding paracancer tissue (4.55 ± 1.3) (Fig. 1D) (Figure S2). The association between pathological characteristics and ERα expression in HCC patients was examined based on TCGA database, Table 3 shows a significant association between the patients' pathological T stage, age, sex, and ERα expression. Furthermore, Kaplan–Meier survival curve analysis for HCC patients demonstrated that patients with high ERα expression had significantly longer survival times than patients with lower ERα expression ($P < 0.001$) (Fig. 1E). In order to further understand the effect of ERα on the prognosis of HCC with different etiologies, HCC patients in TCGA-LIHC were divided into viral infected HCC and non-viral infected HCC, and survival curve analysis was performed between high and low expression of ERα. Results showed that ERα could significantly prolong the 5-year overall survival time in both viral infection-HCC and non-viral HCC (Fig. 1F). Similarly, we also investigated the correlation between high and low expression of ERα and HCC progression in different genders. It can be seen that high ERα expression has a longer survival time compared with low expression in both men and women, indicating that the expression of ERα is closely related to the prognosis of HCC (Fig. 1G).

Table 3 Relationship between clinical pathological features and ERα expression in HCC (TCGA)

Characteristics	Low ESR1	High ESR1	χ2	*p* value
n	187	187		
Gender, n (%)			5.387711103	**0.02***
Male	116 (45.8%)	137 (54.2%)		
Female	71 (58.7%)	50 (41.3%)		
Age, n (%)			8.116021437	**0.004***
<= 60	102 (57.6%)	75 (42.4%)		
> 60	84 (42.9%)	112 (57.1%)		
Pathologic T stage, n (%)			7.264436529	**0.026***
T1	80 (43.7%)	103 (56.3%)		
T2	51 (53.7%)	44 (46.3%)		
T3&T4	56 (60.2%)	37 (39.8%)		
Pathologic N stage, n (%)			1.930530942	0.165
N0	133 (52.4%)	121 (47.6%)		
N1	4 (100%)	0 (0%)		
Pathologic M stage, n (%)			1.791825528	0.181
M0	144 (53.7%)	124 (46.3%)		
M1	4 (100%)	0 (0%)		

Note The result was analyzed by the Pearson chi-square test. * $P < 0.05$

Overall, bioinformatics screening of ERα revealed that it acted as a marked tumor suppression gene can decrease HCC progression.

ERα functions as a tumor suppression gene to inhibit HCC

We determined the mRNA levels of ERα in a hepatic stellate cell line (LX2) and in four HCC cell lines (HepG2, SMMC-7721, HuH7, and Hep3B). Expression of ERα in HCC cell lines was significantly lower than that in LX2 cells. Among the HCC cell lines, HepG2 cell had the highest ERα expression, whereas Hep3B cell had the lowest (Figure S3). The HepG2 and HuH7 cells were selected for further experiments. Quantitative PCR and western blotting were performed on HepG2 and HuH7 cells with ERα knockdown and overexpression, respectively. siRNA and overexpression plasmids of ERα showed good performance (Fig. 2A, B). MTS experiments demonstrated that ERα overexpression prevented tumor cell proliferation, whereas ERα knockdown enhanced tumor cell growth (Fig. 2C, D). ERα overexpression significantly accelerated HCC cell death by flow cytometry analysis (Fig. 2E, F) and inhibited HepG2 and HuH7 cells in S phase (Fig. 2G, H). Accordingly, knockdown of ERα in HepG2 and HuH7 cells significantly increased HCC invasion, as indicated by the Matrigel-coated Transwell assay. Similarly, ERα overexpression in liver cancer cells decreased HCC invasion. The addition of 17-β estradiol (E2) (10 nM) to the culture medium partly reversed the increase in HCC invasion induced by ERα knockdown (Fig. 2I, J).

In conclusion, these functional experiments demonstrated that tumor cell apoptosis was promoted and the growth and invasion of HCC cells were significantly suppressed by ERα up-regulation.

ERα-transfected HCC cell lines inhibited Mφ to M2 phenotype transition

To better understand the role of ERα in the tumor microenvironment (TME), the correlation between ERα expression and all immune cell types was investigated using ssGSEA and Spearman's correlation. We found that ERα was highly correlated with macrophages, with a correlation coefficient of 0.33 (Fig. 3A, B). Correlation coefficients between ERα and other immune cells are shown in Figure S4. CD11b expression was detected after THP-1 cells were polarized with PMA (100 ng/mL)

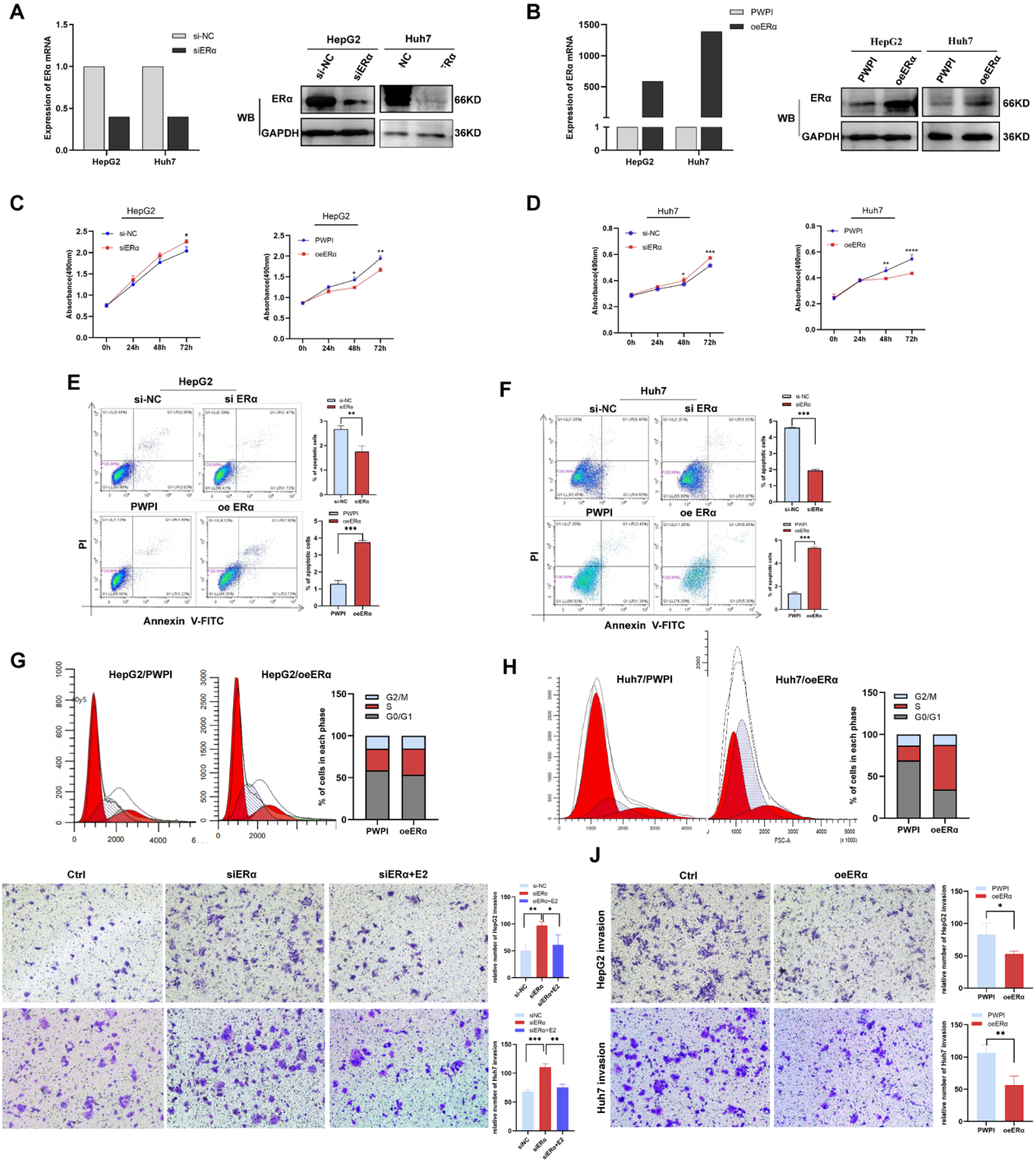

Fig. 2 ERα gain-of-function and loss-of-function experiments in HepG2 and huh7 cells. **(A-B)** Confirming knockdown and overexpression ERα mRNA expression by RT-PCR and protein expression by WB. **(C-D)** Measurement of proliferation in the group of si-NC and siERα and in the group of PWPI and oeERα transfected HepG2 and huh7 cells by MTS assay. **(E-F)** Cell apoptosis was analyzed with Annexin V-FITC/PI double-staining assays by flow cytometry.**(G-H)** Cell cycle phase was analyzed by ModFit, histogram statistics were used to cell cycle distribution. **(I-J)** Transwell® coated Matrigel were applied to detect invasive abilities of HCC cells treated ERα. SiERα cells added E2 (10nM) can decrease HCC cells invasion. Experiments were done at least in 3 replicates. Data are presented as mean ± SD; *P* -value was calculated using two-tailed Student's t test. One-way ANOVA tests were used for comparison among the three or more groups. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

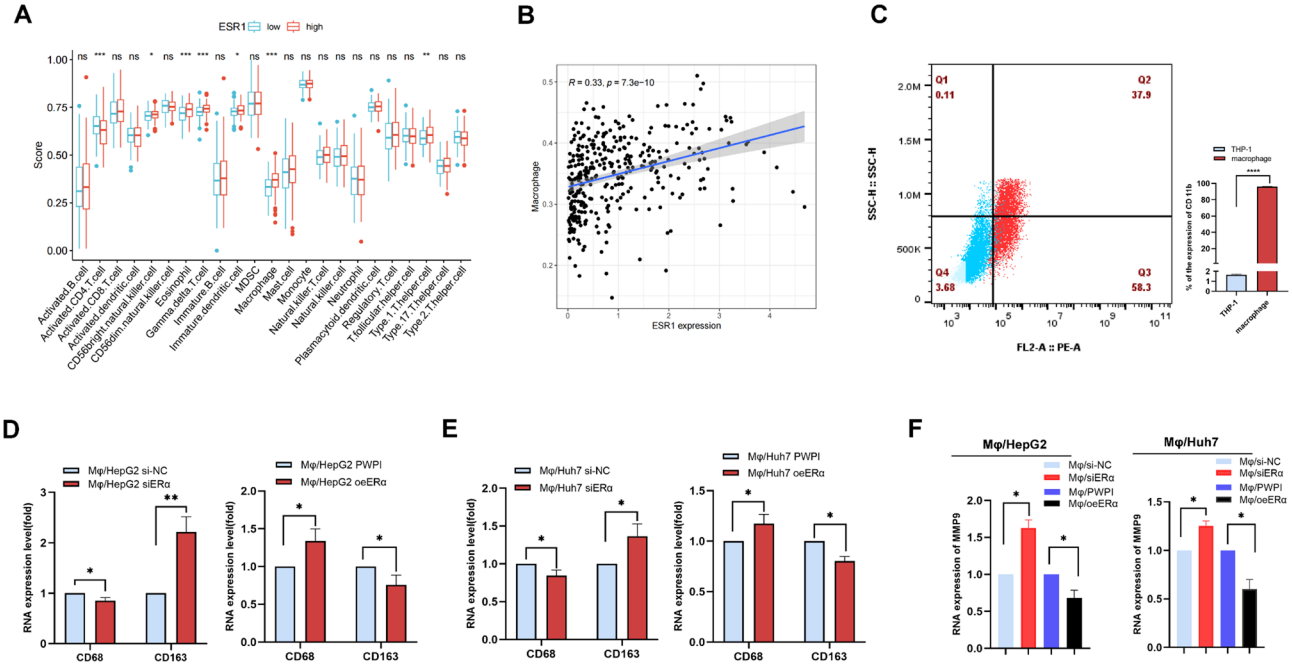

Fig. 3 ERα in liver cancer cells can inhibit the M2 polarization and MMP9 production **(A)** Association between ERα expression and immune cells by ssGSEA in the TCGA-LIHC dataset. **(B)** Correlation between ERα expression and number of macrophages. **(C)** Expression of CD11b in PMA-polarized THP-1 and THP-1 cells by flow cytometry. **(D-E)** Mφ were collected after co-culture with HepG2 and Huh7 cells treated with si-NC, siERα, PWPI, and oeERα to test changes in the expression of CD68 and CD163. **(F)** Change in MMP9 expression was tested in Mφ collected after coculture with HepG2 and Huh7 cells treated with si-NC, siERα, PWPI, and oeERα. Experiments were performed with at least three replicates. The Student's t-test was used to compare the means between groups. * $P < 0.05$

for 48 h. Flow cytometry analysis showed that the polarized THP-1 cells significantly expressed CD11b, indicating that THP-1 cells were polarized to Mφ (Fig. 3C). We established a co-culture system of HCC cells and Mφ, and tested the expression of cytokines in macrophages using qPCR. When Mφ were co-cultured with HCC cells overexpressing ERα, CD68 (a hallmark of the M1 phenotype) was upregulated, whereas CD163 (a sign of the M2 phenotype) was downregulated. In contrast, a decrease in ERα expression induced a reduction in CD68 and increased CD163 expression (Fig. 3D, E). According to previous studies, TAMs can produce large amounts of matrix metalloproteinases (MMPs), particularly MMP9, an essential enzyme that degrades the extracellular matrix. When MMP9 is elevated, the extracellular basement membrane accelerates disassembly and promotes tumor invasion. Accordingly, we examined the expression of MMP9 in Mφ co-culture with HCC cells. Our data showed that decreased ERα levels in HepG2 and HuH7 cells induced Mφ to increase MMP9 mRNA expression (Fig. 3F).

Taken together, ERα alters the status of macrophages in the TME and its reduction promotes the development and invasion of HCC.

ERα decreased infiltrating M2 macrophages by lowering CCL2

Transwell migration assays of Mφ revealed that decreasing ERα led to increased Mφ recruitment (Fig. 4A), and increasing ERα led to reduced Mφ recruitment (Fig. 4B). The addition of E2 (10 nM) to the co-culture medium with low-ERα HCC cells could also reduce Mφ recruitment (Fig. 4A). To elucidate the mechanism by which ERα in HCC cells suppresses M2 macrophage invasion, we investigated the cytokines associated with macrophage recruitment [22, 23]. The HepG2 and HuH7 cells with overexpressed or knocked down ERα were tested through qPCR. Modifying ERα expression in HepG2 and HuH7 cells may result in changes in the mRNA expression levels of CCL2, IFN-γ, TNF-α, CX3CL1, CSF, CCL18, IL-4, IL-6, and IL10 (Fig. 4C, D). In ERα-overexpressing HCC cells, the expression of CCL2, IL-6, and IFN-γ was decreased, whereas that of TNF-α was upregulated. Conversely, HCC cells with lower ERα levels induced higher CCL2, CSF, IL-4, and IL10 levels, and lower levels of IL-6 (Fig. 4C, D). Simultaneously, cytokine levels in the co-culture medium of HepG2 cells with treated ERα and Mφ were determined by ELISA to screen for key cytokines. CCL2 showed the same trend as qPCR that the expression of CCL2 decreased at high levels of ERα and increased at low ERα levels (Fig. 4E). Western blotting also showed that in HepG2 and HuH7

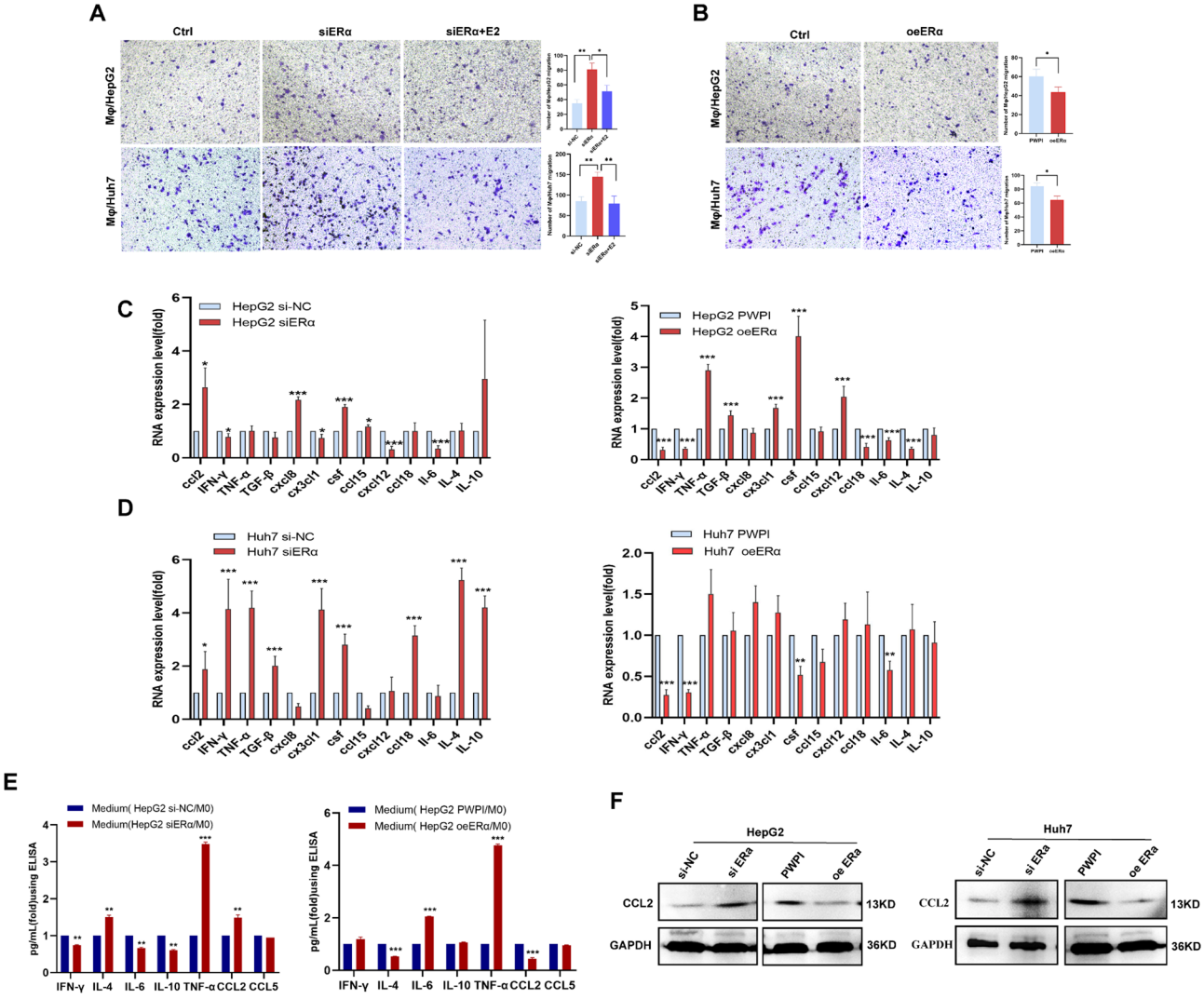

Fig. 4 ERα in liver cancer cells could decrease CCL2 expression to inhibit Mφ infiltration **(A-B)** Migrated Mφ co-cultured with HepG2 and Huh7 cells treated with si-NC and siERα or PWPI and oeERα were examined after 36 h of incubation with the 24-well transwell migration system. SiERα cells treated with E2 (10nM) decreased Mφ recruitment(A). **(C-D)** A group of chemokines and cytokines related to macrophage recruitment and polarization was tested by qPCR through overexpression and knockdown of ERα in HepG2 and Huh7 cells, respectively. **(E)** Some chemokines and cytokines related to macrophages were tested by ELISA. **(F)** Western blotting confirmed that CCL2 expression correlated with ERα expression in HepG2 and Huh7 cells. Experiments were performed with at least three replicates. Data are presented as mean ± SD, *P*-value were calculated using a two-tailed Student's t-test. One-way ANOVA tests were used for comparison among the three or more groups.*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

cells more ERα can decrease CCL2 production, and less ERα increased expression of CCL2 (Fig. 4F).

In summary, the results presented in Fig. 4 indicate that the ERα alters the infiltration of M2 macrophages through CCL2, thereby decreasing liver cancer cell invasion.

ERα decreased CCL2 expression by promoting Hippo signaling activation

To evaluate the mechanism of action of ERα on CCL2, we first explored whether ERα regulated CCL2 at the protein level. ERα markedly promoted Hippo signaling activation through GSEA (Fig. 5A). The effect of ERα on Hippo/YAP signaling pathway activation was investigated in HepG2 and HuH7 cells. qPCR revealed that YAP mRNA expression was not altered in HepG2 and HuH7 cells with ERα knockdown or overexpression (Fig. 5B). Similarly, YAP protein levels were not significantly changed in HCC cells under ERα treatment, but p-YAP was upregulated by ERα overexpression and decreased by low ERα levels (Fig. 5C). Furthermore, IF staining indicated that ERα overexpression was accompanied by high p-YAP levels in the cytoplasm of the HepG2 cells (Fig. 5D). Simultaneously, ERα overexpression also promoted the upstream activation of Hippo signaling involving p-MST and P-LATS. In contrast, ERα reduction induced low levels of p-MST and

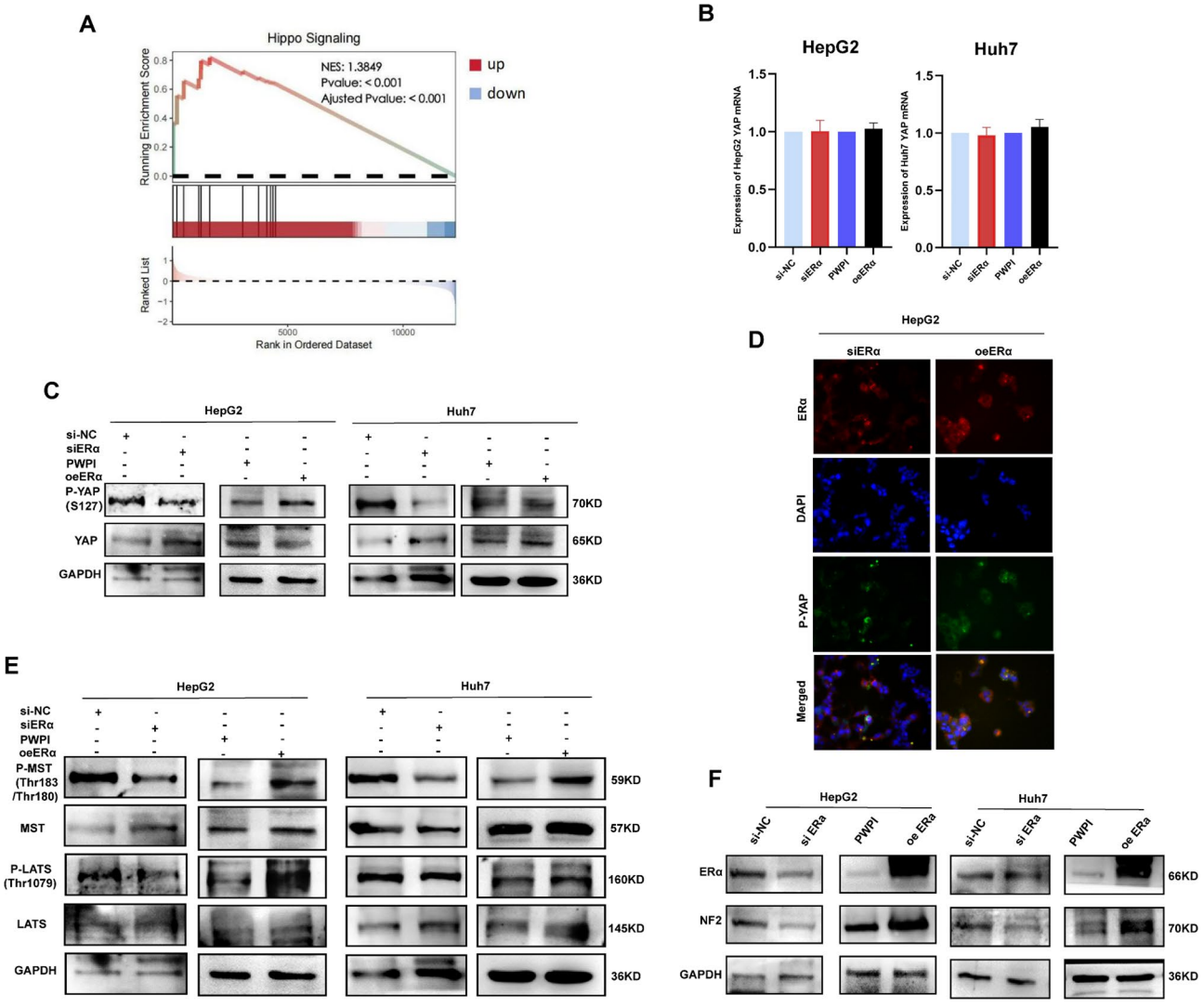

Fig. 5 ERα activated HIPPO signaling by promoting NF2 **(A)** GSEA of ERα regulation in the HIPPO pathway. **(B)** Expression of YAP in HCC cells was assessed by qPCR. **(C)** WB was used to analyze the activation status of YAP and p-YAP in HCC cells treated with siERα and oeERα. **(D)** IF staining of ERα (red) and p-YAP (green) in HepG2 cells. **(E)** phosphorylation status of MST and LATS in HCC cells treated with siERα or oeERα. **(F)** NF2 expression was promoted by ERα by WB. Data are presented as mean ± SD, *P*-value were calculated using a two-tailed Student's t-test

P-LATS (Fig. 5E). NF2 is an important upstream component of Hippo and plays a critical role in the Hippo pathway during tumorigenesis [24, 25]. NF2 was significantly up-regulated by ERα overexpression and was suppressed by low ERα expression (Fig. 5F). All in all, ERα activated Hippo signaling by promoting its upstream factor NF2.

ERα promoted P-YAP and inhibited tumorigenesis by upregulating 14-3-3theta

Abnormal ERα expression regulated CCL2 via the Hippo pathway. To investigate whether ERα can influence Hippo signaling in other ways, potential ERα-binding proteins were identified using mass spectrometry (MS) and isolated using Co-IP to explore other potential pathways. According to the MS analysis, HepG2 and HuH7 cells had 50 co-binding proteins, among which only 14-3-3theta was associated with ERα and YAP (Fig. 6A). previous studies showed 14-3-3 protein binded p-YAP and inhibited YAP nuclear translocation [26]. 14-3-3theta was confirmed to be linked with ERα by Co-IP in HuH7, indicating that 14-3-3theta formed a complex with ERα (Fig. 6B). ERα overexpression upregulated 14-3-3theta. Conversely, lower ERα expression induced a low the 14-3-3theta expression(Fig. 6C). ERα overexpression reduced YAP nuclear translocation (Fig. 6D). Furthermore, IHC demonstrated that YAP expression in the cytoplasm was higher in ERα (+) HCC samples than in ERα (-) samples (Fig. 6E). Together, these results indicate that ERα promoted the activation of Hippo signaling by upregulating NF2 and prevents YAP from moving to

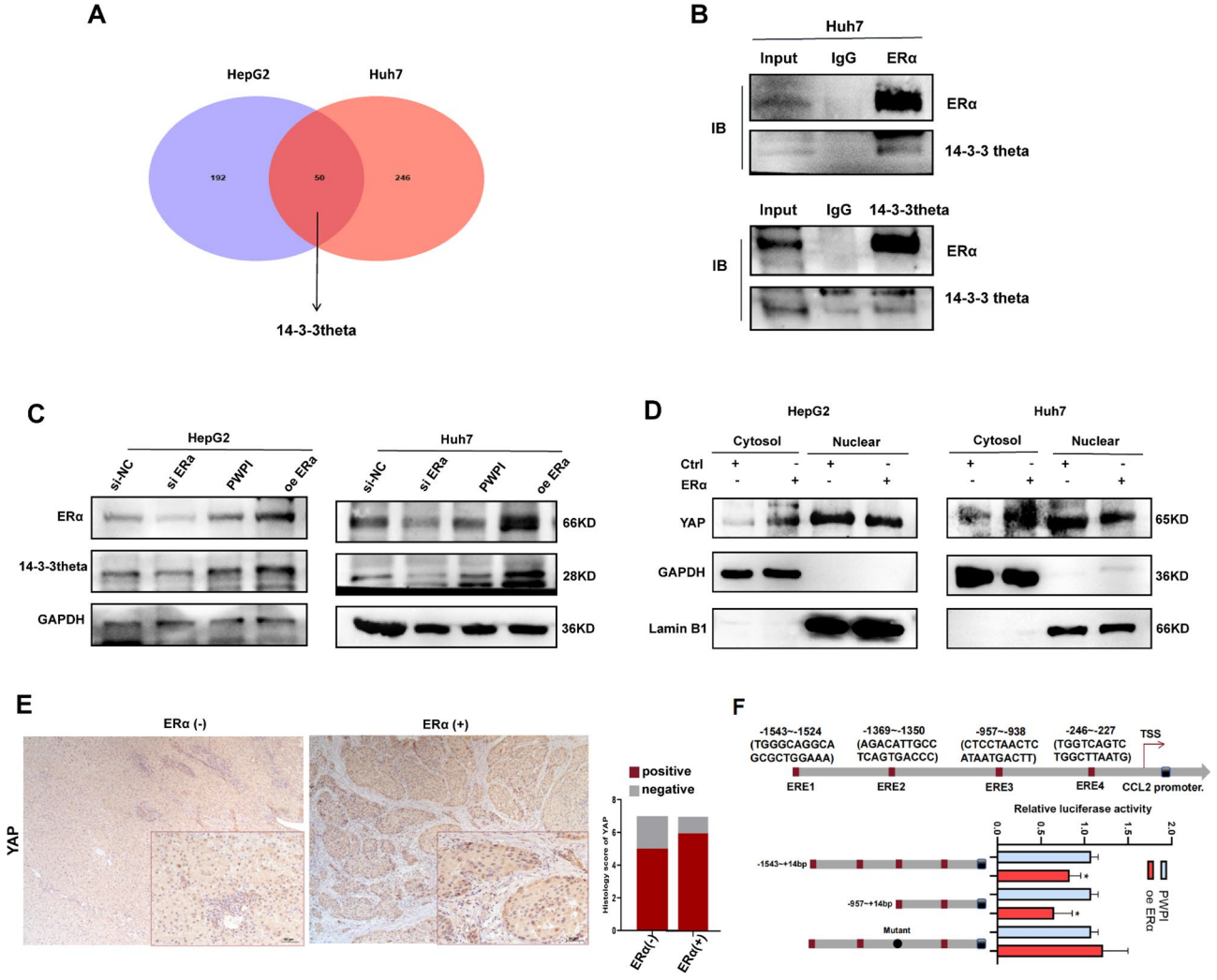

Fig. 6 ERα attenuated the nuclear translocation of YAP through upregulating 14-3-3theta **(A)** Venn diagram showing the potential binding protein of ERα, and 14-3-3theta was identified by MS analysis of Co-IP isolated proteins in both HepG2 and Huh7 cells. **(B)** IB was used to confirm 14-3-3theta protein obtained by co-IP in Huh7 cells. **(C)** 14-3-3theta expression was positively correlated with ERα expression by WB. **(D)** WB was used to detect the cytoplasmic and nuclear expression of YAP. The cells were treated with either siERα or oeERα. **(E)** IHC analyze YAP cytoplasmic expression in ERα (–) and ERα (+) HCCs. **(F)** Luciferase activity assay was conducted in HepG2 cells. Data are presented as mean ± SD from biological replicates. *P*-value were calculated using two-tailed Student's t-test. *, $P < 0.05$

the cytoplasm via nuclear translocation by upregulating 14-3-3theta in HCC cells.

ERα can change the transcriptional regulation to reduce CCL2 expression

Because ERα can decrease CCL2 expression at the mRNA level, we performed a dual-luciferase reporter assay to investigate whether ERα can alter CCL2 expression through transcriptional regulation. Previous studies identified four transcription factor-binding sites for the estrogen response element (ERE) in the CCL2 promoter region. ERα promoted CCL2 activity by binding to ERE3 in the promoter region in lung cancer [9]. We focused on examining whether ERα could modulate CCL2 expression at the transcriptional level in HCC. When co-transfected with the CCL2 promoter plasmid, CCL2 luciferase activity was higher in PWPI plasmids than in ERα (+) plasmids. Furthermore, increasing ERα expression in HepG2 cells decreased luciferase activity for the pGL3 reporter plasmids containing wild-type ERE3, but luciferase activity did not change in plasmids containing mutant ERE3, indicating that ERα combined with ERE3 of CCL2 promoted the reduction of CCL2 (Fig. 6F). In conclusion, these findings indicated that ERα reduced Mϕ invasion in HCC through transcriptional control of CCL2 expression. The mechanism by which ERα decreased CCL2 expression to reduce M2-type macrophage infiltration in HCC is shown in Fig. 7.

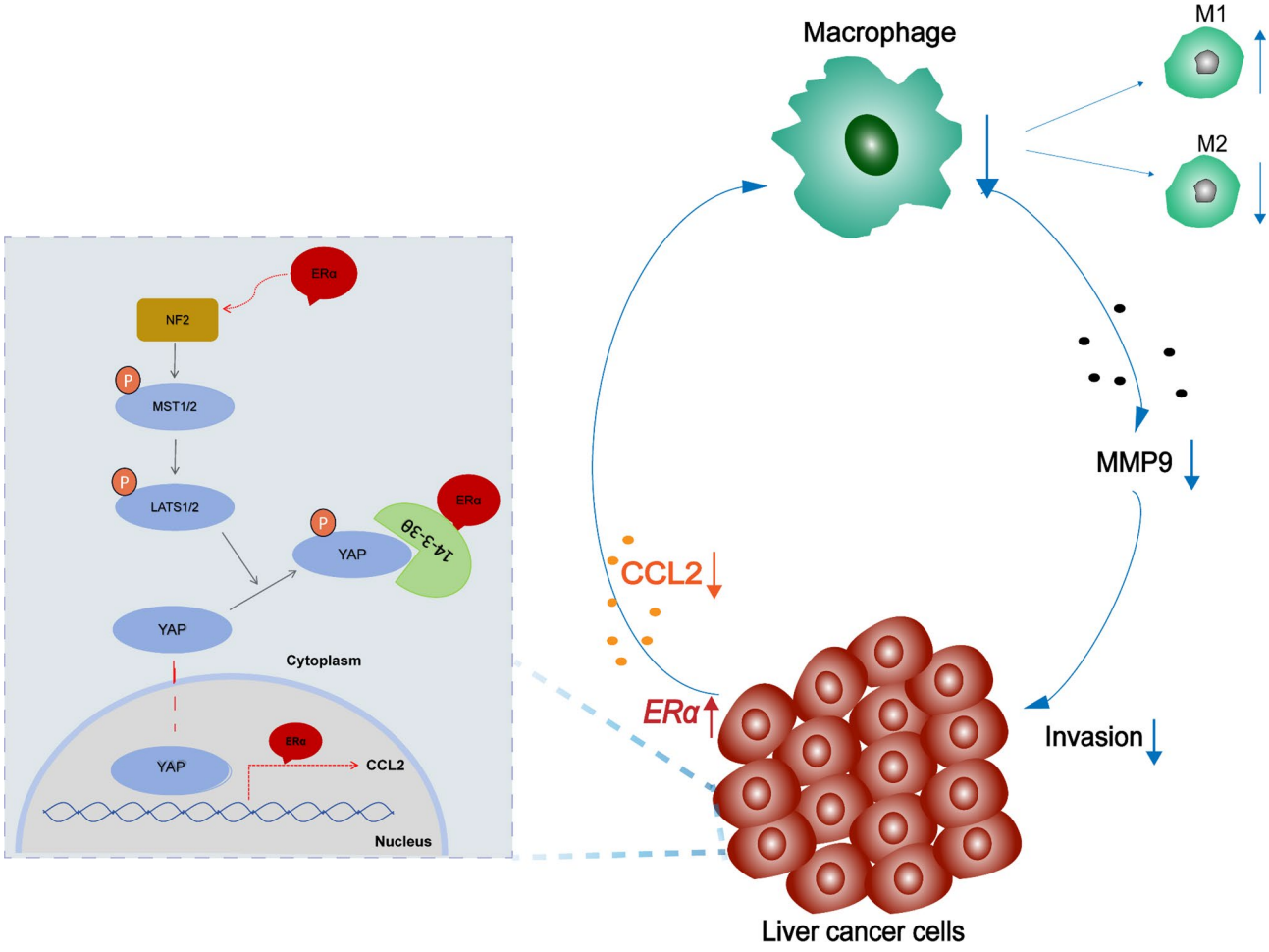

Fig. 7 A map of the molecular mechanism is presented

Discussion

As the liver possesses the highest number of sex-biased genes in addition to reproductive organs, it is considered the most transcriptionally dimorphic organ, showing marked sex differences [2, 27, 28]. Males vs. females and premenopausal vs. postmenopausal women show many differences in progression of viral- and non-viral liver diseases [29–34].

The estrogen receptor is a nuclear receptor family that includes ERα, estrogen receptor β, and membrane estrogen receptor G protein-coupled receptor 30. ERα (also known as ERα66) has two main isoforms: ERα46 and ERα36 [35], which functions in genomic and non-genomic signaling pathways. It is predominantly located in the nucleus and functions in genomic activity [36]. ERα has been widely studied in gonadal organs and in some other organ tumors, such as breast, prostate, and lung cancers, with distinct functions.In liver, it is present in non-tumoral and early cirrhotic liver tissue. But is reduced or undetectable in the HCC tissues [37]. One reason for this decline is that persistent liver tissue injury and an inflammatory microenvironment in cirrhosis and HCC lead to ERα mutations, epigenetic modifications, or post-translational modifications [38]. In addition, enhancing the aromatase-induced inflammatory microenvironment may promote changes in ERα-splice variants from ERα66 to ERα36 [39].

Men are more likely to develop HCC than premenopausal women, but the difference between men and women disappears after the women's menopause [32]. By analyzing Table 3 about clinical information, we also reached the same conclusion: there were significant differences in the expression of ERα between different genders and those aged over 60 years and under 60 years.At the same time, we also found that there were also significant differences in the expression of ERα in different clinicopathological T stages, but no differences in different N and M stages. Therefore, we believe that HCC patients without lymph node and distant metastasis in women younger than 60 years of age may have a better prognosis and targeted ERα therapy may have a good therapeutic effect in this population.

Previous studies have shown that ERα is involved in liver gene expression patterns and the regulation of liver immune responses. ERα suppresses HCC occurrence and development by inhibiting Wnt/β-catenin and activating Hippo signaling [6, 7] and controls IL-6 via NF-κB or JAK/STAT signaling to attenuate fibrosis and chronic liver disease progression [40, 41]. Moreover, we collected clinical information of 10 cases from GSE215011 dataset to evaluate the response of ERα to Nivolumab immunotherapy. It can be seen that the expression of ERα in the responsive group was higher than that in the non-responsive group, patients with high ERα expression may have a better trend of immunotherapy response($p = 0.15$) (Figure. S5). However, it is unknown how ERα acts on immune cells to inhibit HCC development in the tumor microenvironment.

Our bioinformatics analysis revealed that ERα was most strongly associated with macrophages among the 23 immune cells in HCC. Macrophages are particularly abundant in the liver, which are mainly derived from liver-resident macrophages (Kupffer cells KCs) and monocyte-derived macrophages. KCs persist in the body for a long time and rely on local proliferative signaling molecules for self-renewal [42]. When the liver is damaged, KCs is significantly reduced and a large number of monocyte-derived macrophages are recruited in liver [43]. Contribution of macrophages of different origin to tumors is of great different, Monocyte-derived macrophages are more likely to aggravate liver injury than KCs [44]. Therefore, reducing the recruitment of monocyte derived macrophages is of great significance for targeting tumor-associated macrophages in the tumor microenvironment.

The phenotypes of KCs in response to different stimuli were classified as M1(pro-inflammatory, marked CD68 or CD86) and M2(anti-inflammatory, marked CD163 or CD 206). In the early stages of tumor, M1 macrophages are infiltrating into tumors to suppress tumor progression. With cancer progression, more M2 macrophages are infiltrated into the tumor immune microenvironment [45]. In our vitro study, overexpression of ERα in HCC cells decreased macrophage polarization to M2 type, while ERα depletion promoted macrophage polarization to M2 type, indicating anti-tumor effects of ERα by inhibiting M2-type polarization of macrophages. Among the various macrophage chemokines, ERα significantly suppresses CCL2 expression in HCC. CCL2 is a key chemokine of the C-C chemokine family that regulates the migration and infiltration of monocyte-derived macrophages [46]. Previous studies have shown that ERα promotes expression of CCL2 by binding to the ERE3 promoter region of CCL2 in lung cancer [9]. We conducted a dual-luciferase reporter assay to validate the effects of ERα on CCL2 transcription in HCC cells. However, ERα inhibited CCL2 through the promoter ERE3 region of CCL2. The different effects of ERα on CCL2 may be because ERα played different roles in various cells, showing tissue specificity.

Hippo signaling plays an important role in the promotion of hepatocarcinogenesis [47]. It mostly consists of MST, LATS, and YAP/TAZ. YAP/TAZ, as hub genes in this pathway, promote the expression of a number of genes involved in cell proliferation [48]. Abnormalities in YAP drive the onset of diseases from hepatitis to liver cancer via the Hippo regenerative pathway [49–51]. YAP promoted HCC progression and was closely related to poor prognosis of HCC [52]. As a target gene of YAP, CCL2 is regulated by YAP. YAP/TEAD4 promoted angiogenesis of liver cancer by regulating the expression of CCL2 [19]. Similarly, in a study of cardiac dysfunction, YAP increased macrophage infiltration by promoting CCL2 expression [17].

ERα is deeply involved in regulating Hippo signaling in breast cancer. A study showed that YAP/TAZ represses ESR1 transcription by NCOR2/SMRT in ERα(+) breast cancer [53]. But, other studies have shown that YAP, as an ERα cofactor, promoted growth in ERα(+) breast cancer [54]. However, ERα activated Hippo signaling in the HCC [6]. Our results showed that ERα inhibited the expression of CCL2 by acting on NF2 to trigger Hippo phosphorylation and weaken the nuclear translocation of YAP. However, whether ERα is regulated by YAP in the liver is still unknown and requires further investigation. The other hand, using Co-IP and MS, 14-3-3theta was identified bound to ERα.14-3-3 protein is closely related to YAP and inhibit YAP nuclear localization [55]. ERα further inhibited YAP nuclear localization through upregulating 14-3-3theta.

ERα plays an important role in the regulation of many liver diseases. This study mainly focuses on the effect of ERα on the tumor immune microenvironment in HCC. This study on the effect of ERα on macrophages infiltration reduction in HCC has only been conducted in vitro, and further verification in animal experiments is required.

Conclusion

This study showed that ERα is an important gene associated with sex differences, and is involved in the regulation of macrophages in HCC. It inhibits CCL2 expression by activating Hippo signaling by acting on NF2 and binding to 14-3-3theta, and subsequently reducing M2 cell infiltration. At the same time, ERα also reduced the expression of CCL2 on the transcriptional level. These results imply that ERα is a prospective therapeutic target and possible prognostic biomarker for HCC, especially for women younger than 60 years of age with early HCC, targeting ERα may have a better therapeutic effect.

Estradiol can enhance the expression of ERα, and some clinical studies have confirmed that E2 inhibits the development of HCC [56]. However, estrogen not only acts on ERα66, but also on ERβ and ERα36 receptors, which have a different biological function from ERα66 and may affect the treatment efficacy of estrogen [57]. Moreover, estrogen is limited in the treatment of liver cancer to a certain extent due to the potential increase in the risk of breast cancer, endometrial cancer, and other tumors. Given the important role of ERα in liver tumors, the effect of estrogen replacement on ERα and its isoforms in patients with HCC requires further investigation. Furthermore, ERα significantly influences the Hippo pathway in HCC. Activated Hippo signaling decreases macrophage infiltration by reducing cytokine CCL2. Thus, targeting YAP may be a promising therapeutic approach for individuals with ERα (-) HCC.

Abbreviations

HCC	Hepatocellular carcinoma
ERα	Estrogen receptor alpha
MS	Mass spectrum
Mφ	Macrophage
E2	17-β estradiol
IHC	Immunohistochemistry
TAMs	Tumor-associated macrophages
TME	Tumor microenvironment
ERE	Estrogen response element
KCs	Kupffer cells

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12885-025-13676-1.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Supplementary Material 6

Supplementary Material 7

Supplementary Material 8

Author contributions

D-H W wrote the original draft; D-W H provided conceptualization; T-T L designed the Methodology; X-K Z performed data analysis; Z-J L participated in the interpretation of the data; Z-Y W and D-W H revised the manuscript and performed project administration.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

All methods were carried out in accordance with relevant guidelines and regulations, and informed consent to participate was obtained from all of the participants in the study.

Consent for publication

The authors consent to publish this manuscript.

Competing interests

The authors declare no competing interests.

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