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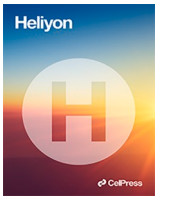

Research article

NCLN as a potential prognosis biomarker in endometrial cancer

Huang-jin Luo [a,b,c,d,1], Li-tong Zhu [a,1], Yu Dai [a], Yun Ma [a], Kai Wang [a], Lei Zhang [a], Qiu-xia Li [a], Ping Jin [a,*]

[a] Department of Gynecology, Shenzhen Maternity and Child Healthcare Hospital, Guangdong, China
[b] Department of Gynecology, Shengli Clinical Medical College of Fujian Medical University, Fujian Provincial Hospital, Fuzhou, Fujian, China
[c] Department of Gynecology, Fujian Provincial Hospital, Fuzhou, Fujian, China
[d] Department of Gynecology, Fuzhou Affiliated Provincial Hospital, Fuzhou, Fujian, China

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ABSTRACT

Endometrial cancer (ECs) stands as one of the three major malignancies impacting females globally, with its incidence steadily increasing. ECs can be categorized into two types based on their clinicopathological features. Type I ECs typically exhibit low stage, favorable histological types, and low histological grade, correlating with a more favorable prognosis. Conversely, Type II ECs present with advanced-stage disease, aggressive behavior, and poorer histological types, resulting in a worse prognosis.

The expression level of progesterone receptors (PR) holds significant importance in determining the prognosis of patients with ECs. Elevated levels of PR are linked to a more favorable prognosis, primarily attributed to progesterone's inhibitory influence on cancer cell proliferation and invasion. Moreover, progesterone promotes cell cycle inhibition through its regulation of PR, further contributing to improved outcomes in ECs.

Nicalin (NCLN) plays a crucial role in facilitating the translocation of multichannel membrane proteins to the endoplasmic reticulum membrane and is implicated in embryonic development. Structurally akin to NODAL Modulator (NOMO), NCLN antagonizes NOMO during embryogenesis, forming a complex that antagonizes the Nodal pathway, thereby influencing mesoderm development. However, the precise relationship between NCLN and ECs remains incompletely understood.

Our research findings reveal that NCLN actively stimulates the proliferation of ECs cells and exhibits a positive correlation with PR, albeit without impacting ER. Moreover, the expression levels of NCLN in ECs demonstrate associations with distinct histological types. These observations suggest that NCLN could emerge as a promising marker in the histological classification of ECs.

1. Introduction

ECs stands out as a significant concern among gynecological cancers, originating from the endometrium and ranking among the top three malignancies affecting the female reproductive system [1]. Its incidence is on the rise globally. According to the 2022 cancer

* Corresponding author. Shenzhen Maternity and Child Healthcare Hospital, Guangdong, China.
E-mail address: pingjin66@126.com (P. Jin).
[1] These authors contributed equally to this study.

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incidence report released by the China Cancer Center, there were approximately 77,700 new cases of endometrial cancer, making it the second most common cancer of the female reproductive system, and the number of deaths was around 13,500, ranking it third in terms of mortality among female reproductive system cancers [2]. Similarly, in the United States in 2022, the American Cancer Society documented approximately 65,950 new cases, leading to about 12,550 deaths among women [3]. Clinically, ECs can be categorized into two main types based on their distinct clinicopathological characteristics. Type I ECs, known as endometrioid adenocarcinomas, typically present with lower stages, favorable histological types, and lower histological grades, contributing to a relatively better prognosis. In contrast, Type II ECs tend to manifest as advanced-stage cancers with aggressive behavior and poorer histological types, leading to a worse prognosis [4]. Notably, Type I ECs often exhibit significant expression of ER and PR, whereas Type II ECs typically lack these receptors.

The endometrial cycle is intricately regulated by the interplay of estrogen and progesterone [5]. ER and PR serve as crucial mediators in the pathophysiology of ECs [6]. Estrogen binds to ER, stimulating epithelial proliferation, while progesterone inhibits endometrial growth and fosters cell differentiation through PR activation. Notably, a higher level of PR expression correlates with improved better prognosis in ECs. Progesterone exerts its antineoplastic effects by suppressing the proliferation and invasion of endometrial cancer cells and promoting cell cycle arrest through PR regulation [7]. Moreover, PR exerts its inhibitory effects on endometrial carcinogenesis by down-regulating the transcriptional activity of the activating protein-1 (AP-1) transcription factor family. Progesterone significantly inhibits AP-1 induction, thereby mitigating the development of ECs [8]. Additionally, PR and its associated complexes induce the expression of A20 and ABIN-2, which subsequently attenuate NF-κB (NFkappaB) transcriptional activity in ECs cells [9].These intricate regulatory mechanisms highlight the multifaceted roles of progesterone and its receptor in the pathogenesis and potential therapeutic targeting of ECs.

NCLN, a key component of the ribosome-associated translocator complex, plays a vital role in facilitating the translocation of multi-pass membrane proteins to the endoplasmic reticulum membrane [10]. Structurally akin to NOMO, NCLN interacts with NOMO during embryonic development to form a complex that antagonizes the Nodal pathway, thereby impacting mesoderm development [11,12]. Importantly, the antagonistic effect of NCLN on the Nodal signaling pathway is contingent upon its dosage [13]. Furthermore, NCLN regulates cellular autophagic activity by modulating the LC3-interacting region (LIR) of ATG8 [14].

Despite these well-documented roles, the literature currently lacks detailed insights into the potential involvement of NCLN in gynecological cancers. Therefore, this study aims to elucidate whether NCLN plays a significant role in the pathogenesis of endometrial cancers. By investigating the function of NCLN in the context of ECs, this research endeavors to uncover potential novel pathways or mechanisms underlying the development and progression of this malignancy.

2. Materials and methods

2.1. Data acquisition

The information of 552 patients with ECs and 35 normal samples are sourced from The Cancer Genome Atlas (TCGA) database (https://portal.gdc.cancer.gov/). All data analysis procedures adhered to the publication guidelines outlined by TCGA (https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga/using-tcga).

2.2. Differentially expressed gene (DEGs) analysis

The expression data (HTseq-Counts) was stratified into high and low expression cohorts using the median expression level of NCLN as the dividing point. Subsequently, unpaired Student's t-test was employed to scrutinize the expression patterns within the DESeq2 R package (version 3.6.3) [15]. Criteria for identifying DEGs included adjusted p-values of less than 0.05 and absolute log2-fold changes greater than 1.5.

2.3. Enrichment analysis

The Gene Ontology (GO) functional enrichment analysis was conducted using the ClusterProfiler package in R (version 3.6.3) [16]. The GO analysis encompassed the exploration of cellular components (CC), molecular functions (MF), and biological processes (BP). Additionally, the normalized enrichment score (NES) and adjusted *p*-value were employed to prioritize the enriched pathways (Hallmark gene sets). Significance thresholds for pathway enrichment were set at a false discovery rate (FDR) below 0.25 and adjusted *p*-values less than 0.05.

2.4. Endometrium tissue

We isolated and cultured primary cells from the endometrium of normal patients due to the absence of commercially available normal cell lines. All enrolled normal patients met stringent inclusion criteria. These criteria included undergoing hysteroscopy at Shenzhen Maternity and Child Healthcare Hospital between September 2019 and June 2020, being aged between 20 and 35 years old, refraining from hormone and immune-related drug treatments for at least six months, displaying no abnormalities on pelvic imaging and cervical screening, having no history of organ transplantation, endometriosis, gynecologic endocrine disorders, or secretable hormone diseases, and not undergoing uterine operations or being pregnant within the preceding six months. Moreover, tissue samples were scrutinized for any abnormalities based on pathology, and any identified abnormalities led to the exclusion of the tissue sample.

The protocol received approval from the institutional ethics committee (Identifier: SIAT-IRB-170315-H0157), and all patients provided written informed consent prior to participation.

2.5. Extracting human endometrial cells

We extracted the cells according to the method of isolation of mesenchymal cells [17]. Endometrial tissue with the size of 1 cm*1 cm was collected by hysteroscope and dissociated using mechanical and enzymatic digestion with a few modifications. Shortly, the tissue pieces were washed three times in D-Hanks (Gibco, 14170112, USA) with penicillin/streptomycin (Gibco, 15140122, USA) and cut up by aseptic scissors before dissociation in DMEM/F-12 (Gibco, 11320033, USA) containing 1 % penicillin/streptomycin, 0.5 % collagenase I (Gibco, 17100017, USA), 20UI DNase I (Invitrogen, 18047019, USA) for 60 min at 37 °C in an incubator (Themo Scientific, USA).

The resulting cell solution was filtered through a sterile 100-μm cell strainer (Biologix, 15–1100, China) to separate single cells from undigested tissue fragments. The filtering cell solution was filtered through a sterile 70-μm cell strainer (Biologix, 15–1070, China) to separate epithelial cells and mesenchymal cells. Endometrial epithelial cells are above the filter and endometrial mesenchymal cells are in the cytosol. After washing with a complete medium with DMEM/F-12 and 10 % fetal bovine serum (Gibco, 10099141C, USA) and 1 % penicillin/streptomycin, and centrifugation for 5 min at 1000×*g*, the resulting cell pellets were resuspended in D-Hanks, respectively.

2.6. Cell cultures

All cell lines were from ATCC (USA). Ishikawa and RL95-2 were cultured in DMEM/F-12 with 10 % FBS, and 1 % penicillin/streptomycin. The RL95-2 is an epithelial-like cell that was isolated from the endometrium of a White, 65-year-old patient with carcinoma. The Ishikawa is a well-differentiated endometrial adenocarcinoma cell line, from a 39-year-old Japanese patient. Immortal cells were maintained in DMEM/F-12 without phenol red (Gibco, 21041025, USA) with 10 % FBS, and 1 % penicillin/streptomycin. Cells were cultured at 37 °C in an incubator containing 5 % CO_2.

2.7. Construction of the over expression of NCLN and shNCLN cell lines

To establish the Ishikawa and RL95-2 NCLN-knockdown (sh) cell lines and the immortal NCLN-overexpression cell lines, we used the lentivirus designed by HanBio Company (Shanghai, China) to transfect the cells. We used the lentivirus empty vector for the control group (nc).

2.8. Quantitative real-time PCR (qPCR)

TRIzol kit (Invitrogen, 15596018CN, USA) is used to isolate total RNA and the Prime-Script RT kit (Takara, RR037A, Japan) to transcribe to cDNA immediately. Quantitative real-time PCR was carried out in 96-well plates using the StepOne Plus Real-Time PCR System (BioRad, USA). SYBR Green PCR Master Mix (Takara, RR037A, Japan) amplified cDNA after reverse transcription (Roche, Switzerland). Experiments were performed in five copies. B-actin was the control group. The sequences of primer are shown in Table S1 (Table S1).

2.9. Western blot

The primary antibodies included anti-NCLN (Abcam, ab170100), anti-estrogen receptor (Abcam, ab108398), anti-progesterone receptor (Abcam, ab32085), anti-GAPDH (Abcam, ab8245), Anti-FoxM1 (Abcam, ab207298), Anti-MAPK (Cell Signaling Technology, 9926T) and Anti-Erk (Cell Signaling Technology,4370S). The total protein lysates and details of the Western blot were according to the Abcam supports.(https://www.abcam.com/protocols/general-western-blot-protocol).The immunoreactive bands were visualized by the ECL Plus system (BioRad, USA).

2.10. MTT and CCK8

The cells were seeded in 96-well plates. Each cell line was seeded in 20 wells and five plates. MTT Cell Proliferation and Cytotoxicity Assay Kit (Sigma, CGD1, USA) evaluated the absorbance at 490 nm at 24 h, 48 h, 72 h, 96 h, and 120 h, respectively. The cells were seeded in 96-well plates. Each cell line was seeded in 20 wells and seeded in five plates. Cell Counting Kit-8 (Dojindo, CK04, Japan) evaluated the absorbance at 450 nm at 24 h, 48 h, 72 h, 96 h, and 120 h.

2.11. Immunofluorescence staining

The cells were seeded in six-well plates. Cells were incubated with anti-CK 7 antibody (Abcam, ab68459), anti-vimentin (Abcam, ab92547), and DAPI-Fluoromount-G (Abcam, ab104139). The protocol was described in the Abcam supports(https://www.abcam.com/protocols/immunocytochemistry-immunofluorescence-protocol).

Table 1
Demographic and clinical characteristics of patients with endometrial carcinoma in TCGA-UCEC, stratified by high and low NCLN expression.

Characteristic	Low expression of NCLN	High expression of NCLN	*p*	$\chi 2$
n	271	272		
Clinical stage, n (%)			<0.001	20.095
Stage I	144 (26.5 %)	195 (35.9 %)		
Stage II	33 (6.1 %)	18 (3.3 %)		
Stage III	76 (14 %)	48 (8.8 %)		
Stage IV	18 (3.3 %)	11 (2 %)		
Histological type, n (%)			<0.001	44.280
Endometrioid	171 (31.5 %)	236 (43.5 %)		
Mixed	12 (2.2 %)	10 (1.8 %)		
Serous	88 (16.2 %)	26 (4.8 %)		
Histologic grade, n (%)			0.051	5.937
G1	43 (8.1 %)	55 (10.3 %)		
G2	51 (9.6 %)	69 (13 %)		
G3	169 (31.8 %)	145 (27.3 %)		
Tumor invasion (%), n (%)			0.739	0.181
<50	125 (26.6 %)	134 (28.5 %)		
≥50	106 (22.6 %)	105 (22.3 %)		
Menopause status, n (%)			0.143	3.894
Pre	12 (2.4 %)	23 (4.6 %)		
Peri	8 (1.6 %)	9 (1.8 %)		
Post	229 (46.1 %)	216 (43.5 %)		
Diabetes, n (%)			0.174	2.149
No	150 (33.9 %)	171 (38.7 %)		
Yes	66 (14.9 %)	55 (12.4 %)		
OS event, n (%)			0.003	9.744
Alive	212 (39 %)	240 (44.2 %)		
Dead	59 (10.9 %)	32 (5.9 %)		
DSS event, n (%)			0.018	6.300
Alive	230 (42.5 %)	251 (46.4 %)		
Dead	39 (7.2 %)	21 (3.9 %)		
PFS event, n (%)			0.018	6.135
Alive	197 (36.3 %)	222 (40.9 %)		
Dead	74 (13.6 %)	50 (9.2 %)		
Age, median (IQR)	65 (59, 73)	62 (55, 69)	<0.001	
BMI, median (IQR)	31.65 (26.49, 38.51)	32.83 (26.34, 39.45)	0.649	

2.12. HE (Hematoxylin-eosin staining) and Papanicolaou staining

All cell lines were seeded in 24-wells plate with cell crawling. After 24 h, cells were fixed in 4 % paraformaldehyde at room temperature for 60 min. HE stain kit (Solarbio, G1120, China) and Papanicolaou stain kit (Solarbio, G1614, China) to validate the nuclear heterotype.

2.13. Statistical analysis

The statistical data with TCGA database was analyzed by R 3.6.3. Wilcoxon rank-sum test and Wilcoxon signed-rank test was compared the expression of NCLN in ECs and the normal group. Welch one-way ANOVA was used to analyze the association of NCLN expression and the grade of clinicopathological factors. We applied the univariate logistic regression, the Fisher exact test, and normal and adjusted Pearson κ^2 tests to validate the effect of clinicopathological factors on NCLN expression. Furthermore, we combined univariate Cox regression analysis and multivariate Cox regression analysis to evaluate the prognostic value of NCLN expression and other clinicopathological factors on overall survival (OS). The pROC package depicted the receiver operating characteristic (ROC) [18], with the value range of the calculated area under the curve (AUC) from 0.5 to 1.0 indicated the discrimination ability of 50%–100 %. Kaplan–Meier (K-M) analysis was applied to analyze survival analysis. The data with CCK8 or MTT were analyzed by GraphPad Prism 9 or SPSS 25.0 to evaluate the differences between groups with the two-tailed *t*-test. *P < 0.05 in a two-tailed test was considered significant. $^{**}P < 0.01$, $^{***}P < 0.001$.

3. Result

3.1. Clinical information

The data from 543 patients with endometrial cancer and 5 enrolled patients receiving primary cells were analyzed separately. The information includes the clinical stage, histological type, histological grade, tumor invasion, menopause status, diabetes, OS event, disease-specific survival (DSS) event, progression-free survival (PFS) event, age, and BMI in ECs patients (Table 1), and age, weight, height, and body mass index (BMI) in enrolled patients (Table 2). NCLN expression showed significant correlations with clinical stage

Table 2
Patient demographics for endometrial epithelium Extraction.

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Mean
Age (Years)	32	30	32	29	33	31.20 ± 2.7
Weight (Kg)	57.00	54.50	52.00	52.00	51.00	53.30 ± 6.0
Height (cm)	163.00	160.50	159.50	162.00	160.00	161.00 ± 2.1
BMI (Kg/m^2)	21.45	21.16	20.44	19.81	19.92	20.56 ± 0.53

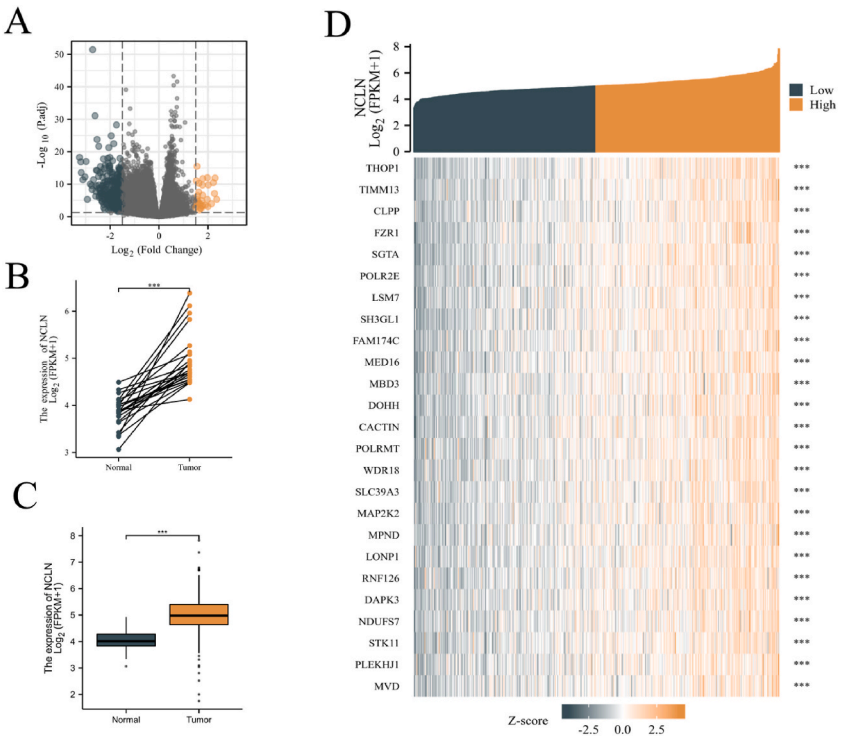

Fig. 1. Results of Differential Expression Analysis of Genes (DEGs)
(A) Volcano plot illustrating differentially expressed RNAs. (B) Differential expression of NCLN between ECs and the normal group in paired differential expression analysis. (C) Differential expression of NCLN between ECs and the normal group in unpaired differential expression analysis. (D) Heat map displaying the 25 genes correlated with NCLN.

($p < 0.001$), histological type ($p < 0.001$), OS event ($p = 0.003$), DSS event ($p = 0.018$), PFS event (p = 0.018) and age ($p < 0.001$). Other clinical parameter showed not significant associations with NCLN expression. The enrolled patient is aged between 29 and 33 years, with a BMI ranging from 19.81 to 21.45 kg/m^2. All enrolled patients met the inclusion criteria.

3.2. Differential expression analysis of NCLN in ECs

We implemented a cut-off criterion of $|\log FC| < 1.5$ and $p < 0.05$ for the analysis of the HTSeq-counts data pertaining to NCLN-related genes in TCGA. This analysis unveiled 753 upregulated and 776 downregulated DEGs. The volcano plot illustrates the expression of DEGs(Fig. 1A). Furthermore, through both unpaired and paired differential expression analyses, we observed a significant elevation in NCLN expression in tumors in contrast to normal tissue(Fig. 1B–C). This finding underscores the potential relevance of NCLN in the context of tumorigenesis. Moreover, the heatmap presented showcases the expression profiles of the 25 genes most strongly associated with NCLN (Fig. 1D). This visualization offers insights into the co-expression patterns and potential regulatory networks involving NCLN and its related genes.

3.3. Functional analysis

GO analysis of DEGs associated with NCLN revealed significant regulations in several key pathways, including neuroactive ligand-receptor interaction, G protein-coupled receptor binding, hormone activity, neuropeptide hormone activity, epidermis development, skin development, keratinocyte differentiation, and neuropeptide signaling pathway(Fig. 2A). These findings shed light on the diverse

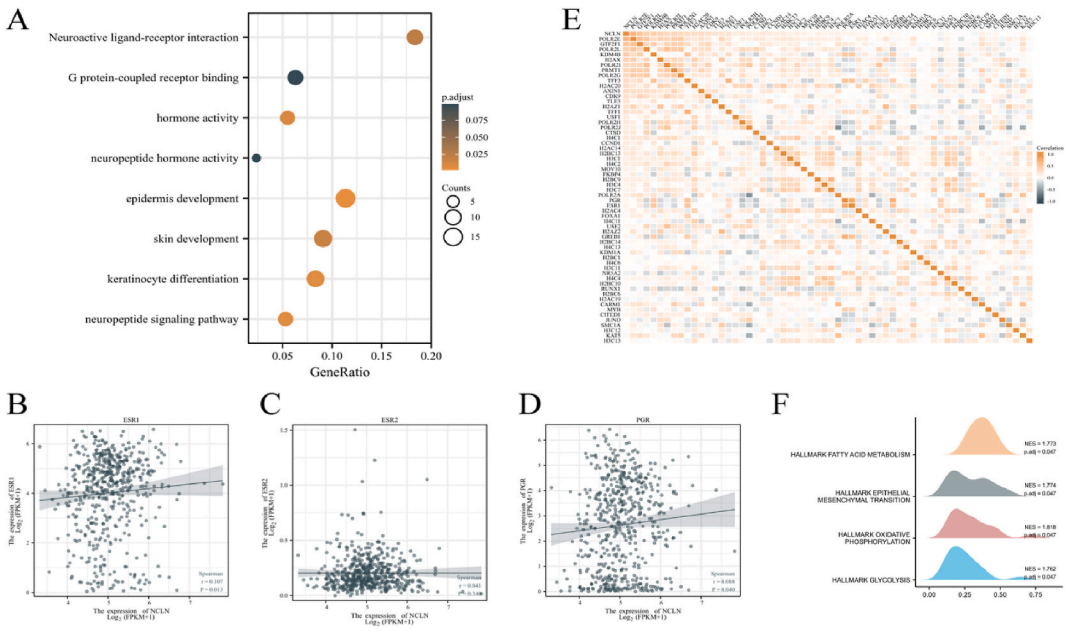

Fig. 2. Functional analysis of NCLN in ECs
(A) Biological process enriched related to NCLN-related genes. (B) Correlation analysis of NCLN and ESR1. (C) Correlation analysis of NCLN and ESR2. (D) Correlation analysis of NCLN and PGR. (E) The correlation heat map of the genes regulating estrogen and progesterone to NCLN. (F) The results of enrichment analysis from GSEA.

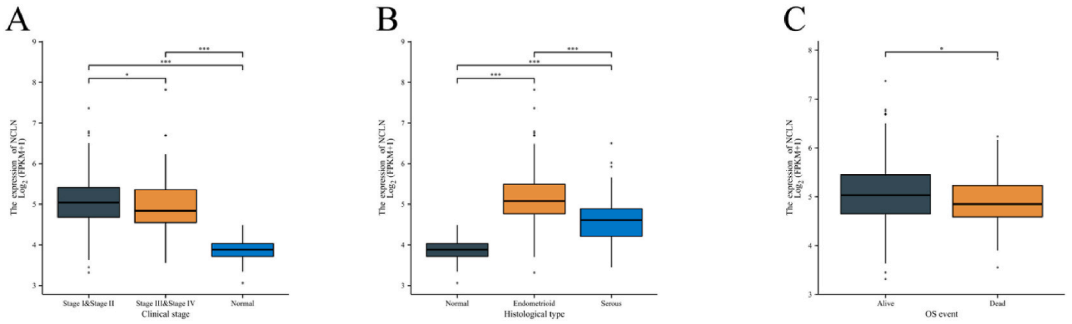

Fig. 3. Correlations in NCLN Expression and Clinicopathologic Variables
(A)Correlations in NCLN Expression and clinical stages. (B) Correlations in NCLN Expression and histological type. (C) Correlations in NCLN Expression and OS event.

Table 3
Correlation of NCLN expression with clinicopathological information analyzed by logistic regression.

Characteristics	Total(N)	Odds Ratio (OR)	P value
Clinical stage (Stage III & Stage IV vs. Stage I & Stage II)	543	0.522 (0.355–0.762)	<0.001
Histological type (Serous vs. Endometrioid)	521	0.214 (0.130–0.341)	<0.001
Histologic grade (G2&G3 vs. G1)	532	0.760 (0.487–1.180)	0.224
Tumor invasion (%) (≥50 vs. <50)	470	0.924 (0.642–1.329)	0.670
BMI (>30 vs.≤30)	512	1.218 (0.856–1.735)	0.274

biological processes potentially influenced by NCLN. Given the pivotal role of estrogen in endometrial physiology, we conducted correlation analysis to explore the relationship between NCLN expression and estrogen receptors.The results indicated a positive correlation between NCLN expression and estrogen receptor 1 (ESR1) ($r = 0.107$, $p = 0.013$) and progesterone receptor (PGR) ($r = 0.088$, $p = 0.040$), while no significant correlation was observed with estrogen receptor 2 (ESR2) ($r = 0.041$, $p = 0.340$)(Fig. 2B–D). This suggests a potential involvement of NCLN in estrogen and progesterone regulation.The correlation heatmap illustrated NCLN's potential involvement in estrogen and progesterone regulation(Fig. 2E). GSEA analysis suggested NCLN's potential participation in

Table 4
Clinicopathological Variables related to overall survival (OS) in ECs patients analyzed by univariate and multivariate analyses.

Characteristics	Total(N)	Univariate analysis		Multivariate analysis	
		Hazard ratio (95 % CI)	P value	Hazard ratio (95 % CI)	P value
Clinical stage	551				
Stage I	341	Reference			
Stage II&Stage III&Stage IV	210	3.270 (2.145–4.984)	**<0.001**	2.928 (1.678–5.107)	**<0.001**
Histological type	527				
Endometrioid	409	Reference			
Serous	118	2.646 (1.726–4.057)	**<0.001**	1.364 (0.749–2.482)	0.310
Histologic grade	540				
G1	98	Reference			
G2&G3	442	11.604 (2.855–47.167)	**<0.001**	5.280 (1.258–22.170)	**0.023**
Tumor invasion (%)	473				
<50	259	Reference			
≥50	214	2.813 (1.744–4.535)	**<0.001**	1.889 (1.094–3.264)	**0.023**
Age	549				
≤60	206	Reference			
>60	343	1.847 (1.160–2.940)	**0.010**	1.659 (0.904–3.046)	0.102
BMI	518				
≤30	211	Reference			
>30	307	0.967 (0.636–1.470)	0.876		
Diabetes	451				
No	328	Reference			
Yes	123	1.172 (0.731–1.878)	0.510		
NCLN	551	0.756 (0.580–0.984)	**0.038**	1.200 (0.842–1.710)	0.314

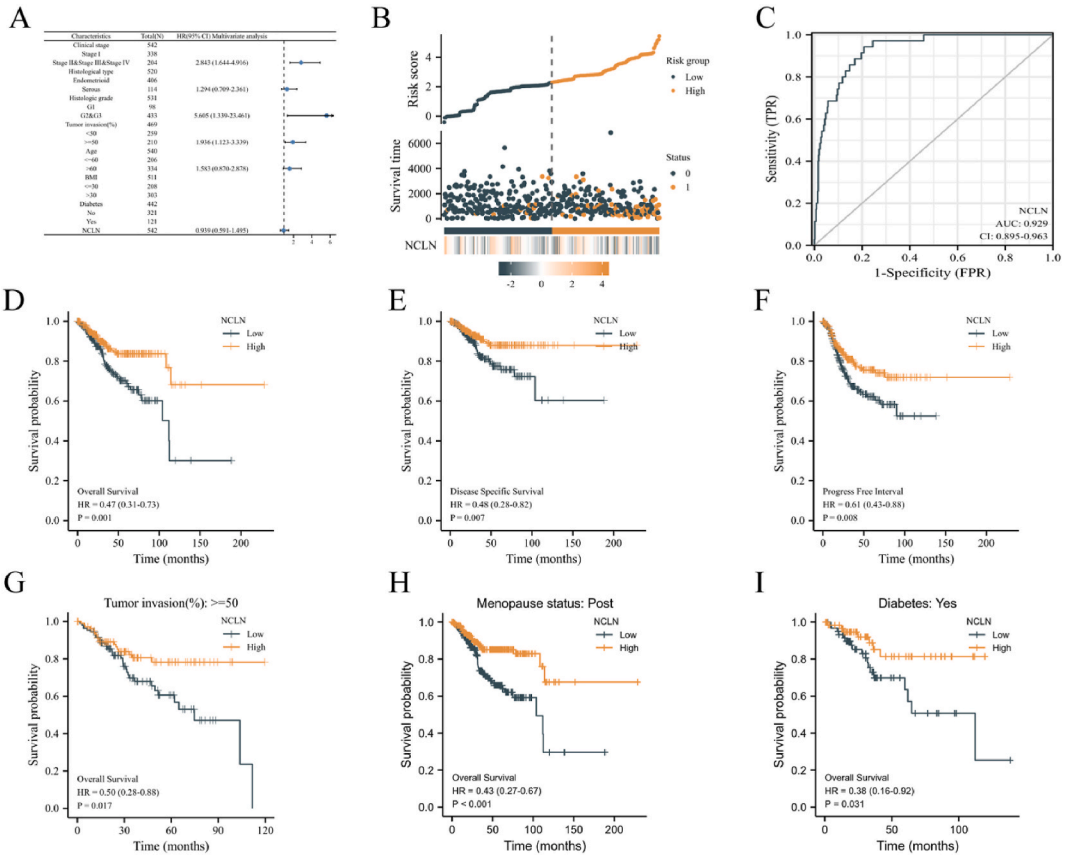

Fig. 4. The prognostic value of NCLN in ECs
(A)The forest plot analyzing Multivariate Cox regression. (B) The risk of survival status and NCLN expression. (C) The ROC curve of NCLN. (D) The prognostic value of NCLN in OS. (E) The prognostic value of NCLN in DSS. (F) The prognostic value of NCLN in PFI. (G–I) The prognostic value of NCLN with different risk factors in OS.

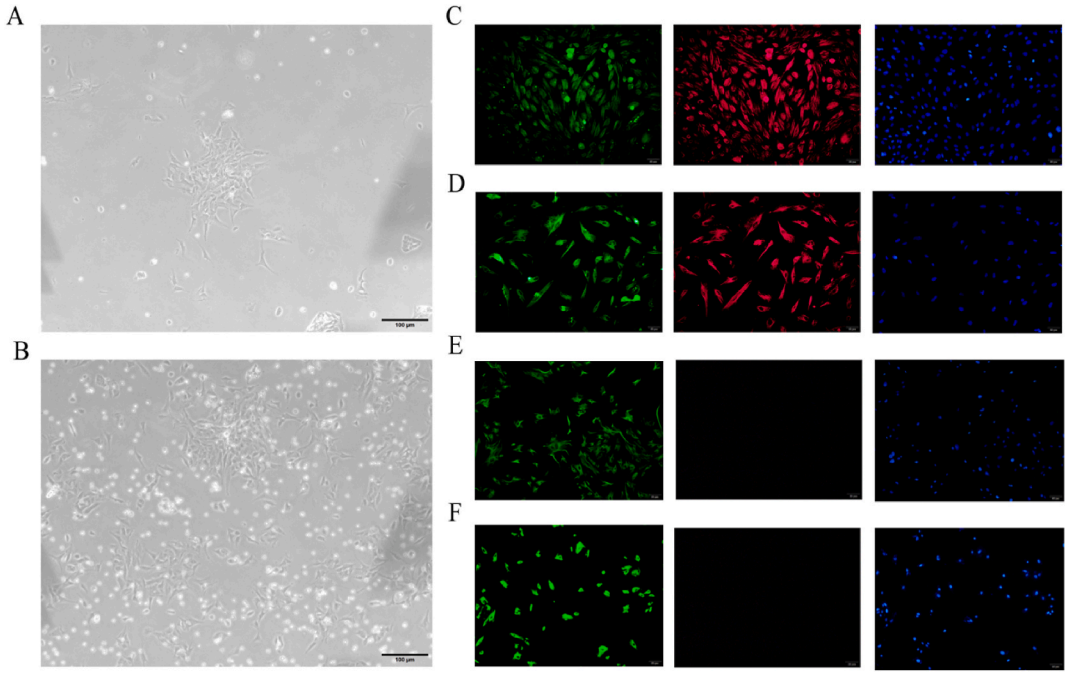

Fig. 5. Extraction and Identification of Endometrial Epithelium
(A) Human endometrial epithelial cells in generations zero. (B) Human endometrial mesenchymal cells in generations zero. (C) Immunofluorescence image in Ishikawa (Green-Vimentin, Red-CK-7, Blue-DAPI). (D) Immunofluorescence image in human endometrial epithelial cells (Green-Vimentin, Red-CK-7, Blue-DAPI). (E) Immunofluorescence image in HESC (Green-Vimentin, Red-CK-7, Blue-DAPI). (F) Immunofluorescence image in human endometrial mesenchymal cells (Green-Vimentin, Red-CK-7, Blue-DAPI).

fatty acid metabolism, epithelial mesenchymal transition (EMT), oxidative phosphorylation, and glycolysis(Fig. 2F). These findings provide further insights into the multifaceted functions of NCLN in endometrial physiology and cancer progression.

3.4. Correlations in NCLN Expression and Clinicopathologic Variables

The correlation analysis conducted revealed a significant association between NCLN expression and clinical stages, histological type, and OS events(Fig. 3). Subsequent logistic regression analysis confirmed that NCLN was significantly linked with both clinical stages ($p < 0.001$) and histological types ($p < 0.001$) (Table 3). Within the Cox regression model, various factors including clinical stages ($p < 0.001$), histological type ($p < 0.001$), grade ($p < 0.001$), tumor invasion ($p < 0.001$), and age ($p < 0.01$) were identified as correlated with poor prognosis in ECs(Table 4). However, in the multivariate Cox regression analysis, after adjusting for potential confounders, only the clinical stage ($p < 0.001$), histological grade ($p = 0.023$), and tumor invasion ($p = 0.023$) remained as independent prognostic factors for OS(Fig. 4A), while NCLN did not exhibit significance($p = 0.314$).

In Fig. 4B, the distribution of NCLN expression, survival status, and corresponding expression profiles is depicted. Surviving ECs patients are represented by blue dots, while deceased patients are denoted by orange dots. The median of the risk score is depicted as the upper line, dividing the low-risk score group (on the left side of the line) characterized by low NCLN expression from the high-risk score group (on the right side of the line) characterized by high NCLN expression. With an increase in the risk score, there's a gradual rise in the number of orange dots, indicating a higher proportion of deceased ECs patients. This observation suggests that patients in the high-risk group exhibit poorer survival outcomes and a higher risk of death. Moreover, the diagnostic value of NCLN was assessed using the ROC curve, which revealed a significant area under the curve (AUC = 0.929, 95 % CI: 0.895–0.963)(Fig. 4C). This indicates that NCLN possesses diagnostic potential in predicting outcomes related to ECs.

The K-M survival curve was employed to evaluate the prognostic significance of NCLN in the OS of ECs. Patients were stratified into high and low-expression groups based on NCLN levels. Notably, the high expression group exhibited a strong correlation with improved OS (HR = 0.47 (0.31–0.73), $p = 0.001$), DSS (HR = 0.48 (0.28–0.82), $p = 0.007$), and PFS (HR = 0.61 (0.43–0.88), $p = 0.008$) (Fig. 4D–F). Additionally, factors such as diabetes, menopause, and tumor infiltration beyond half of the muscle layer were identified as high-risk factors for prognosis. Subsequently, K-M survival curves were employed to evaluate the prognostic outcomes within each high-risk group. Remarkably, in every high-risk group, high NCLN expression was associated with improved OS(Fig. 4G–I). These findings underscore the potential prognostic value of NCLN expression levels in predicting outcomes across different risk categories in ECs patients, suggesting its utility as a prognostic biomarker for guiding clinical decision-making.

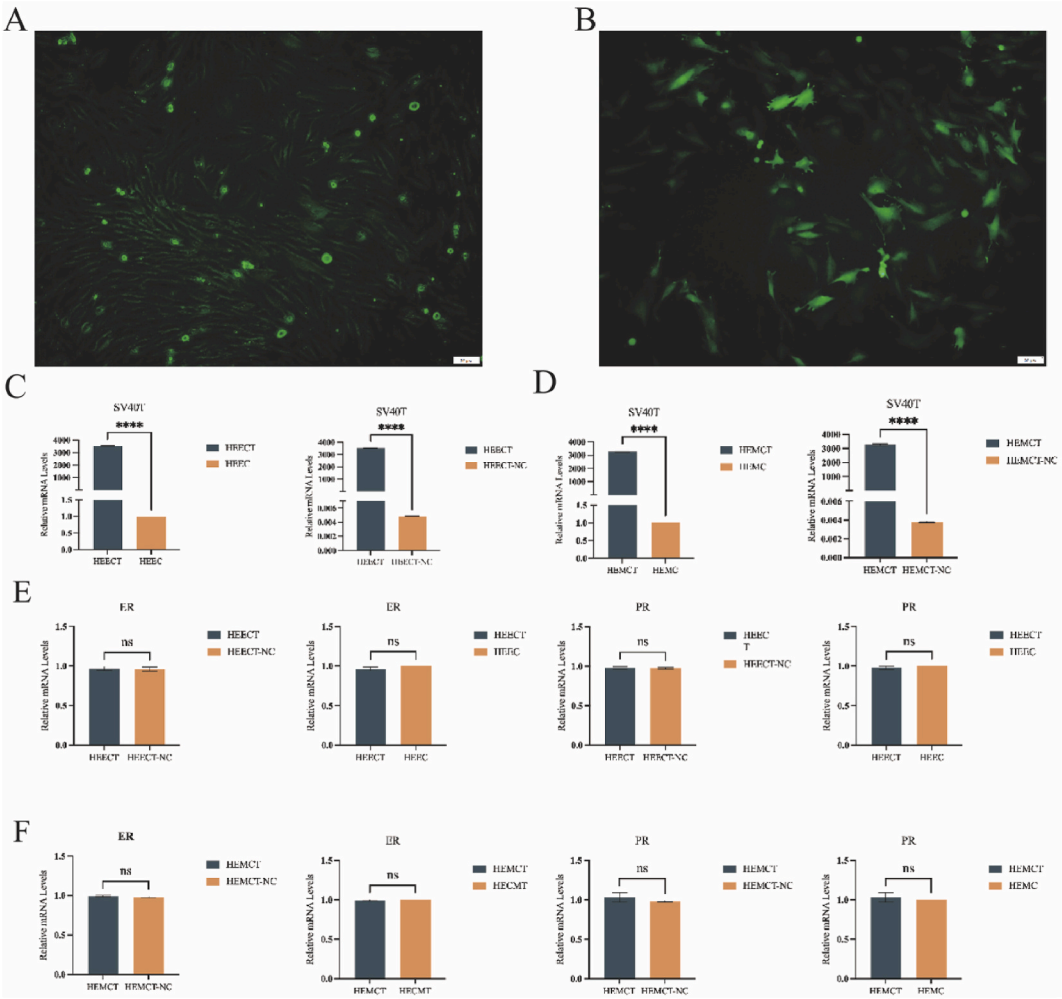

Fig. 6. Exploration Characteristics of Immortal Cells
(A)Fluorescent images of immortal human endometrial epithelial cells. (B) Fluorescent images of immortal human endometrial mesenchymal cells. (C) mRNA expression in SV40-T in immortal human endometrial epithelial cells. (D) mRNA expression in immortal human endometrial mesenchymal cells. (E) ER and PR expression in mRNA of immortal human endometrial epithelial cells. (F) ER and PR expression in mRNA of immortal human endometrial mesenchymal cells.

3.5. Extraction and Identification of Endometrial Epithelium and immortal cell lines

Fig. 5A–B depict primary epithelium and mesenchymal cells at Generation 0. Fig. 5C–F illustrate the purity of primary cells across three generations using immunofluorescence techniques. In these figures, red represents CK-7, green represents Vimentin, and blue represents DAPI. To validate the purity of the primary cells in culture, we utilized endometrial epithelial cancer cells Ishikawa and endometrial mesenchymal cells HESC as positive and negative control groups, respectively. The primary endometrial epithelial cells consistently stained with Ishikawa cells, showing positive for both CK-7 and Vimentin. Conversely, the primary mesenchymal cells consistently stained with HESC cells, exhibiting positive for Vimentin while being negative for CK-7. These findings confirm the successful isolation and culture of primary endometrial epithelial and mesenchymal cells, demonstrating their purity and suitability for further experimentation and analysis.

To establish immortal human endometrial epithelium, we employed lentivirus carrying SV40-T to enhance SV40-T expression in both human endometrial epithelial and mesenchymal cells. The lentivirus also carried a green fluorescent marker and anti-Puromycin resistance. Cells were cultured in a complete medium containing Puromycin, and their verification was performed using a Fluorescence Microscope (Fig. 6A–B). qPCR further confirmed stable SV40-T expression in the primary cells(Fig. 6C–D). Notably, the expression levels of ER and PR did not exhibit significant differences between SV40-T labeled and unlabeled cells (Fig. 6E–F). These findings indicate successful SV40-T upregulation without altering the expression of ER and PR.

The in vitro proliferative capacity of different groups was assessed using MTT assay (Fig. 7A). Interestingly, the proliferative capacity of mesenchymal cells was initially higher than that of epithelial cells in the group without SV40-T. However, in the presence of

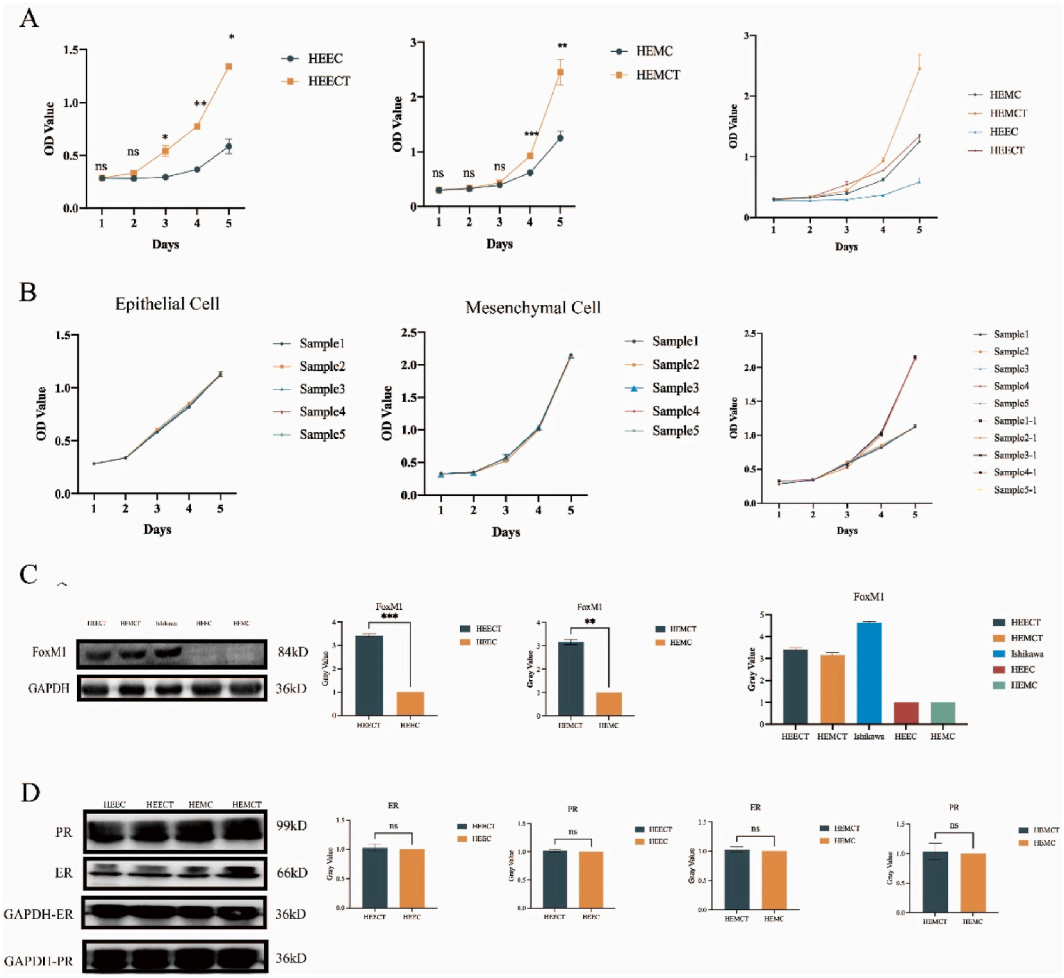

Fig. 7. Analysis of Proliferation in Different Groups in *vitro*
(A) The proliferation of different groups in *vitro* by MTT. (B) The proliferation of different groups in *vitro* by CCK8. (C) FoxM1 expresses differently between different groups by Western blot. (D) ER and PR express differently between different groups by Western blot. *Note: The cells with the suffix T are immortalized cell lines after SV-40T infection.*

SV40-T, both groups showed consistent proliferation rates, and notably, cells with SV40-T exhibited higher proliferation compared to those without SV40-T. Further exploration of the proliferative differences between mesenchymal cells and epithelial cells with SV40-T from patients was conducted using CCK8 assay(Fig. 7B). Results indicated that while there was no significant difference in proliferation among five epithelial cell lines and five mesenchymal cell lines, mesenchymal cell lines generally displayed higher proliferation compared to epithelial cell lines. Western blot analysis corroborated these findings by confirming protein levels of Forkhead Box M1 (FoxM1), ER, and PR. (Fig. 7C–D and Fig. S1). Notably, SV40-T appeared to augment proliferative capacity without altering cellular characteristics, including ER and PR expression. This implies that SV40-T may stimulate proliferation through FoxM1 upregulation, indicating a potential underlying mechanism for its proliferative effects.

We employed HE and Papanicolaou staining to assess the nuclear atypia of immortal cells. HE staining confirmed the immortal cells even after 20 generations post-SV40-T labeling, with no discernible nuclear atypia (Fig. 8A–B). Similarly, Papanicolaou staining, performed every five generations post-labeling, showed no evidence of nuclear atypia(Fig. 8C). These findings suggest that although SV40-T bolstered proliferation, it did not provoke cellular carcinogenesis, as indicated by the absence of nuclear atypia in the immortalized cells.

3.6. NCLN enhances cell proliferation in vitro

To substantiate the variance in NCLN expression between endometrial cancer and normal epithelium, qPCR analysis unveiled no significant correlation between SV40-T expression and NCLN levels. Moreover, NCLN expression was notably elevated in Ishikawa and RL95-2 cells post-SV40-T labeling compared to normal epithelium(Fig. 9A). Subsequent experiments utilized lentivirus carrying NCLN to modulate NCLN expression levels. Specifically, NCLN expression was augmented in immortal epithelial cells and diminished in

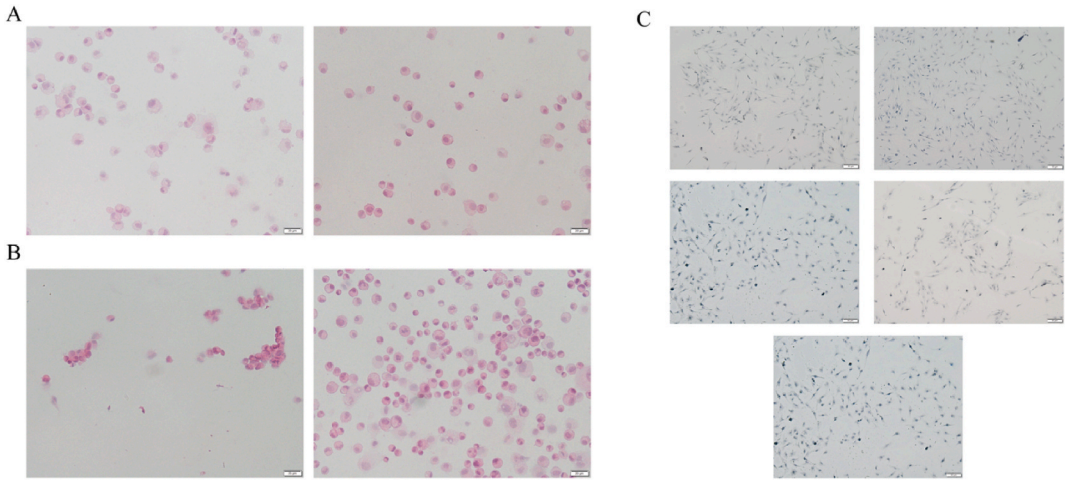

Fig. 8. Validation of carcinogenesis in immortal cells
(A) Validation of carcinogenesis in immortal human endometrial epithelial cells by HE stain. The left is immortal cells, the right is no-immortal cells. (B) Validation of carcinogenesis in immortal human endometrial mesenchymal cells by HE stain. The left is immortal cells, the right is no-immortal cells. (C) Validation of carcinogenesis in immortal human endometrial epithelial cells by Papanicolaou stain. The first picture is generation 25. The second is generation 50. The third is generation 75. The fourth is generation 100. The fifth is no-immortal cells.

Ishikawa and RL95-2 cells(Fig. 9B).

CCK8 experiments unveiled that NCLN overexpression heightened proliferation in immortal cells, whereas reducing NCLN expression curtailed proliferation in Ishikawa and RL95-2 cells(Fig. 9C). This underscores a direct relationship between NCLN levels and cell proliferation rates. Additionally, this proliferation is closely linked to alterations in the cell cycle. Western blot analysis confirmed a significant correlation between NCLN and the MAPK/Erk pathway(Fig. 9D and Fig. S2). Specifically, NCLN was observed to stimulate MAPK phosphorylation, triggering Erk activation and consequently resulting in a shortened cell cycle and intensified cell proliferation. These findings offer mechanistic insights into how NCLN influences cellular processes by modulating the MAPK/Erk pathway.

3.7. Correlation of NCLN with PR and ER

We employed both qPCR and WB analyses to validate observations from the TCGA database regarding alterations in ER and PR expression among various groups. Our analysis unveiled a substantial correlation between NCLN and PR, while no significant correlation was detected with ERα and ERβ(Fig. 10A and B and Fig.S3). These results mirror those from the TCGA analysis, offering additional confirmation for the link between NCLN and PR expression levels.

4. Discussion

As far as we know, ECs incidence has been on the rise annually. From the molecular profiling conducted by TCGA since 2014 to the present ProMise model, there has been a concerted effort to delve into the molecular intricacies of ECs. This endeavor aims to understand the diverse molecular characteristics exhibited by ECs, paving the way for more tailored and effective clinical treatment decisions based on molecular markers [19,20].

Previous research has established NCLN's involvement in embryonic development through the regulation of Nodal signaling, which antagonizes NOMO and significantly impacts mesodermal development [11,12]. Given the origin of the female reproductive system from embryonic mesoderm, we postulated that NCLN might also play a role in the carcinogenic processes of ECs.Analysis of data from the TCGA database supported this hypothesis, revealing elevated expression of NCLN in patients with ECs and its correlation with various clinicopathological features. Notably, NCLN displayed higher expression in endometrioid carcinoma compared to serous carcinoma, with higher levels observed in early-stage disease than in advanced stages. These findings suggest that NCLN holds promise as a prognostic marker for ECs. To delve deeper into the functional role of NCLN, we conducted GO analysis using TCGA data. This analysis highlighted NCLN's potential involvement in hormone activity and various metabolic processes such as fatty acid metabolism, EMT, oxidative phosphorylation, and glycolysis, as revealed by GSEA. Overall, these findings underscore the multifaceted role of NCLN in ECs, potentially influencing hormone activity and critical metabolic pathways crucial for cancer progression. Further investigations are warranted to validate these findings and elucidate the precise mechanisms by which NCLN contributes to endometrial carcinogenesis.

The MAPK/Erk pathway is integral to numerous cellular processes, including proliferation, differentiation, apoptosis, and stress response [21,22]. Aspirin has been shown to inhibit the growth and metastasis of ECs by regulating MAPK/Erk activation [23]. Additionally, cannabinoid receptor 1 (CNR1) has been implicated in promoting progesterone resistance in ECs by activating Erk [24].

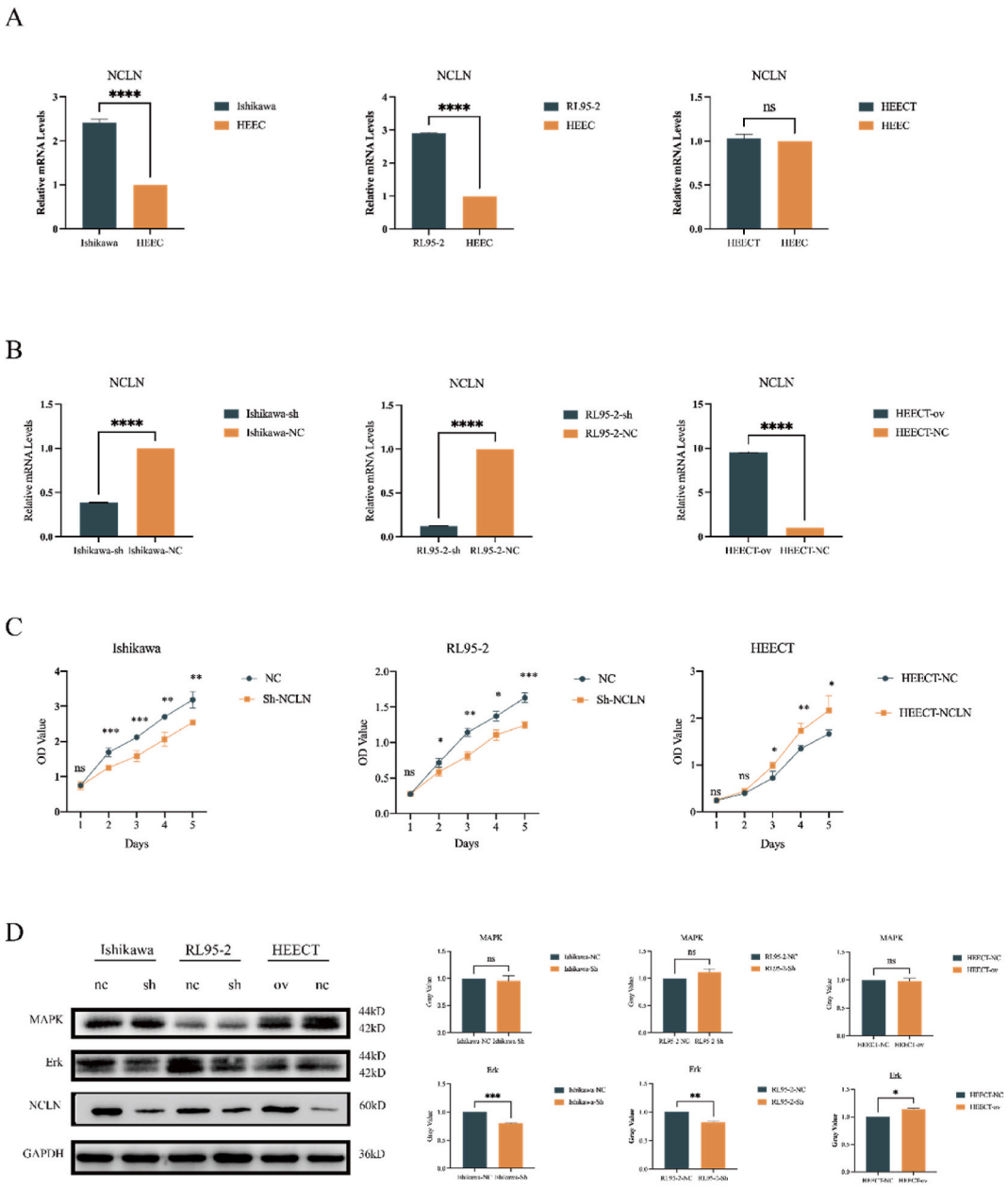

Fig. 9. Analysis of Proliferation in Different Groups with NCLN in *vitro*

Analysis of Proliferation in Different Groups with NCLN in *vitro:*(A) The difference in NCLN in epithelial cells and endometrial cancer. (B) Validation of NCLN by qPCR after transfection. (C) CCK8 analyzed the proliferation of immortal human endometrial epithelial cells, RL95-2, and Ishikawa after transfection. (D) MAPK and Erk were detected by Western blot with NCLN.

Obesity, a major risk factor for ECs, is associated with dysregulated lipid metabolism, wherein MAPK/Erk plays a role in regulating natural killer (NK) and natural killer T (NKT) cells, potentially facilitating immune escape by cancer cells [25]. Upregulation of NCLN in human endometrial epithelial cells has been demonstrated to enhance proliferation in *vitro*, while downregulation in ECs cells inhibits proliferation in *vitro*. Moreover, the expression of Erk is positively correlated with NCLN, suggesting that NCLN may modulate proliferation by regulating the MAPK/Erk pathway. However, further studies are warranted to substantiate this relationship.

Estrogen and progesterone are key co-regulator for Cyclic changes in the endometrium. Estrogen binds ER to promote endometrium proliferation [26], while progesterone binds to PR promotes endometrium differentiation [27]. ECs are classified into two types based on pathological features. Type I ECs, which express abundant ER and PR and have a better prognosis with hormone-targeted therapy, and Type II ECs, which lack these receptors and have a poorer prognosis [28]. The status of ER and PR can predict prognosis and metastasis in ECs patients, regardless of stage [29,30]. PR inhibit ECs proliferation independently of ER status, and its expression is

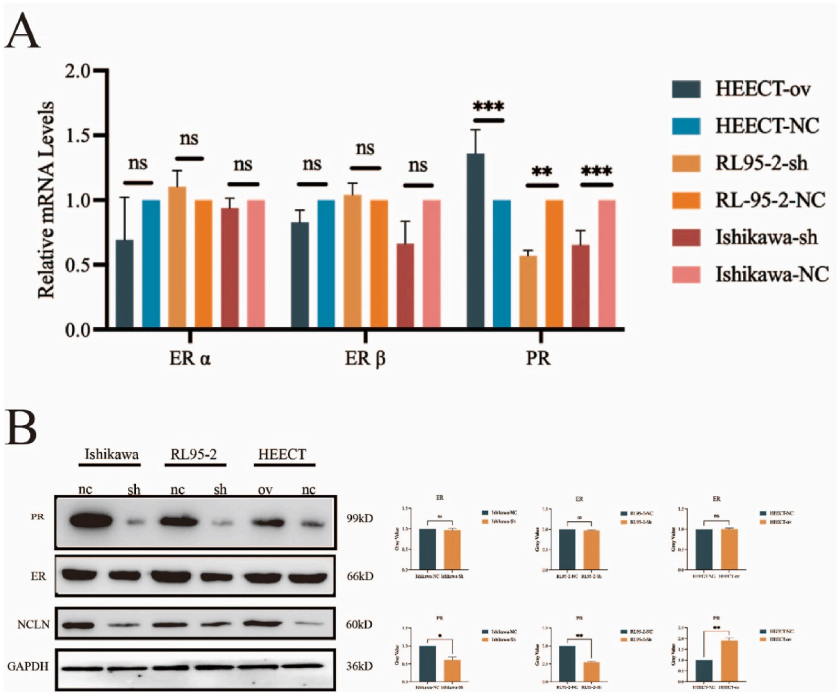

Fig. 10. The correlation between PR, ER and NCLN
(A) The expression of PR and ER was analyzed by qPCR between different groups; (B) PR and ER were detected by Western blot with NCLN.

associated with favorable clinical outcomes in EC patients wishing to preserve their fertility [31].PR is a member of the nuclear hormone receptor superfamily of ligand-activated transcription factors and interacts with transcription factors such as SP1, AP1, FOXO1, and the p65 subunit of NF-κB [32]. The complex of PR and Src kinase can activate MAPK signaling and inhibit other transcriptional procession [33,34]. Re-induction of PR expression increases the sensitivity of hormone receptor-silenced ECs to hormone therapy [35]. PR deficiency weakens the expression of Cyclin G1 protein, promoting carcinogenesis and development in ECs [36]. PR has two main isoforms, PRA and PRB [37]. Clinical evidence has shown that an imbalance of these isoforms can predict the prognosis of EC patients [38]. Downregulation of PRA or/and PRB is highly associated with advanced clinical stages [39]. The sensitivity of ECs to progestin therapy can be regulated by PRB [40]. Our in vitro studies validated a positive correlation between PR and NCLN. Endometrioid carcinoma, which expresses abundant ERs and PRs, also shows higher expression of NCLN compared to serous carcinoma, as indicated by TCGA data analysis. This suggests that NCLN could serve as a potent prognostic biomarker in the histological assessment of ECs. Although the interaction between PR and NCLN is beyond the scope of this article, our team plans to investigate this relationship further.

In our study, we observed that NCLN expression was higher in normal tissue compared to ECs. Additionally, we found a correlation between NCLN expression and histology, suggesting that NCLN levels may vary based on the histological subtype of ECs. Moreover, our findings suggest a potential regulatory role of NCLN in PR expression. It is possible that NCLN influences PR, which may have implications for progestin resistance in ECs. Modulating NCLN to reverse progestin resistance could potentially lead to improved prognosis for EC patients. These results suggest that NCLN may serve as a promising marker for predicting the prognosis of patients with ECs. Further research and clinical validation are warranted to explore the full potential of NCLN as a prognostic marker and its role in modulating progestin resistance in ECs.

5. Conclusion

NCLN has been shown to enhance endometrial cancer cell proliferation in vitro while also increasing the expression of progesterone receptors. Analysis of data from the TCGA database has revealed that NCLN exhibits high expression levels in endometrioid carcinoma, which is associated with a more favorable prognosis, whereas its expression is relatively low in serous carcinoma. Furthermore, the observation of higher NCLN expression in early stages compared to advanced stages in clinical staging suggests that NCLN may serve as a promising prognostic indicator in conjunction with histological assessment. This indicates the potential utility of NCLN as a prognostic marker that could help predict outcomes and guide treatment decisions for endometrial cancer patients, especially when considering the histological subtype and clinical stage of the disease.

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Data availability statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval

The protocol was approved by the institutional ethics committee (Identifier: SIAT-IRB-170315-H0157). All patients provided written informed consent.

CRediT authorship contribution statement

Huang-jin Luo: Writing – original draft, Methodology, Data curation, Conceptualization. **Li-tong Zhu:** Writing – review & editing, Methodology. **Yu Dai:** Formal analysis. **Yun Ma:** Formal analysis. **Kai Wang:** Formal analysis. **Lei Zhang:** Funding acquisition. **Qiu-xia Li:** Formal analysis. **Ping Jin:** Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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