YAP1 affects the prognosis through the regulation of stemness in endometrial cancer

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Background: Endometrial cancer stem-like cells (ECSCs) had been proven to be responsible for recurrence, metastasis, and drug-resistance in patients with endometrial cancer. The HIPPO pathway had been shown to play an important role in the development and maintenance of stemness in a variety of tumors. While there was less research about its function in ECSCs. The aim of this study was to explore the role of YAP1, a core molecular of HIPPO pathway, on the stemness of endometrial cancer and to reveal its influence on prognosis. **Methods:** We collected specimens and clinical data from 774 patients with endometrial cancer to analyze the correlation between YAP1 expression and prognosis. We then examined the expression of YAP1 in ECSCs and EC cell lines (Ishikawa; HEC1-A) in vitro experiments. Changes in the stemness of cell lines were detected after YAP1 silencing by siRNA. Finally, high-throughput sequencing was used for predicting the potential molecular interactions and mechanisms of YAP1's effect on stemness. **Result:** Down-regulation of YAP1 significantly suppresses the stemness of EC cell lines. High expression of YAP1 leads to poor prognosis in EC by regulation of stemness. **Conclusion:** YAP1 plays an important role in prognosis of patients with EC by regulation of stemness.

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Abstract

Background:

Endometrial cancer stem-like cells (ECSCs) had been proven to be responsible for recurrence, metastasis, and drug resistance in patients with endometrial cancer. The HIPPO pathway had been shown to play an important role in the development and maintenance of stemness in a variety of tumors. While there was less research about its functions in ECSCs. The aim of this study was to explore the role of YAP1, a core molecular of the HIPPO pathway, in the stemness of endometrial cancer and to reveal its influence on prognosis.

Methods:

We collected specimens and clinical data from 774 patients with endometrial cancer to analyze the correlation between YAP1 expression and prognosis. We then examined the expression of YAP1 in ECSCs and EC cell lines (Ishikawa; HEC1-A) in vitro experiments. Changes in the stemness of cell lines were detected after YAP1 silencing by siRNA. Finally, high-throughput sequencing was used for predicting the potential molecular interactions and mechanisms of YAP1's effect on stemness.

Result:

Down-regulation of YAP1 significantly suppresses the stemness of EC cell lines. High expression of YAP1 leads to poor prognosis in EC by regulation of stemness.

Conclusion:

YAP1 plays an important role in the prognosis of patients with EC by regulation of stemness.

Keywords: Endometrial cancer; Stemness; YAP1; Prognosis; Stem cell.

Introduction

Endometrial cancer (EC) is a common gynecological cancer with a rising incidence, especially in high-income countries(Lu & Broaddus 2020). An increase in the aged population, obesity, and other metabolite diseases may contribute to its high incidence(Ferlay et al. 2018). Many patients with EC were diagnosed at an early stage, and with appropriate treatment good survival was achieved(Brooks et al. 2019). However, postoperative recurrence and metastasis greatly influence the survival of patients with advanced endometrial cancer and remain great challenges for surgeons. Meanwhile, the molecular mechanisms of postoperative recurrence and metastasis of EC were still unclear. Recent research had proposed the concept of cancer stem-like cells (CSCs) in EC(Giannone et al. 2019), which contributes to specific self-renewal and differentiation in

73 cancer cells. Their features and role in progression, invasiveness, and metastasis were
74 identified(Babaei et al. 2021; Shin & Cheong 2019). CD44 was related to EC stemness reported
75 by another research (Elbasateeny et al. 2016; Park et al. 2019). Also, spheres generated from EC
76 cell lines showed the co-expression of CD44 and CD133 in ECSCs(Mirantes et al. 2013; Rutella
77 et al. 2009). Besides, several studies had linked NANOG to ECSCs(Zhou et al. 2011). OCT4 and
78 SOX2 were reported to have great impacts on the deregulation of NANOG and also were found to
79 be associated with the self-renewal capacity in ECSCs(Hubbard et al. 2009; Zhou et al. 2011).
80 Also, the tumor spheres which had a higher proliferation potential than differentiated cells showed
81 the co-expression of NONAG, OCT4, and SOX2 mRNA(Zhou et al. 2011). Thus, these proteins
82 including OCT4, SOX2, NANOG, CD44, and CD133 had been widely defined as stemness-related
83 markers(Mirantes et al. 2013; Rutella et al. 2009; Szymonik et al. 2021). And it is of great meaning
84 to conducting further research on ECSCs because it would probably provide new insight into the
85 understanding of recurrence and metastasis in EC.

86 Studies had illustrated that several pathways, such as the Notch signaling pathway, Wnt
87 pathway, PI3K/AKT pathway, TGF-beta signaling, and HIPPO signaling pathway, are involved
88 in the process of stemness. Research showed that activation of the HIPPO pathway would lead to
89 chemotherapy resistance and increasing of stemness in ovarian cancer(Muñoz-Galván et al. 2020).
90 A similar study also identified PFKFB3 as a novel target to regulate stem cells in small lung
91 carcinoma through HIPPO pathway(Thirusangu et al. 2022). And it had been proven that MCM2
92 promoted the stemness and sorafenib resistance of hepatocellular carcinoma cells via hippo
93 signaling(Zhou et al. 2022). Also, by regulating the HIPPO pathway, exosomal miR-4800-3p
94 influenced the stemness in hepatocellular carcinoma(Lin et al. 2022). YAP1, as a key role in the
95 pathway, was also identified as a regulator of stemness in glioblastoma(Castellan et al. 2021).
96 Researchers also claimed similar findings in colorectal cancer(Tian et al. 2020), ovarian cancer(Li
97 et al. 2020), and osteosarcoma(Shi et al. 2021), etc. Nowadays, few studies are focusing on the
98 role of the HIPPO pathway in ECSCs. And we still have not revealed the link between YAP1 and
99 stemness in EC.

100 Therefore, our study aimed to investigate the effect of YAP1 on the prognosis of patients with
101 EC and the function of YAP1 on the stemness of EC cell lines. Our study would provide new
102 insight into the study of the HIPPO pathway in EC stemness and reach the goal of finding the
103 potential biological target for the recurrence and metastasis of EC.
104

Materials & Methods

106 All procedures were performed by relevant guidelines and were in line with the World Medical
107 Association Declaration of Helsinki. This study had been approved by the Ethics Committee of
108 the First Affiliated Hospital of Chongqing Medical University (2021-676).

109 **1.1 Cases and follow-up.**

110 A total of 774 patients from 2015 to June 2020 who accepted primary surgery at the First
111 Affiliated Hospital of Chongqing Medical University were enrolled in this study. Tissue samples

112   were obtained from postoperative specimens after the definitive surgery, with at least 5cm of the
113   tumor margin. The clinical data of cases should include at least the followings: age, BMI, FIGO
114   staging, histotype, cervical stromal invasion, myometrial invasion, lymph vascular invasion
115   (LVSI), etc. The procedure was approved by the Ethics Committee of the First Affiliated Hospital
116   of Chongqing Medical University.
117   The postoperative follow-up was mainly carried out by outpatient services and telephone. The
118   follow-up frequency was as follows: every 3 months for the first two years, then every half of a
119   year for the following three years, and once a year after five years. The content of the follow-up
120   mainly contained a physical examination, necessary imaging examination, and biochemical
121   examination. Recurrence was confirmed by physical examination and imaging examination,
122   including X-ray, CT, PET/CT, MRI and so on(Ouldamer et al. 2016). Patient survival was
123   confirmed by telephone interviews. Recurrence-free survival (RFS) is defined as the time between
124   the date of surgery and the date of recurrence (proved by imaging or histology)(Huijgens &
125   Mertens 2013). Death is defined as death due to postoperative recurrence. And overall survival
126   (OS) is defined as the time between the date of surgery and the patient's date of death(Ouldamer
127   et al. 2018).
128
129   **1.2 Immunohistochemistry and Evaluation.**
130   The specimens were sent to the Department of Pathology at Chongqing Medical University
131   (CQMU) for further processing. All the procedures were conducted by the same standards(Jiang
132   et al. 2020). Paraffin was used to embed the postoperative specimens. The tissues would be made
133   into 2-3 micrometers slices. H&E staining was used to identify the most cancerous part of the
134   specimens. The histological parameters were recorded including histological type, size of lesion,
135   grade, and invasion depth. These were first judged by the junior pathologists.
136   Immunohistochemistry (IHC) was performed by an automated Immuno-Stainer (manufactured by
137   Leica Bond-Max) and followed the instructions(Yang et al. 2016). The slices were dried for 12
138   hours at a temperature of 60°C. After deparaffinized, the slices were hydrated in 70% methanol
139   for 10 minutes. Antigen retrieval was carried out by microwaved heating for 5 minutes in 0.1mol/l
140   citrate buffer. Endogenous peroxide was blocked by 3% H_2O_2 in methanol. After that, the slices
141   were incubated with YAP1 antibody (1:400, 14074, Cell Signaling Technology), ER (SP1, in
142   1:50), PR (MX009, in 1:500), Ki67 (MX006, in 1:300), and P53 (MX008, in 1:200) (Maixin
143   Biotech, China) for 1 hour, and then incubated with anti-rabbit secondary antibody (manufactured
144   by Leica) at a room temperature for 30 minutes. 3,3'-diaminobenzidine tetrahydrochloride (DAB
145   Substrate System, DAKO) and hematoxylin were used to color the slices.
146   Referring to other studies, we set the cutoff value of four factors as follows: ERα, 5%; PR, 5%;
147   Ki67, 40%(Jia et al. 2021; Jiang et al. 2021; Jiang et al. 2020). P53 was classified as normal or
148   abnormal. On this basis, we defined the patient as having high or low expression. The evaluation
149   of staining was as follows(Jiang et al. 2021): briefly, the average percentage of positive-stained
150   cells in five random scopes was recorded. Slices with more than 50% positive-stained cells were

151 considered as high expression. Research had identified that nuclear localization of YAP1 was the
152 activated form of YAP1 which would induce the subsequent mechanism and thus we were
153 focusing on the nuclear staining of YAP1(Zanconato et al. 2015). The frequency of YAP1 staining
154 in nuclear was graded from 0 to 4 by the percentage of positive cells as follows: grade 0, < 3%;
155 grade 1, 3–25%; grade 2, 25–50%; grade 3, 50–75%; grade 4, > 75%. Grades 0, 1, and 2 were
156 considered the low expression, and grades 3 and 4 were high expression(Ballout et al. 2022). Two
157 pathologists would evaluate the same slice (blind). If the results differed by less than 10%, the
158 average of the two results was considered the ultimate result. Otherwise, they would reevaluate
159 the slice and then reach a consensus (unblind).
160
161 **1.3 Cell culture and transfection.**
162 Ishikawa, HEC1-A, and RL95-2 were purchased from Cellbook Technology (Guangzhou,
163 China). Short tandem repeat (STR) analysis was performed in all cell lines. Ishikawa and HEC1-
164 A were cultured in DMEM (Gibco) supplemented with 10% FBS while RL95-2 was cultured in
165 DMEM/F12 (Gibco) with 10% FBS.
166 ECSCs were grown in DMEM/F12 (Gibco) with 10ng/ml hFGF (Peperotech), 20ng/ml hEGF
167 (Peperotech), and B27 supplement (17504044, Gibco)(Chen et al. 2020). 0.5ml of all the above
168 mediums was added to ECSCs cultured plates every other day. All the cell lines were grown at
169 37°C in a 95% humidified atmosphere and 5% CO_2 And the ECSCs obtained from Ishikawa and
170 HEC1-A were described as ECSCs[Ishikawa] and ECSCs[HEC1-A], respectively.
171 siRNA targeting human YAP1 mRNA (siRNA#1, siRNA#2, which were shown in
172 Supplementary File 1) and a non-specific scramble siRNA sequence (Control, which was shown
173 in Supplementary File 1) were synthesized (Genepharma, Shanghai, China) and then transfected
174 into Ishikawa and HEC1-A using Lipofectamine 2000 (Invitrogen, Carlsbad, USA) according to
175 the manufacturer's instruction. Assays were performed after 2 days, which confirmed the
176 efficiency of transfection.
177
178 **1.4 ECSCs sphere formation.**
179 After confirming the transfection of siRNA targeting human YAP1 in Ishikawa and HEC1-A,
180 cells were followingly cultured in DMEM/F12 (Gibco) with 10ng/ml hFGF (Peperotech), 20ng/ml
181 hEGF (Peperotech) and B27 supplement (17504044, Gibco) in 6 wells plates respectively, with a
182 density of 20,000 cells for each well. The differences in sphere formation between siRNA#1,2 and
183 siNC were compared on the 7th day. The spheres (>50 μm in diameter) were counted under a light
184 microscope. And the number of spheroids in each group was then counted in three random fields
185 of view under the light microscope for statistical analysis. The average number represents the
186 result. Each sample was counted three times(Suzuki et al. 2021).
187
188 **1.5 RNA extraction and quantitative reverse transcription PCR.**

189   Total RNA was extracted by using TRIzol® RNA Isolation Reagents (Invitrogen, Carlsbad,
190   USA). Quantitative reverse transcription PCR (qRT-PCR) was used to detect the difference in
191   mRNA expressions. mRNA analysis was performed in Bio-rad CFX Connect Real-time PCR
192   Instrument by using SYBR green master mix (Takara, Dalian, China). All used primers were
193   shown in Supplementary File 2. The PCRs were performed at least three times independently for
194   each experiment. The relative expression of indicated genes compared with β-actin was calculated
195   using the $2^{-\Delta\Delta CT}$ method.
196
1.6 Western blotting assays.
198   Western blotting assays were used to identify the different expressions of protein. Modified
199   RIPA lysis buffer was used to collect the total cell lysates. BCA Protein Assay Kit (Beyotime,
200   China) was used to standardize the concentration of different samples. Proteins were separated by
201   SDS-PAGE, transferred to a polyvinylidene fluoride (PVDF) membrane by using Mini-
202   PROTEAN® Tetra (Bio-rad, USA), and probed with primary antibodies respectively. The primary
203   antibodies were as follows: YAP (D8H1X) XP® Rabbit mAb (1:1000, 14074S, Cell signaling
204   technology), SOX2 Polyclonal antibody (1:1000, 11064-1-AP, Proteintech), OCT4 Polyclonal
205   antibody (1:1000, 11263-1-AP, Proteintech), NANOG Polyclonal antibody (1:1000, 14295-1-AP,
206   Proteintech), CD44 Polyclonal antibody (1:2000, 15675-1-AP, Proteintech), CD133 Polyclonal
207   antibody (1:2000, 18470-1-AP, Proteintech), GAPDH Monoclonal antibody (1:50000, 60004-1-
208   lg, Proteintech), Alpha Tubulin Polyclonal antibody (1:2000, 11224-1-AP, Proteintech). After that
209   the membranes were incubated with corresponding secondary antibodies: HRP-Goat anti-rabbit
210   IgG (H+L) (RS0002, Immunoway); HRP-Goat anti-mouse IgG (H+L) (RS0001, Immunoway).
211   Blots were detected using an enhanced chemiluminescence system (S6, manufactured by CLiNX,
212   Shanghai, China).
213
1.7 Online database
215   Kaplan-Meier Plotter (KM Plotter; http://kmplot.com/analysis/) is a website that can assess the
216   correlation between all genes (mRNA) and survival in pan-cancer(Lánczky & Győrffy 2021).
217   STRING (Version 11.5; https://cn.string-db.org/) is a database that can show the protein-protein
218   interaction (PPI) networks, which are based on current studies(Szklarczyk et al. 2021).
219
1.8 High-throughput sequencing of the transcriptome
221   ECSCs[Ishikawa] that we cultured were collected on the eighth day. High-throughput sequencing
222   was conducted by LC-Biotechnology (Hangzhou, China). The detailed protocols and data analysis
223   were recorded in Supplementary File 3.
224
1.9 Statistics
226   The differences in clinical features between the two groups as well as the univariate and
227   multivariate analyses were performed in SPSS 26.0. The column graphs were plotted with

228   GraphPad Prism 5. R language was used for analysis and visualization of high throughput
229   sequencing. Cytoscape (3.9.1) was used for visualizing the PPI network. Student's t-test was used
230   to test the difference.
231

Results

233   **2.1 Characteristic features of patients**
234   All cases were grouped by the expression level of YAP1 (low expression: n=626; high
235   expression: n=148). Figure 1A-B showed the samples of 2 different levels of YAP1 expression in
236   the CQMU cohort. And the differences in characteristic features of the two groups were compared.
237   Age, BMI, myometrial invasion, and PR had a p-value greater than 0.05 respectively, which
238   indicated that no difference exists in these features between the two groups. While there were
239   significant differences in FIGO staging, cervical stromal invasion, histotypes, lymph vascular
240   invasion, ER, Ki67, and P53 (p-value less than 0.05). Also, the RFS and OS were significantly
241   different. There were 460, 58, and 108 cases in FIGO I, II, and III stages in the low expression
242   group, respectively. While in the high expression group, 85, 16, and 47 cases were in FIGO I, II,
243   and III stages, respectively. 96 patients had cervical stromal invasion in the low expression group
244   while it was 37 in the high expression group. In the low expression group, 73.6% of cases were
245   type I. While 62.8% of cases were type I in the high expression group. In addition, 22.5% and
246   31.8% of cases had LVSI in low and high expression groups respectively. There were 16.1%, and
247   61.2% of low expressions of ER, and Ki67 respectively in the low YAP1 expression group. While
248   the same terms were 23.6%, and 48.6% in the high YAP1 expression group. 63.6% of patients had
249   a normal expression of P53 in the low-expression group and it was 56.8% in the high expression
250   group. The details were shown in Table 1.
251

252   **2.2 Univariate and multivariate analyses for recurrence.**
253   We performed the univariate analysis with the common variables for recurrence in EC. The
254   factors that have statistical significance were further analyzed by multivariate analysis. The details
255   were shown in Table 2. These remaining factors had significant differences: myometrial invasion
256   (p=0.006), histotype (p=0.007), LVSI (p=0.001), ER (p=0.021), Ki67 (p=0.017), P53(p=0.014),
257   and YAP1 (p=0.005).
258

259   **2.3 High expression of YAP1 was related to poorer prognosis.**
260   We draw the Kaplan-Meier curves using the data from KM Plotter and the cohort from CQMU.
261   The curves obtained from KM Plotter (which was shown in Figure 1C-D) indicated that based on
262   mRNA expressions, 314 and 108 patients were in low and high expression groups in the KM
263   Plotter dataset. Although there was no significant difference between the two groups in RFS and
264   OS (RFS: log-rank P=0.24; OS: log-rank P=0.32), the patients in the high group showed to have a
265   poorer result in RFS, which was consistent with our hypothesis. Most of the recurrences of EC
266   occurred within 3 or 5 years after surgery. It is substantial to note that patients with cancer were
267   generally older, so there is a precipitous decline in OS after 100 months when patients may pass

268 away due to other causes (e.g., other diseases). At the same time, there were significant differences
269 in RFS and OS in the cohort from CQMU (which was shown in Figure 1E-F). The cases in the
270 high expression group based on the results of IHC (n=148) had a poorer prognosis reflected by
271 RFS and OS (RFS: log-rank P<0.001; OS: log-rank P<0.001).
272
273 **2.4 Identification of ECSCs and detecting the expression of YAP1.**
274   As we had previously mentioned, OCT4, SOX2, NANOG, CD44, and CD133 had been widely
275 defined as stemness-related markers (Mirantes et al. 2013; Rutella et al. 2009; Szymonik et al.
276 2021). High expression of these genes is important evidence for the identification of endometrial
277 cancer stem cells. We cultured and isolated ECSCs from Ishikawa, HEC1-A, and RL952. We
278 recorded the morphological changes of ECSCs by using microscopy on the third, fifth, and seventh
279 days, which were shown in Figure 2A. It could be seen that the cells gradually formed spheres
280 starting from the third day. Among them, Ishikawa and HEC1-A showed the highest efficiency in
281 spheric formation and proliferation, which is related to the most stem cell-like state. Therefore, we
282 used these two cell lines for the subsequent experiments. To further validate the ECSCs that we
283 isolated, bulky ECSCs spheroids were collected on the eighth day. And we examined the
284 molecules associated with ECSCs as previously mentioned. As it's expected, qRT-PCR suggested
285 that mRNA levels of SOX2, OCT4, CD44, CD133, and NANOG, which had been defined as
286 stemness-related molecules, were significantly increased in ECSCs^{Ishikawa} and ECSCs^{HEC1-A},
287 compared with its original cell lines. The results were shown in Figure 2B-C. Subsequently, we
288 further confirmed the elevated expression of these molecules at the protein level by western blot
289 assay, which was shown in Figure 2D-E. These results confirmed that the stem-like cells we
290 cultured and isolated on the eighth day were ECSCs, which had the characteristics of ECSCs.
291   Also, the examination of the expression of YAP1 in EC cell lines and ECSCs was performed at
292 the same time. The expression of YAP1 in ECSCs^{Ishikawa} and ECSCs^{HEC1-A} showed a significant
293 increase compared with Ishikawa and HEC1-A respectively, which is confirmed by qRT-PCR and
294 western blot assay (the results were shown in Figure 2F-G). This was consistent with our previous
295 speculation.
296
297 **2.5 Down-regulation of YAP1 suppressed the stemness in Ishikawa and HEC1-A**
298   The changes in stemness of Ishikawa and HEC1-A were evaluated after YAP1 knockdown by
299 siRNA. The knockdown of YAP1 expression was confirmed by qRT-PCR, which was shown in
300 Figure 3A-B. Then, we further cultured the cells after YAP1 expression knockdown in the stem
301 cell medium as described previously. After YAP1 knockdown, the cells still had the ability of
302 spheric formation (same shape as ECSCs). However, it showed a reduced trend of sphere-forming
303 in number and size. Figure 3C-F showed the changes in the sphere-forming ability of stem cells in
304 different groups on the seventh day. The number of spheres in the siRNA#1 and siRNA#2 groups
305 was less compared with the control group.
306   Also, we tested the expression of stemness molecules 48 hours after YAP1 knockdown.
307 Significant decreases in SOX2, OCT4, CD44, CD133, and NANOG were observed in qRT-PCR.
308 Western blot also demonstrated the same trend. The graphs were displayed in Figure 3 G-J. This

309 result confirmed that down-regulation of YAP1 would suppress the stemness in Ishikawa and
310 HEC1-A.
311   In addition, we also detected changes in the target genes (CTGF, CYR61, ANKRD1, AMOTL2)
312 of YAP1 by qRT-PCR in YAP1 knockdown Ishikawa and HEC1-A cells, and we obtained similar
313 results to other studies. Similar results were obtained by knockdown of TEAD-1 and the use of
314 YAP-TEAD1 inhibitor (Peptide17). The results were shown in Supplementary File 4.
315
316 **2.6 High-throughput sequencing of transcriptome revealed the potential pathways in ECSCs**
317   The column graph and volcanic graph were displayed in Figure 4A-B, which indicated that 2675
318 genes were significantly increased in ECSCs^{Ishikawa} (cultured in DMEM/F12 with 10ng/ml hFGF,
319 20ng/ml hEGF, and B27 supplement) compared with Ishikawa (cultured in DMEM with 10%
320 FBS). While 253 genes were decreased. The heatmap in Figure 4C showed the Top100 genes with
321 the most significant changes, which included YAP1 (The details were shown in Supplementary
322 File 5). Gene ontology (GO) enrichment analysis indicated that the most related pathways were
323 plasma membrane, membrane, integral component of the plasma membrane, integral component
324 of membrane, extracellular space, extracellular region, extracellular matrix organization,
325 extracellular matrix, and cell surface. Kyoto Encyclopedia of Genes and Genomes (KEGG)
326 pathway enrichment indicated that typical pathways in cancer were involved, including the Wnt
327 signaling pathway, and the p53 signaling pathway. Also, the HIPPO signaling pathway had a
328 significant difference between ECSCs^{Ishikawa} and Ishikawa (p-value=0.000227292). The details
329 were shown in Figure 4D-E.
330
331 **2.7 Protein-protein interactions network was constructed**
332   Based on the previous result of high throughput sequencing, the protein-protein interactions
333 network was constructed by STRING. The hub genes were displayed in Figure 5A (The details
334 were shown in Supplementary File 6). We further analyzed the core molecules related to YAP1,
335 which were shown in Figure 5B. These genes had close correlation with YAP1 based on our result:
336 LEF1, FAT1, JUN, AMOTL2, DKK4, GJA1.
337

Discussion

339   Many studies started to focus on the role that cancer stem-like cells played in recurrence, drug
340 resistance, and metastasis(Huang et al. 2020; Najafi et al. 2019). In our study, we focused on the
341 area of ECSCs which remained unclear. After grouping 774 patients by their expression levels of
342 YAP1, we found a significant difference in prognosis between high and low expression groups.
343 The data from KM Plotter also confirmed the same trend. Subsequently, we explored the
344 underlying mechanism behind it and found that YAP1, a core molecule of the HIPPO pathway,
345 played an important role in the development and maintenance of CSCs' stemness(Ma et al. 2019).
346 Many studies have also confirmed this view. After the down-regulation of YAP1 in vitro, the
347 stemness of Ishikawa and HEC1-A was significantly inhibited. This confirmed our view that
348 YAP1 may influence the prognosis of patients with EC by regulating stemness.

349First, YAP1 had now been shown to play a key role in many types of tumors. Researchers had
350illustrated the function of YAP1 as a prognosis marker in advanced gallbladder cancer(García et
351al. 2020). Overexpression of YAP1 was also proved to be associated with poorer prognosis in
352breast cancer(Guo et al. 2019). Our study also drew a similar result: patients with EC who had a
353high expression level of YAP1 tended to have a poorer prognosis. In parallel, we found that
354immunohistochemical staining suggests that patients with different expressions of YAP1 have
355different prognoses. This suggests the potential of YAP1 as an immunohistochemical index in the
356prediction of recurrence. Especially in developing countries, immunohistochemistry can be
357applied to more patients conveniently and economically. Our results suggested that YAP1 was a
358promising molecule for predicting the prognosis of patients with EC and was also a potential target
359for relapse prevention therapy, which was certainly meaningful and interesting.

360Secondly, studies had suggested YAP1 had a strong impact on the development and maintaining
361stemness in many cancers. YAP1 overexpression contributed to the induction of cancer stemness
362in prostate cancer(Lee et al. 2021). Therefore, in this study, we also focused on whether YAP1 had
363made an influence on the stemness of EC cell lines. As it's expected, the knockdown of YAP1 in
364vitro significantly suppressed the stemness of Ishikawa and HEC1-A, which revealed a potential
365mechanism for YAP1 to affect the prognosis of EC patients, by regulating the stemness of EC.
366Further study of the role that YAP1 acts in stemness will provide the basis for subsequent drug
367development, etc.

368Third, the existing studies were not well understood for ECSCs. Although the important role of
369several pathways (such as Wnt pathway, NOTCH pathway(Götte et al. 2011; Wang et al. 2017))
370in ECSCs had been proposed. The pathways behind ECSCs had not been systematically described.
371Therefore, we performed high-throughput transcriptome sequencing of ECSCs. YAP1, as well as
372the HIPPO pathway, were proven to be crucial in regulating stemness. Our results might provide
373new insight for others to further investigate ECSCs. More experiments should focus on the role of
374the HIPPO pathway played in ECSCs. Notably, we found that there were still many genes in stem
375cells that differ from normal cell lines, and these need to be further investigated by scholars.

376There were a few limitations in our research:① This study was a single-center study, and the
377sample size was limited by the follow-up time. More cases were needed to get more accurate
378results. ② Although our results were sufficient to confirm the role YAP1 played in ECSCs, more
379experiments in vivo would furtherly confirm our findings. ③ The potential pathways and
380molecules in ECSCs were revealed by high through-put sequencing. But they should be verified
381in the following studies.

382

Conclusions

384Our study indicated that YAP1 affected the prognosis of patients with EC by regulating
385stemness. High expression of YAP1 resulted in poorer outcomes. Knockdown of YAP1 would
386significantly inhibit the stemness of Ishikawa and HEC1-A. HIPPO pathway had a strong impact
387on ECSCs. It can be concluded that YAP1 was a promising biomarker for predicting the prognosis
388of EC and was a potential molecule for targeting the recurrence and metastasis of EC.

389

Abbreviations

391   EC, endometrial cancer; CQMU, Chongqing Medical University; CSCs, cancer stem-like cells;
392 ECSCs, endometrial cancer stem-like cells; YAP1, Yes-associated protein 1; FIGO, Federation
393 International of Gynecology and Obstetrics; BMI, body mass index; LVSI, lymph vascular
394 invasion; RFS, recurrence-free survival; OS, overall survival; qRT-PCR, quantitative reverse
395 transcription PCR; WB, western blot; hEGF, human epidermal growth factor; hFGF, human
396 fibroblast growth factor; SOX2, SRY-box containing gene 2; OCT4, octamer-binding transcription
397 factor 4; CD44, cluster of differentiation-44; CD133, cluster of differentiation-133.

398

Declarations

400 **Authors' contributions**

401   This study was designed by Rui Yuan. Wei Kong and Yuzhen Huang participated in the writing
402 of the manuscript. Wei Kong, Yuzhen Huang, Peng Jiang, Yuan Tu, Ning Li, Jinyu Wang
403 participated in statistical analysis, cell culture, qRT-PCR, and western blot, etc. Qian Zhou,
404 Yunfeng Zheng, Shikai Gou, and Chenfan Tian participated in collecting data. All the authors had
405 approved the final version of the manuscript.

406 **Consent to participate**

407   Written informed consent was obtained from the patients for their anonymized information to
408 be published in this article.

409 **Data availability**

410   The data would be available from the corresponding author upon reasonable request.
411

412 **Conflict of interest**

413   The authors declared no conflict of interest.
414

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417

418 **References**

419 Babaei G, Aziz SG-G, and Jaghi NZZ. 2021. EMT, cancer stem cells and autophagy; The three main axes of
420   metastasis. *Biomedicine & Pharmacotherapy = Biomedecine & Pharmacotherapie* 133:110909.
421   10.1016/j.biopha.2020.110909
422 Ballout F, Lu H, Chen L, Sriramajayam K, Que J, Meng Z, Wang TC, Giordano S, Zaika A, McDonald O, Peng D,
423   and El-Rifai W. 2022. APE1 redox function is required for activation of Yes-associated protein 1 under
424   reflux conditions in Barrett's-associated esophageal adenocarcinomas. *Journal of Experimental & Clinical*
425   *Cancer Research : CR* 41:264. 10.1186/s13046-022-02472-5
426 Brooks RA, Fleming GF, Lastra RR, Lee NK, Moroney JW, Son CH, Tatebe K, and Veneris JL. 2019. Current
427   recommendations and recent progress in endometrial cancer. *CA: a Cancer Journal For Clinicians* 69:258-
428   279. 10.3322/caac.21561
429 Castellan M, Guarnieri A, Fujimura A, Zanconato F, Battilana G, Panciera T, Sladitschek HL, Contessotto P, Citron
430   A, Grilli A, Romano O, Bicciato S, Fassan M, Porcù E, Rosato A, Cordenonsi M, and Piccolo S. 2021.
431   Single-cell analyses reveal YAP/TAZ as regulators of stemness and cell plasticity in Glioblastoma. *Nature*
432   *Cancer* 2:174-188. 10.1038/s43018-020-00150-z
433 Chen G, Liu B, Yin S, Li S, Guo Ye, Wang M, Wang K, and Wan X. 2020. Hypoxia induces an endometrial cancer
434   stem-like cell phenotype via HIF-dependent demethylation of SOX2 mRNA. *Oncogenesis* 9:81.
435   10.1038/s41389-020-00265-z
436 Elbasateeny SS, Salem AA, Abdelsalam WA, and Salem RA. 2016. Immunohistochemical expression of cancer
437   stem cell related markers CD44 and CD133 in endometrial cancer. *Pathology, Research and Practice*
438   212:10-16. 10.1016/j.prp.2015.10.008
439 Ferlay J, Colombet M, Soerjomataram I, Dyba T, Randi G, Bettio M, Gavin A, Visser O, and Bray F. 2018. Cancer
440   incidence and mortality patterns in Europe: Estimates for 40 countries and 25 major cancers in 2018.
441   *European Journal of Cancer (Oxford, England : 1990)* 103:356-387. 10.1016/j.ejca.2018.07.005
442 García P, Rosa L, Vargas S, Weber H, Espinoza JA, Suárez F, Romero-Calvo I, Elgueta N, Rivera V, Nervi B,
443   Obreque J, Leal P, Viñuela E, Aguayo G, Muñiz S, Sagredo A, Roa JC, and Bizama C. 2020. Hippo-YAP1
444   Is a Prognosis Marker and Potentially Targetable Pathway in Advanced Gallbladder Cancer. *Cancers* 12.
445   10.3390/cancers12040778
446 Giannone G, Attademo L, Scotto G, Genta S, Ghisoni E, Tuninetti V, Aglietta M, Pignata S, and Valabrega G. 2019.
447   Endometrial Cancer Stem Cells: Role, Characterization and Therapeutic Implications. *Cancers* 11.
448   10.3390/cancers11111820
449 Götte M, Greve B, Kelsch R, Müller-Uthoff H, Weiss K, Kharabi Masouleh B, Sibrowski W, Kiesel L, and
450   Buchweitz O. 2011. The adult stem cell marker Musashi-1 modulates endometrial carcinoma cell cycle
451   progression and apoptosis via Notch-1 and p21WAF1/CIP1. *International Journal of Cancer* 129:2042-
452   2049. 10.1002/ijc.25856

453   Guo L, Chen Y, Luo J, Zheng J, and Shao G. 2019. YAP1 overexpression is associated with poor prognosis of
454   breast cancer patients and induces breast cancer cell growth by inhibiting PTEN. *FEBS Open Bio* 9:437-
455   445. 10.1002/2211-5463.12597
456   Huang T, Song X, Xu D, Tiek D, Goenka A, Wu B, Sastry N, Hu B, and Cheng S-Y. 2020. Stem cell programs in
457   cancer initiation, progression, and therapy resistance. *Theranostics* 10:8721-8743. 10.7150/thno.41648
458   Hubbard SA, Friel AM, Kumar B, Zhang L, Rueda BR, and Gargett CE. 2009. Evidence for cancer stem cells in
459   human endometrial carcinoma. *Cancer Research* 69:8241-8248. 10.1158/0008-5472.CAN-08-4808
460   Huijgens ANJ, and Mertens HJMM. 2013. Factors predicting recurrent endometrial cancer. *Facts, Views & Vision*
461   *In ObGyn* 5:179-186.
462   Jia M, Jiang P, Hu J, Huang Z, Deng Y, and Hu Z. 2021. The optimal cut-off value of immunohistochemical
463   parameter P53 for predicting recurrence of endometrial cancer. *International Journal of Gynaecology and*
464   *Obstetrics: the Official Organ of the International Federation of Gynaecology and Obstetrics* 153:344-350.
465   10.1002/ijgo.13498
466   Jiang P, Huang Y, Tu Y, Li N, Kong W, Di F, Jiang S, Zhang J, Yi Q, and Yuan R. 2021. Combining
467   Clinicopathological Parameters and Molecular Indicators to Predict Lymph Node Metastasis in
468   Endometrioid Type Endometrial Adenocarcinoma. *Frontiers In Oncology* 11:682925.
469   10.3389/fonc.2021.682925
470   Jiang P, Jia M, Hu J, Huang Z, Deng Y, Lai L, Ding S, and Hu Z. 2020. Prognostic Value of Ki67 in Patients with
471   Stage 1-2 Endometrial Cancer: Validation of the Cut-off Value of Ki67 as a Predictive Factor. *OncoTargets*
472   *and Therapy* 13:10841-10850. 10.2147/OTT.S274420
473   Lánczky A, and Győrffy B. 2021. Web-Based Survival Analysis Tool Tailored for Medical Research (KMplot):
474   Development and Implementation. *Journal of Medical Internet Research* 23:e27633. 10.2196/27633
475   Lee H-C, Ou C-H, Huang Y-C, Hou P-C, Creighton CJ, Lin Y-S, Hu C-Y, and Lin S-C. 2021. YAP1 overexpression
476   contributes to the development of enzalutamide resistance by induction of cancer stemness and lipid
477   metabolism in prostate cancer. *Oncogene* 40:2407-2421. 10.1038/s41388-021-01718-4
478   Li Y, Wang D, Liu J, Li Y, Chen D, Zhou L, Lang T, and Zhou Q. 2020. Baicalin Attenuates YAP Activity to
479   Suppress Ovarian Cancer Stemness. *OncoTargets and Therapy* 13:7151-7163. 10.2147/OTT.S254607
480   Lin H, Peng J, Zhu T, Xiong M, Zhang R, and Lei L. 2022. Exosomal miR-4800-3p Aggravates the Progression of
481   Hepatocellular Carcinoma via Regulating the Hippo Signaling Pathway by Targeting STK25. *Frontiers In*
482   *Oncology* 12:759864. 10.3389/fonc.2022.759864
483   Lu KH, and Broaddus RR. 2020. Endometrial Cancer. *The New England Journal of Medicine* 383:2053-2064.
484   10.1056/NEJMra1514010
485   Ma S, Meng Z, Chen R, and Guan K-L. 2019. The Hippo Pathway: Biology and Pathophysiology. *Annual Review of*
486   *Biochemistry* 88:577-604. 10.1146/annurev-biochem-013118-111829
487   Mirantes C, Espinosa I, Ferrer I, Dolcet X, Prat J, and Matias-Guiu X. 2013. Epithelial-to-mesenchymal transition
488   and stem cells in endometrial cancer. *Human Pathology* 44:1973-1981. 10.1016/j.humpath.2013.04.009
489   Muñoz-Galván S, Felipe-Abrio B, Verdugo-Sivianes EM, Perez M, Jiménez-García MP, Suarez-Martinez E,
490   Estevez-Garcia P, and Carnero A. 2020. Downregulation of MYPT1 increases tumor resistance in ovarian
491   cancer by targeting the Hippo pathway and increasing the stemness. *Molecular Cancer* 19:7.
492   10.1186/s12943-020-1130-z

493 Najafi M, Mortezaee K, and Majidpoor J. 2019. Cancer stem cell (CSC) resistance drivers. *Life Sciences*
494   234:116781. 10.1016/j.lfs.2019.116781

495 Ouldamer L, Bendifallah S, Body G, Canlorbe G, Touboul C, Graesslin O, Raimond E, Collinet P, Coutant C,
496   Lavoué V, Lévêque J, Bricou A, Huchon C, Daraï E, and Ballester M. 2018. Change in hazard rates of
497   recurrence over time following diagnosis of endometrial cancer: An age stratified multicentre study from
498   the FRANCOGYN group. *European Journal of Surgical Oncology : the Journal of the European Society of*
499   *Surgical Oncology and the British Association of Surgical Oncology* 44:1914-1920.
500   10.1016/j.ejso.2018.07.053

501 Ouldamer L, Bendifallah S, Body G, Touboul C, Graesslin O, Raimond E, Collinet P, Coutant C, Lavoué V,
502   Lévêque J, Daraï E, and Ballester M. 2016. Predicting poor prognosis recurrence in women with
503   endometrial cancer: a nomogram developed by the FRANCOGYN study group. *British Journal of Cancer*
504   115:1296-1303. 10.1038/bjc.2016.337

505 Park JY, Hong D, and Park JY. 2019. Association between Morphological Patterns of Myometrial Invasion and
506   Cancer Stem Cell Markers in Endometrial Endometrioid Carcinoma. *Pathology Oncology Research : POR*
507   25:123-130. 10.1007/s12253-017-0320-5

508 Rutella S, Bonanno G, Procoli A, Mariotti A, Corallo M, Prisco MG, Eramo A, Napoletano C, Gallo D, Perillo A,
509   Nuti M, Pierelli L, Testa U, Scambia G, and Ferrandina G. 2009. Cells with characteristics of cancer
510   stem/progenitor cells express the CD133 antigen in human endometrial tumors. *Clinical Cancer Research :*
511   *an Official Journal of the American Association For Cancer Research* 15:4299-4311. 10.1158/1078-
512   0432.CCR-08-1883

513 Shi P, Li Y, and Guo Q. 2021. Circular RNA circPIP5K1A contributes to cancer stemness of osteosarcoma by miR-
514   515-5p/YAP axis. *Journal of Translational Medicine* 19:464. 10.1186/s12967-021-03124-6

515 Shin M-K, and Cheong J-H. 2019. Mitochondria-centric bioenergetic characteristics in cancer stem-like cells.
516   *Archives of Pharmacal Research* 42:113-127. 10.1007/s12272-019-01127-y

517 Suzuki I, Yoshida S, Tabu K, Kusunoki S, Matsumura Y, Izumi H, Asanoma K, Yagi H, Onoyama I, Sonoda K,
518   Kohno K, Taga T, Itakura A, Takeda S, and Kato K. 2021. YBX2 and cancer testis antigen 45 contribute to
519   stemness, chemoresistance and a high degree of malignancy in human endometrial cancer. *Scientific*
520   *Reports* 11:4220. 10.1038/s41598-021-83200-5

521 Szklarczyk D, Gable AL, Nastou KC, Lyon D, Kirsch R, Pyysalo S, Doncheva NT, Legeay M, Fang T, Bork P,
522   Jensen LJ, and von Mering C. 2021. The STRING database in 2021: customizable protein-protein
523   networks, and functional characterization of user-uploaded gene/measurement sets. *Nucleic Acids Research*
524   49:D605-D612. 10.1093/nar/gkaa1074

525 Szymonik J, Wala K, Górnicki T, Saczko J, Pencakowski B, and Kulbacka J. 2021. The Impact of Iron Chelators on
526   the Biology of Cancer Stem Cells. *International Journal of Molecular Sciences* 23. 10.3390/ijms23010089

527 Thirusangu P, Ray U, Sarkar Bhattacharya S, Oien DB, Jin L, Staub J, Kannan N, Molina JR, and Shridhar V. 2022.
528   PFKFB3 regulates cancer stemness through the hippo pathway in small cell lung carcinoma. *Oncogene*
529   41:4003-4017. 10.1038/s41388-022-02391-x

530 Tian C, Lang T, Qiu J, Han K, Zhou L, Min D, Zhang Z, and Qi D. 2020. SKP1 promotes YAP-mediated colorectal
531   cancer stemness via suppressing RASSF1. *Cancer Cell International* 20:579. 10.1186/s12935-020-01683-0

532　Wang ZM, Wan XH, Sang GY, Zhao JD, Zhu QY, and Wang DM. 2017. miR-15a-5p suppresses endometrial
533　　　cancer cell growth via Wnt/β-catenin signaling pathway by inhibiting WNT3A. *European Review For*
534　　　*Medical and Pharmacological Sciences* 21:4810-4818.
535　Yang B, Shan B, Xue X, Wang H, Shan W, Ning C, Zhou Q, Chen X, and Luo X. 2016. Predicting Lymph Node
536　　　Metastasis in Endometrial Cancer Using Serum CA125 Combined with Immunohistochemical Markers PR
537　　　and Ki67, and a Comparison with Other Prediction Models. *PloS One* 11:e0155145.
538　　　10.1371/journal.pone.0155145
539　Zanconato F, Forcato M, Battilana G, Azzolin L, Quaranta E, Bodega B, Rosato A, Bicciato S, Cordenonsi M, and
540　　　Piccolo S. 2015. Genome-wide association between YAP/TAZ/TEAD and AP-1 at enhancers drives
541　　　oncogenic growth. *Nature Cell Biology* 17:1218-1227. 10.1038/ncb3216
542　Zhou X, Luo J, Xie H, Wei Z, Li T, Liu J, Liao X, Zhu G, and Peng T. 2022. MCM2 promotes the stemness and
543　　　sorafenib resistance of hepatocellular carcinoma cells via hippo signaling. *Cell Death Discovery* 8:418.
544　　　10.1038/s41420-022-01201-3
545　Zhou X, Zhou Y-P, Huang G-R, Gong B-L, Yang B, Zhang D-X, Hu P, and Xu S-R. 2011. Expression of the stem
546　　　cell marker, Nanog, in human endometrial adenocarcinoma. *International Journal of Gynecological*
547　　　*Pathology : Official Journal of the International Society of Gynecological Pathologists* 30:262-270.
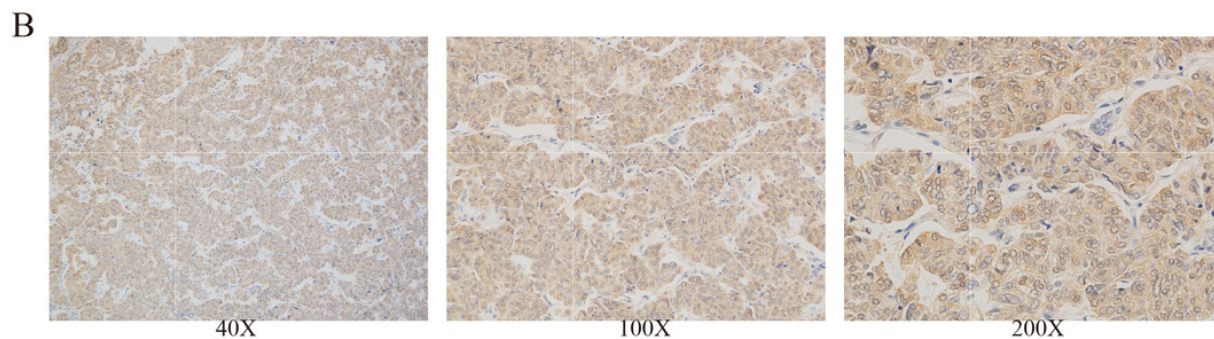548　　　10.1097/PGP.0b013e3182055a1f
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Figure 1

Different expressions of YAP1 from CQMU cohort and Kaplan-Meier Curves from KM Plotter and CQMU cohort

Examples of different expressions of YAP1 from CQMU cohort were displayed. **(A)** Case 140 (without recurrence by the end time of follow-up): High-differentiated EC with low expression of YAP1. **(B)** Case 81 (With recurrence by the end time of follow-up): Low-differentiated EC with high expression of YAP1. Kaplan-Meier curves showed the differences of RFS and OS between high and low expression YAP1 groups. KM Plotter: low expression group, n=314; high expression group, n=108. CQMU cohort: low expression group, n=626; high expression group, n=148. Logrank was used to test the difference. **(C)** RFS of two groups from KM Plotter. (Logrank P=0.24) **(D)** OS of two groups from KM Plotter. (Logrank P=0.32) **(E)** RFS of two groups from CQMU cohort. (Logrank P<0.001) **(F)** OS of two groups from CQMU cohort. (Logrank P<0.001).

A

40X   100X   200X
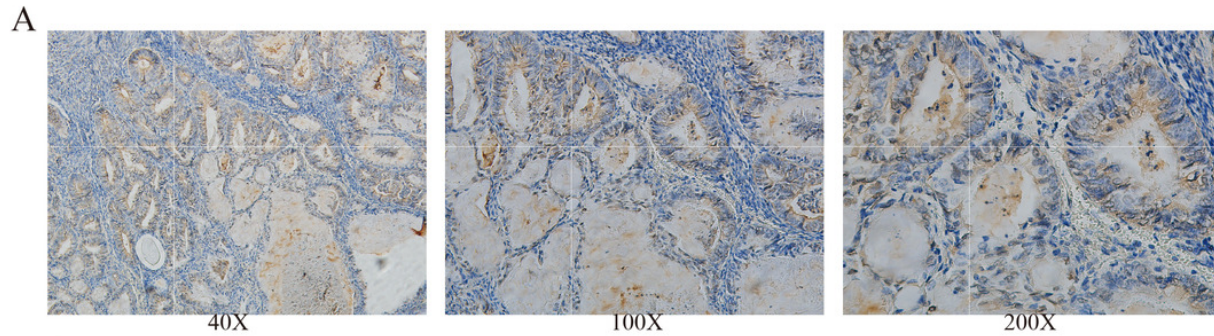
High-differentiated EC tissue sample with low expression of YAP1

B

40X   100X   200X

Low-differentiated EC tissue sample with high expression of YAP1

C

HR = 1.41 (0.8 − 2.48)
logrank P = 0.24

Expression
— low
— high

Number at risk

low	314	91	13	1	1
high	108	24	3	1	0

D

HR = 1.23 (0.81 − 1.86)
logrank P = 0.32

Expression
— low
— high

Number at risk

low	286	89	12	1	1
high	256	58	8	2	0

E

logrank P < 0.001

Expression
— low
— high

F

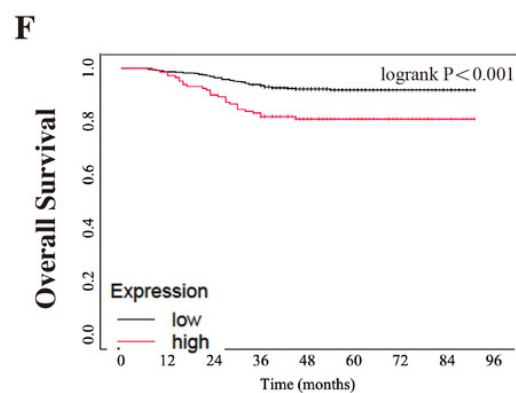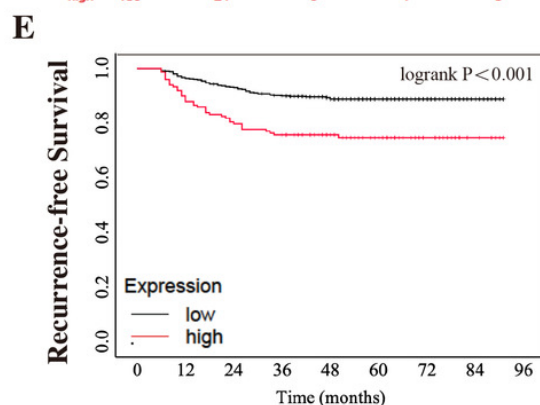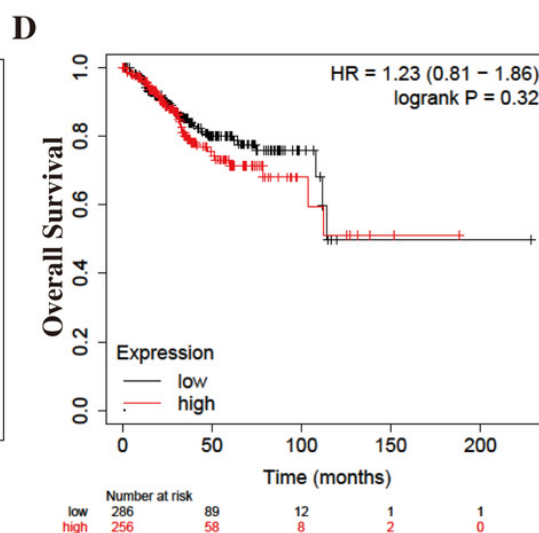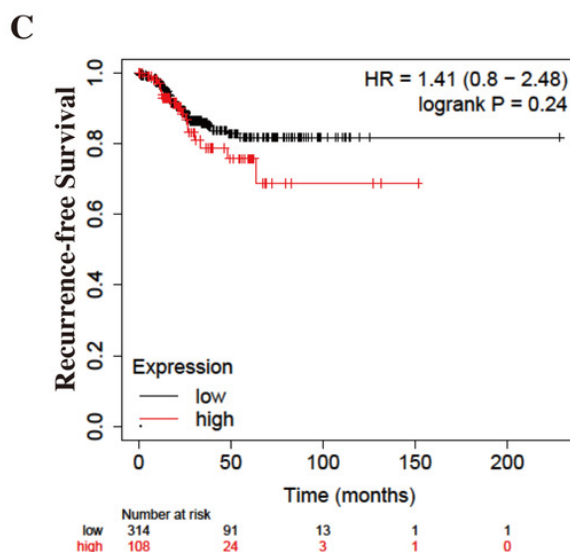logrank P < 0.001

Expression
— low
— high

Figure 2

Identification of ECSCs and detecting expression of YAP1

HEC1-A, Ishikawa, and RL952 were used for culture of ECSCs. And the most stem-like state cell lines were HEC1-A and Ishikawa. These two cell lines were used for the subsequent experiments. Student's t test was used to test the differences between two groups. **(A)** The spheric formation images of HEC1-A, Ishikawa, and RL952 on the 3rd, 5th and 7th day were displayed (100X). **(B-C)** Analysis of the relative mRNA expressions of SOX2, OCT4, NANOG, CD44, and CD133 in different ECSCs, compared with its original cell lines. (qRT-PCR were tested for three times. Mean±SD. *$P<0.05$, **$P<0.01$, *** $P<0.001$.) **(D-E)** Protein expressions of SOX2, OCT4, NANOG, CD44, and CD133 by western blot in different ECSCs and controls. **(F)** Relative mRNA expression of YAP1 detected by qRT-PCR. (qRT-PCR were tested for three times. Mean±SD. *** $P<0.001$) **(G)** Protein expression of YAP1 in different ECSCs, compared with its original cell lines.
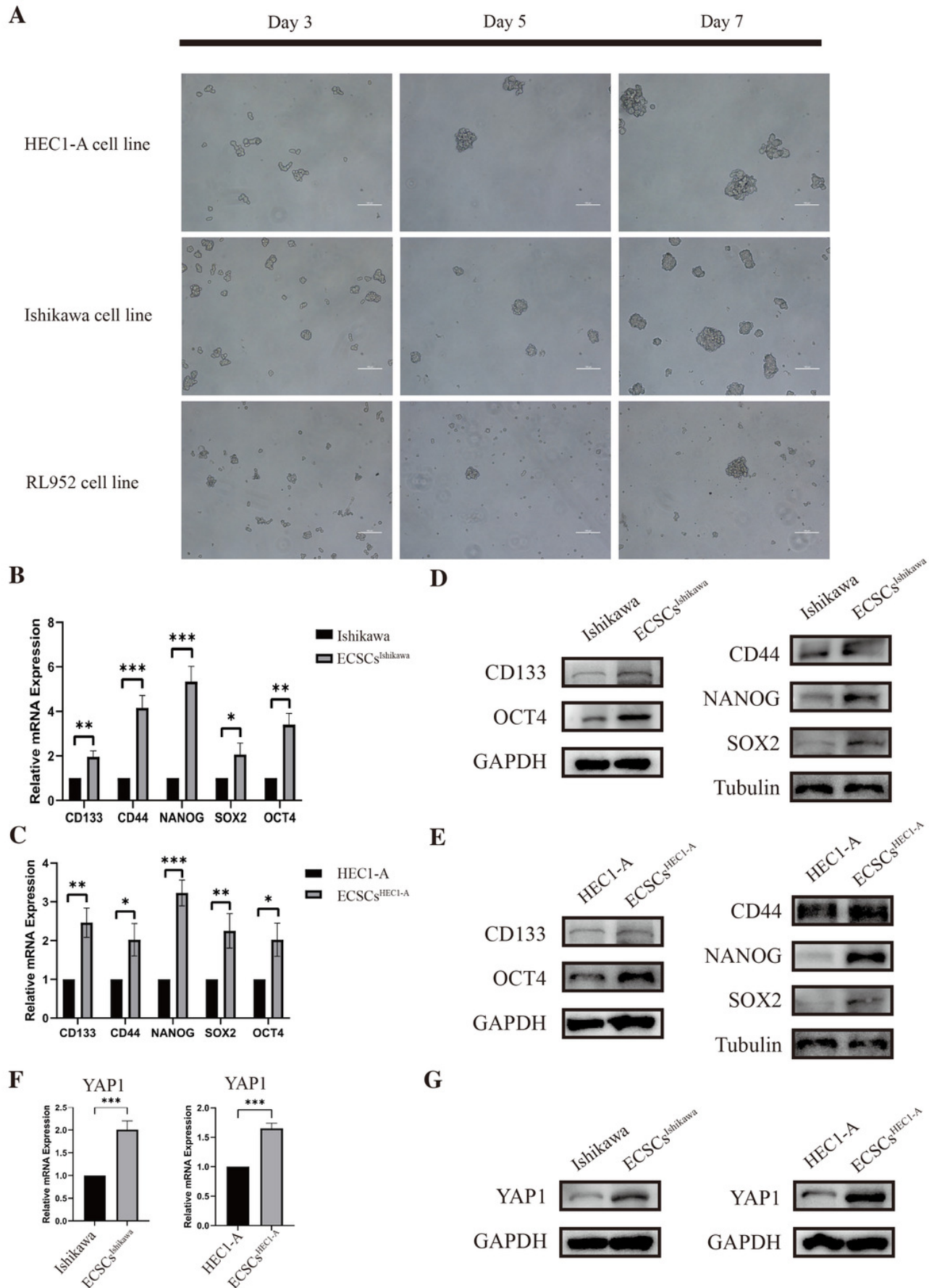
Figure 3

The different abilities of sphere formation after YAP1 knockdown and expressions of SOX2, OCT4, NANOG, CD44, and CD133 after YAP1 knockdown

The efficiency of spheres formation was compared after YAP1 knockdown. **(A-B)** Down-regulation of YAP1 by siRNA was confirmed by qRT-PCR. (qRT-PCR were tested for three times) **(C)** Images of spheres formation of ECSCs^{Ishikawa} after YAP1 knockdown on the 7th day (100X). **(D)** Counting of ECSCs^{Ishikawa} spheres. Mean number of five random scopes stood for the ultimate result. **(E)** Images of spheres formation of ECSCs^{HEC1-A} after YAP1 knockdown on the 7th day (100X). **(F)** Counting of ECSCs^{HEC1-A} spheres. Mean number of five random scopes stood for the ultimate result. After YAP1 knockdown in Ishikawa and HEC1-A, the expressions of SOX2, OCT4, NANOG, CD44, and CD133 were detected by qRT-PCR and western blot. **(G)** mRNA expressions of SOX2, OCT4, NANOG, CD44, and CD133 in Ishikawa were detected by qRT-PCR. (qRT-PCR were tested for three times. Student's t test was used to test the differences between two groups. Mean±SD. *P<0.05, **P<0.01, *** P<0.001, ****P<0.0001.) **(H)** Protein expressions of YAP1, SOX2, OCT4, NANOG, CD44, and CD133 in Ishikawa were detected. **(I)** mRNA expressions of SOX2, OCT4, NANOG, CD44, and CD133 in HEC1-A were detected by qRT-PCR. (qRT-PCR were tested for three times. Student's t test was used to test the differences between two groups. Mean±SD. *P<0.05, **P<0.01, *** P<0.001, ****P<0.0001.) **(J)** Protein expressions of YAP1, SOX2, OCT4, NANOG, CD44, and CD133 in HEC1-A were detected.

A YAP mRNA Expression (Ishikawa cell line)

B YAP mRNA Expression (HEC1-A)

C ECSCs^Ishikawa, Day7, 100X

D ECSCs^Ishikawa, Day7

E ECSCs^HEC1-A, Day7, 100X

F ECSCs^HEC1-A, Day7

G Ishikawa cell line

H Ishikawa cell line
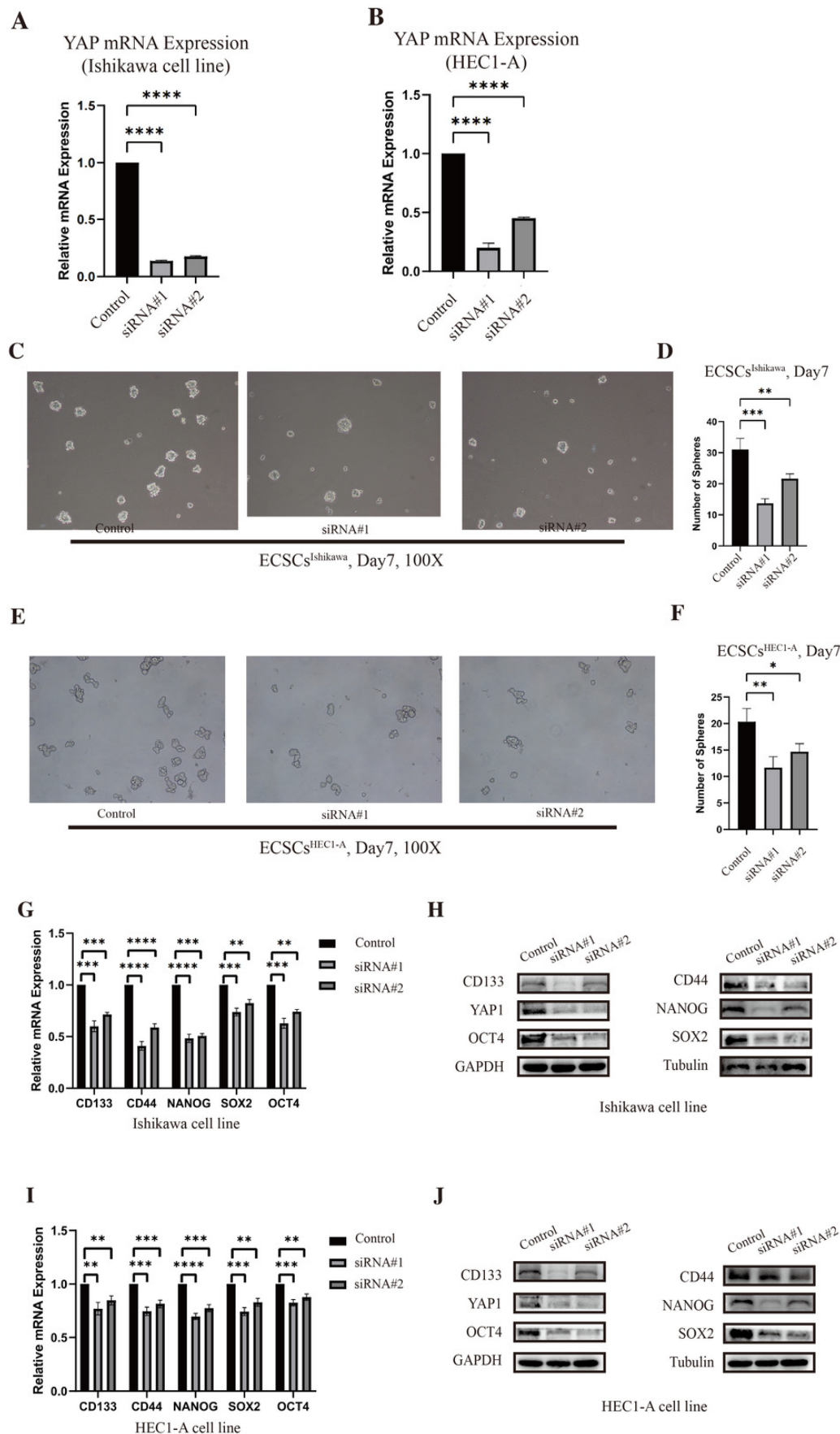
I HEC1-A cell line

J HEC1-A cell line

Figure 4

Outcome of high through-put sequencing

High through-put sequencing was performed by using total RNA of ECSCs[Ishikawa], compared with Ishikawa cell line. **(A-B)** Plots indicated that 2675 genes were up-regulated in ECSCs[Ishikawa] while 253 genes were down-regulated. log2 (fold change) ≥ 1 or log2 (fold change) ≤ -1 and p value < 0.05. **(C)** Heatmap of Top100 significant genes. **(D)** GO enrichment analysis. **(E)** KEGG enrichment analysis.

A — Barplot of differentially expressed genes

B — Volcano plot of differentially expressed genes

C — Heatmap

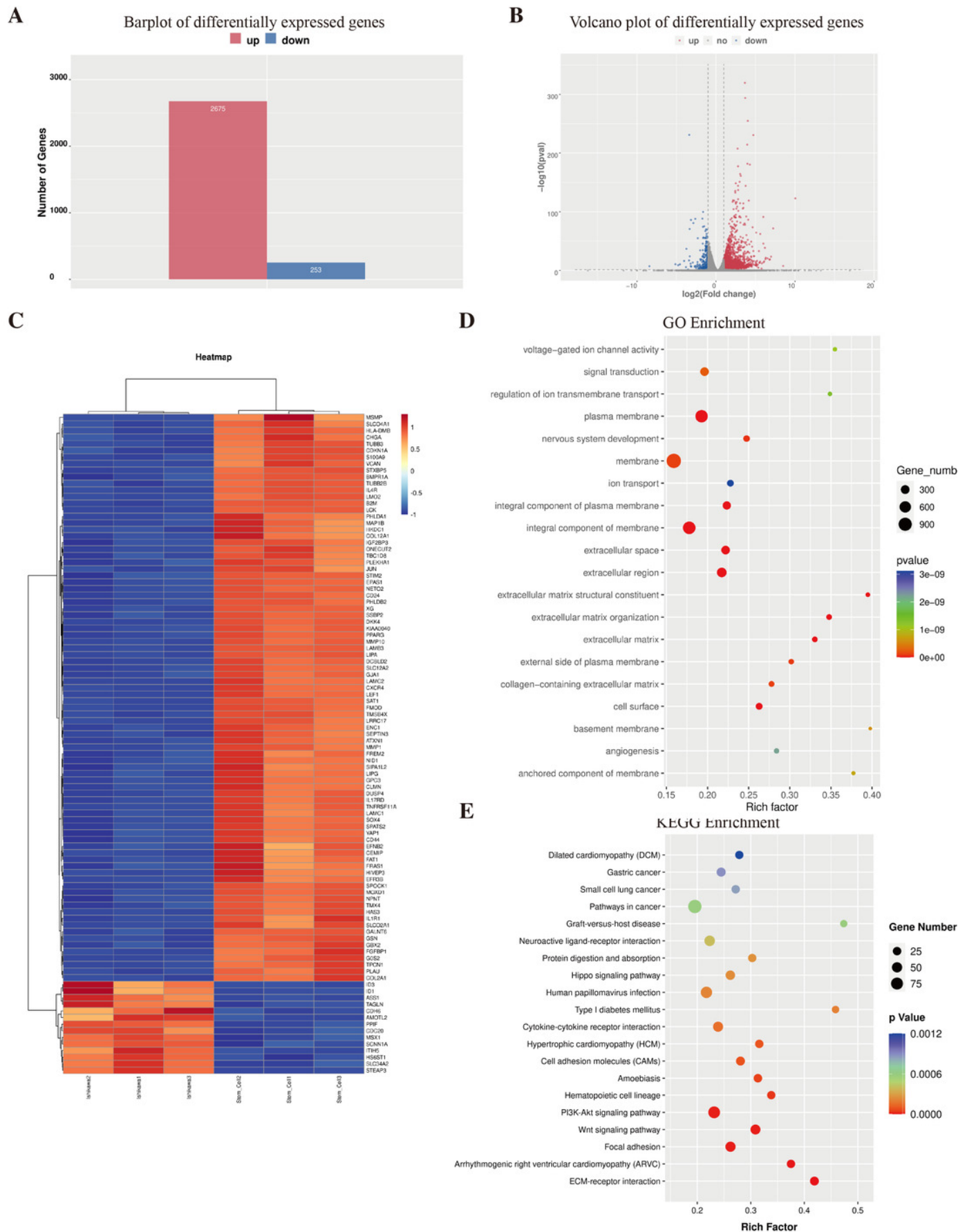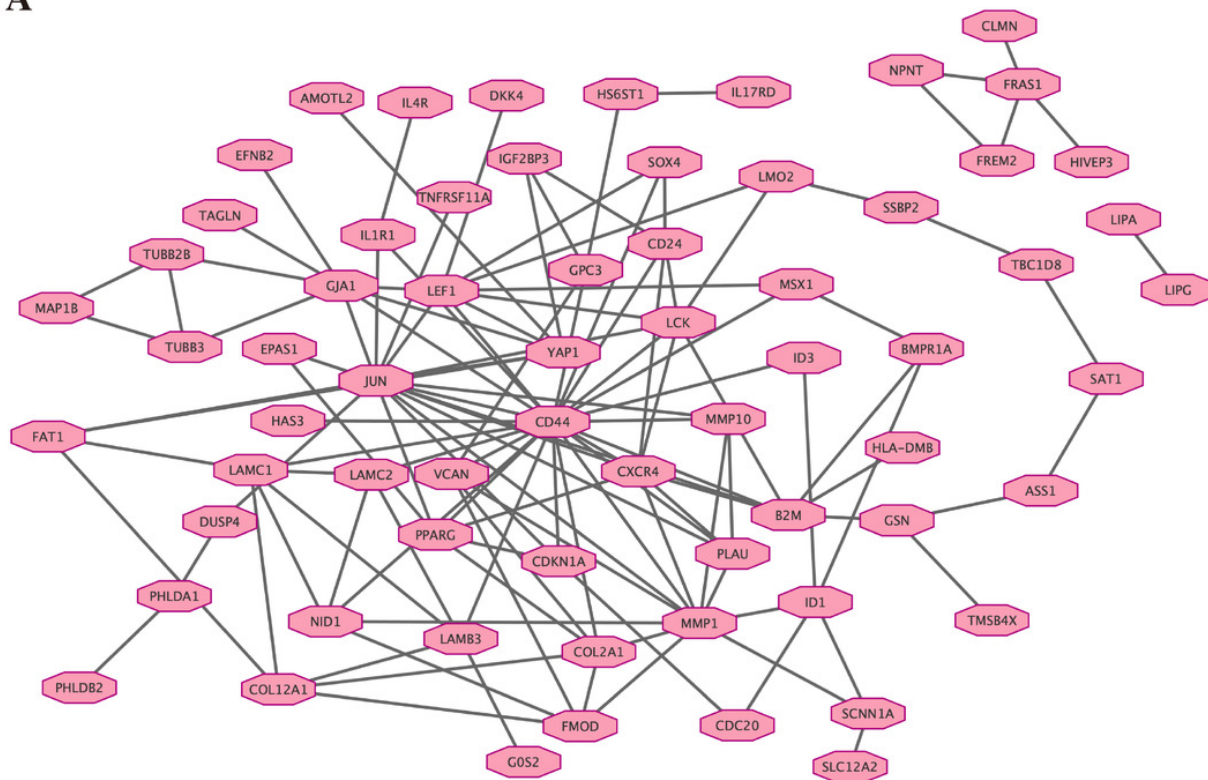D — GO Enrichment

E — KEGG Enrichment

Figure 5

Protein-protein interactions network

Protein-protein interactions (PPI) network was constructed by STRING. **(A)** PPI network. **(B)** PPI network related to YAP1.
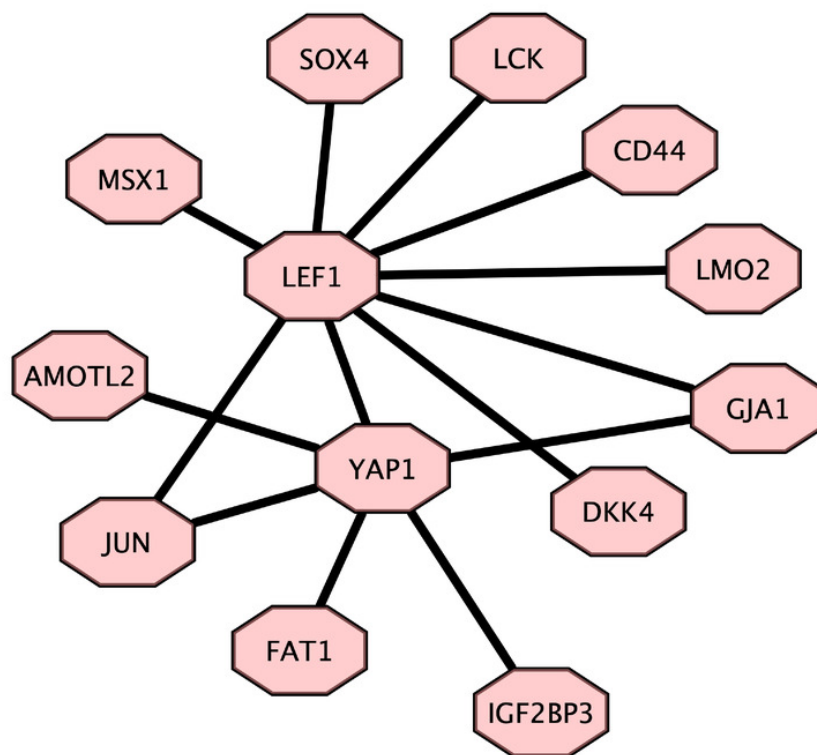
Table 1(on next page)

Table 1 Relationship between YAP1 and clinical characteristics of patients with EC. Table 2 Univariate and multivariate analyses for recurrence

1   **Table 1 Relationship between YAP1 and clinical characteristics of patients with EC**

Clinical characteristics	YAP1 expression (n=774)		P-values
	Low (n=626)	High (n=148)	
Age (years)			0.315
Mean±SD	53.70±9.38	54.55±9.99	
Range	24-81	34-78	
BMI (kg/m²)			0.393
Mean±SD	24.59±3.66	24.88±3.96	
Range	16.35-45.72	17.80-41.87	
FIGO Staging (n, %)			<0.001
FIGO I	460, 73.5%	85, 57.4%	
FIGO II	58, 9.3%	16, 10.8%	
FIGO III	108, 17.3%	47, 31.8%	
Cervical stromal invasion (n, %)			0.005
Yes	96, 15.3%	37, 25.0%	
No	530, 84.7%	111, 75.0%	
Myometrial invasion (n, %)			0.213
Yes	183, 29.2%	51, 34.5%	
No	443, 70.8%	97, 65.5%	
Histotypes (n, %)			0.009
Type I	461, 73.6%	93, 62.8%	
Type II	165, 26.4%	55, 37.2%	
LVSI (n, %)			0.018
Yes	141, 22.5%	47, 31.8%	
No	485, 77.5%	101, 68.2%	
ER (n, %)			0.031
Low	101, 16.1%	35, 23.6%	
High	525, 83.9%	113, 76.4%	
PR (n, %)			0.415
Low	129, 20.6%	35, 23.6%	
High	497, 79.4%	113, 76.4%	
Ki67 (n, %)			0.005
Low	383, 61.2%	72, 48.6%	
High	243, 38.8%	76, 51.4%	
P53 (n, %)			
Normal	398, 63.6%	84, 56.8%	0.001
Abnormal	228, 36.4%	64, 43.2%	
Recurrence (n, %)			<0.001
Yes	68, 10.9%	37, 25%	
No	558, 89.1%	111, 75%	

Recurrence-free survival (months)			0.359
Median	49	49	
Range	6-91	6-91	
Death (n, %)			<0.001
Yes	48, 7.7%	28, 18.9%	
No	578, 92.3%	120, 81.1%	
Overall survival (months)			0.653
Median	51	51.5	
Range	7-91	8-91	

2   **ABBREVIATIONS:** YAP1, Yes-associated protein 1; FIGO, Federation International of
3   Gynecology and Obstetrics; BMI, body mass index; LVSI, lymph vascular invasion; SD, standard
4   deviation; ER, estrogen receptor; PR, progesterone receptor.
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Table 2(on next page)

Table 2 Univariate and multivariate analyses for recurrence

1   **Table 2 Univariate and multivariate analyses for recurrence**

Clinicopathological	Univariate analysis			Multivariate analysis		
	HR	95%CI	p Value	HR	95%CI	p Value
Age (ref. <60)	2.441	1.663-3.584	<0.001	1.453	0.974-2.166	0.067
FIGO			<0.001			0.093
FIGO II (ref. FIGO I)	2.613	1.356-5.035	0.004	1.400	0.620-3.161	0.418
FIGO III (ref. FIGO I)	7.296	4.793-11.107	<0.001	1.804	1.054-3.086	0.031
Cervical stromal invasion (ref. No)	2.973	1.991-4.438	<0.001	1.215	0.727-2.031	0.458
Myometrial invasion (ref. No)	3.792	2.569-5.597	<0.001	1.824	1.191-2.794	0.006
Histotypes (ref. Type I)	5.866	3.908-8.805	<0.001	1.959	1.200-3.198	0.007
LVSI (ref. No)	4.650	3.161-6.841	<0.001	2.070	1.336-3.207	0.001
ER (ref. High)	6.139	4.184-9.008	<0.001	2.023	1.110-3.686	0.021
PR (ref. High)	4.618	3.148-6.774	<0.001	1.045	0.575-1.897	0.886
Ki67 (ref. Low)	2.966	1.982-4.438	<0.001	1.676	1.098-2.566	0.017
P53 (ref. Normal)	1.903	1.298-2.791	0.001	1.643	1.108-2.437	0.014
YAP1 (ref. Low)	2.516	1.686-3.755	<0.001	1.803	1.190-2.731	0.005

2   **ABBREVIATIONS:** YAP1, Yes-associated protein 1; FIGO, Federation International of
3   Gynecology and Obstetrics; BMI, body mass index; LVSI, lymph vascular invasion; ER, estrogen
4   receptor; PR, progesterone receptor; CI, confidence interval; HR, hazard ratio.
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