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Interference of EFNA4 suppresses cell proliferation, invasion and angiogenesis in hepatocellular carcinoma by downregulating PYGO2

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ABSTRACT

Hepatocellular carcinoma (HCC) is the most common type of liver cancer. Ephrin A4 (EFNA4) acts as an oncogene in multiple cancers but is little known in HCC. It is revealed that EFNA4 is highly expressed in patients with HCC and influences the proliferation of HCC cells; however, detailed regulatory mechanism of EFNA4 in HCC needs to be unveiled. Here, we discovered that EFNA4 was highly expressed in HCC cell lines. EFNA4 knockdown greatly suppressed cell proliferation, migration and invasion, as well as inhibiting angiogenesis in Huh7 cells. EFNA4 was demonstrated to interact with pygopus-2 (PYGO2) and positively regulate PYGO2 expression. Gene gain- and loss-of-function experiments revealed that the anti-tumor effect of EFNA4 knockdown was partly abolished by PYGO2 overexpression. Furthermore, EFNA4 knockdown blocked wnt/β-catenin signaling in Huh7 cells, which was then abolished by PYGO2. In conclusion, this study further ensured the oncogenic role of EFNA4 in HCC, and disclosed that EFNA4 knockdown suppressed cell proliferation, invasion, angiogenesis, and wnt/β-catenin signaling in HCC by downregulating PYGO2.

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KEYWORDS
Ephrin A4; pygopus-2; hepatocellular carcinoma; β-catenin

Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent type of malignancies worldwide and ranks as the second leading cause of cancer-related death in men and the sixth in women.[1] Curative surgical resection is the most common treatment for HCC, and the increased detection rate of HCC has improved patients' survival in recent decades, but the 5-y survival rate is unsatisfactory attributed to high rates of recurrence and distant metastasis after curative surgical resection.[2,3] Hence, it is imperative to explore the molecular mechanisms underlying HCC to identify effective molecules for treatment of HCC.

Eph receptor tyrosine kinases and their membrane-bound ligands, the ephrins (EFNs), are well known for their role in regulating cell shape and attachment via regulating cell migration and adhesion.[4–6] In accordance with gene sequence similarity and the binding of ephrin A or B ligands, the EFNs are divided into A and B classes. Ephrin A4 (EFNA4) is widely expressed in multiple organs and involved in the development of various physiological and pathological processes. Recent evidence discloses the involvement of EFNA4 in the proliferation and metastasis of ovarian cancer, glioma, and chronic lymphocyte leukemia, and suggests that EFNA4 may play a functional role in fate decisions of cancer cells.[7–9] For instance, high expression of EFNA4 was associated with poor recurrence-free survival in patients with oral squamous cell carcinoma (OSCC). Meanwhile, EFNA4-induced cell migration and sphere formation of most OSCC cells, indicating that EFNA4 acted as an oncogene in OSCC and might be a useful prognostic biomarker for OSCC.[10] In addition, EFNA4 was broadly overexpressed in triple-negative breast cancer (TNBC) and ovarian cancer, and anti-EFNA4 antibody–drug conjugate (ADC) was found to achieve sustained tumor regressions in TNBC patient-derived xenografts (PDX) and reduced the frequency of tumor-initiating cells.[11] The above studies suggest that EFNA4 expression is highly expressed in multiple cancers and is expected to be an oncogene. However, there is still a shortage of studies exploring the correlation between EFNA4 and HCC. It is revealed that EFNA4 was highly expressed and led to poor prognosis in patients with HCC. EFNA4 influenced the proliferation and migration of HCC cells through a PIK3R2/GSK3β/β-catenin positive feedback loop.[12] Nevertheless, a more detailed regulatory mechanism of EFNA4 in HCC still needs to be unveiled.

To find out the molecular mechanism of EFNA4, we searched LinkedOimcs database (http://www.linkedomics.org/login.php) and discovered lots of EFNA4-associated genes. Among these genes, VPS7 was the top associated with EFNA4, and pygopus-2 (PYGO2) ranked second. The role of VPS7 in HCC is unclear, while PYGO2 is widely up-regulated in various types of cancer and has been recognized as novel biomarker for HCC prognosis.[13] Thus, we speculated that the regulatory mechanism of EFNA4 in HCC might be linked to PYGO2.

In this study, we systematically investigated the role of EFNA4 in HCC, and explored its potential regulatory mechanism, which might provide new insights for the understanding of EFNA4 in HCC and lay a basis for its clinical application in precision diagnosis and therapy for HCC.

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Materials and methods

Cell culture

The immortalized human liver epithelial cell THLE-3, and HCC cell lines (Huh7, MHCC97-H, SNU-449 and HCCLM3) were purchased from American Type Culture Collection and cultured in DMEM (Gibco; Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (Gibco). The cells were cultured at 37°C in an atmosphere of 5% CO_2 in the air.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cells using Trizol (Invitrogen, USA), followed with the synthesis of cDNA using the M-MLV First Strand Kit (Invitrogen). Afterward, the qRT-PCR was conducted with the application of the SYBR Green Master Mix (Thermo, USA) on the ABI 7500 Fast system (Life Technologies, USA). The expression level of each gene was calculated using the $2^{-\Delta\Delta Ct}$ method, and was normalized to β-actin expression.

Western blot

Total protein was isolated from cells using radio immunoprecipitation assay buffer (Thermo Fisher Scientific, USA), followed by protein quantification employing the BCA protein concentration kit (Beyotime Biotechnology, China). Afterward, the equal amount of proteins was subjected to sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) for separation. The separated proteins were subsequently transferred onto a nitrocellulose filter membrane. The membrane was blocked with 5% skimmed milk for 1 h at room temperature, and then probed with primary antibodies at 4°C overnight. On the following day, the membranes were washed three times and hybridized with horseradish peroxidase (HRP)-coupled second antibody for 1 h at room temperature. The bands were visualized with an enhanced chemiluminescence system (Amersham), and quantified with ImageJ version 1.52 software (National Institute of Health).

Cell transfection

Plasmids, including overexpression (oe)-PYGO2, short hairpin (sh) RNA targeting EFNA4 (shRNA-EFNA4-1 and shRNA-EFNA4-2), and their respective negative controls (oe-NC and shRNA-NC) were from GenePharma (Shanghai, China). Huh7 cells were seeded in 24-well plates (1×10^5 cells/well) and cultured at 37°C with 5% CO_2. After reaching to 60–70% confluence, cells were transfected with above plasmids using Lipofectamine 3000 (Thermo Fisher Scientific, China) strictly in line with the guidelines of the manufacture.

Cell proliferation assay

Cell proliferation was determined using cell counting kit-8 (CCK-8) assay and colony formation assay, respectively. For CCK-8 assay, Huh7 cells were seeded into 96-well plates (2×10^3 cells/well) for culture. At 24 h, 48 h, and 72 h, respectively, 10 μl of CCK-8 solution was added to each well, and the cells were incubated for another 3 h. Finally, the absorbance at 450 nm of each well was measured using a microplate reader. For colony formation assay, the cells were seeded into 6-well plates (500 cells/well) and incubated for 2 weeks. The culture medium was changed every 3 d. Finally, the cell colonies were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet for 15 min at room temperature. The images were captured under a light microscopy.

Cell migration and invasion assay

Cell migration and invasion abilities were determined using wound-healing and Transwell assays, respectively. For wound-healing assay, cells were seeded into 6-well plates and cultured in an incubator for 24 h. Subsequently, a single scratch wound was created using a 200 μl pipette tip. The cells were washed with PBS and further incubated with serum-free medium. The images were captured at 0 h and 24 h under a light microscopy. For Transwell assay, the 24-well Transwell (Corning) with Matrigel (BD Biosciences) coating was used. The cells resuspended with serum-free medium was added to the upper chamber of the Transwell (3×10^4 cells/well), and the lower chamber was filled with complete medium with 10% FBS. 24 h afterward, the non-invaded cells were wiped out using cotton swabs, and the invaded cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. The images were captured under a light microscopy.

Tube formation assay

About 80 μl Matrigel (BD Biosciences) was 1:1 diluted by DMEM and dispended into 48-well plates and incubated for 1 h at 37°C for solidification. HUVECs (2×10^4) in 200 μl conditioned medium from tumor cells were added to each well and incubated for 20 h. Images were obtained under a light microscopy.

Co-immunoprecipitation (Co-IP)

The total protein from cells was harvested using radio immunoprecipitation assay buffer (Thermo Fisher Scientific), and incubated with IgG and IP-indicated antibodies, and untreated proteins served as an input control. The mixture was incubated with Protein A/G Agarose (Santa Cruz Biotechnology, Inc.) overnight. Afterward, the mixture was centrifuged for 2 min and washed with PBS five times. The binding proteins were assessed by western blot.

Enzyme-linked immunosorbent assay (ELISA)

The cell culture medium was harvested, and the concentration of vascular endothelial growth factor (VEGF) and VEGF receptor 2 (VEGFR2) was determined using their corresponding ELISA detection kits (Beyotime

Biotechnology) in accordance with the manufacturer's instructions. The absorbance at 450 nm of each well was detected using a microplate reader.

Statistical analysis

All data were presented as mean ± SD, and analyzed using GraphPad Prism 8 (GraphPad Software, CA, USA). Statistical significance was tested by Student's t-test between two groups and one-way analysis of variance (ANOVA) with post-hoc Tukey's test among multiple groups. Differences described as $p < .05$ were considered as statistical significance.

Results

EFNA4 was upregulated in HCC and correlated with poor prognosis

To rapid understand the role of EFNA4 in HCC, the GEPIA database (http://gepia.cancer-pku.cn/) was applied. As shown in Figure 1, a and b, the expression level of EFNA4 in tumor samples of HCC patients was markedly upregulated compared to the normal samples, and was correlated with TNM stages in patients. Meanwhile, a relatively high level of EFNA4 was positively correlated with a low overall survival (OS) and disease-free survival (DFS) (Figure 1, c and d), suggesting that the high EFNA4 expression indicated a poor prognosis in patients with HCC. In addition, CCLE database (https://sites.broadinstitute.org/ccle) was also employed to evaluate the expression of EFNA4 in cancer cells. As shown in Figure 1e, EFNA4 was presented a widely high expression in cancer cells, including HCC. To verify this finding, we also detected the expression of EFNA4 in the immortalized human liver epithelial cell THLE-3 and HCC cell lines (Huh7, MHCC97-H, SNU-449 and HCCLM3). As presented in figure 1, f and g, compared to THLE-3 cells, the mRNA level and protein expression of EFNA4 were greatly increased in HCC cell lines, especially Huh7 cells. Thus, Huh7 cells were applied for the further experiments.

Interference of EFNA4 suppressed Huh7 cell proliferation, invasion and angiogenesis

To clarify the specific role of EFNA4 in HCC, loss-of-function assay was conducted in Huh7 cells with the knockdown of EFNA4 by transfection with shRNA-EFNA4-1/2. shRNA-EFNA4-2 was selected for the following experiments resulting from its relatively high transfection efficacy (Figure 2, a and b). Subsequently, a series of cellular biological activities were assessed using CCK-8, colony formation, wound-healing and Transwell assays. The results revealed that interference of EFNA4 hugely inhibited cell viability, reduced cell colonies, and weakened cell migration and invasion abilities (Figure 2, c–f). The downregulated protein expression of MMP2 and MMP9 from western blot assay following EFNA4 knockdown further verified the decreased invasion ability (Figure 2g). In addition, the results from tube formation assay revealed that well-formed and mature capillary tubes were built in the control and shRNA-NC groups, whereas interference of EFNA4 led to a less well-formed capillary tubes (Figure 3a). Meanwhile, interference of EFNA4 also resulted in a remarkable reduction of VEGF and VEGFR2 (Figure 3, b and c), suggesting that EFNA4 interference hindered angiogenesis *in vitro*.

EFNA4 was positively correlated with PYGO2 in HCC

From LinkedOimcs database (http://www.linkedomics.org/login.php), we could find lots of EFNA4-associated genes. VPS7 was the top 1 gene associated with EFNA4, with PYGO2 ranking second (Figure 4, a–c). However, the role of VPS7 in HCC is unclear, while PYGO2 has been recognized as the novel biomarker for HCC prognosis.[13] Thus, we chose PYGO2 for the following research. The subsequent bioinformatic analysis from LinkedOimcs and GEPIA both revealed a positive correlation between EFNA4 and PYGO2 in HCC (Figure 4, d–e). Afterward, Humanbase website (https://hb.flatironinstitute.org/) predicted the targeting interaction relationship between EFNA4 and PYGO2, which was then verified via Co-IP assay (figure 4, f and g). Finally, interference of EFNA4 exhibited an inhibitory effect on PYGO2 at mRNA level and protein expression level in Huh7 cells (Figure 4, h and i). These results suggested that EFNA4 could bind to PYGO2 and positively regulate PYGO2 expression.

PYGO2 is upregulated in HCC and correlated with poor prognosis

Next, the role of PYGO2 in HCC was identified through the GEPIA and CCLE databases. The results revealed that the expression level of PYGO2 was upregulated in tumor samples of HCC patients compared to the normal samples and was correlated with TNM stages in patients (Figure 5, a and b). Meanwhile, high PYGO2 expression was positively correlated with low OS and DFS (Figure 5, c and d). In addition, PYGO2 was also highly expressed in multiple cancer cells (Figure 5e). Furthermore, the aberrant expression of PYGO2 in HCC was validated in *in vitro* experiments as the mRNA level and protein expression of PYGO2 were greatly increased in Huh7 cells, compared to THLE-3 cells (figure 5, f and g).

The inhibitory effects of EFNA4 knockdown on Huh7 cell proliferation, invasion and angiogenesis were partly abolished by PYGO2 overexpression

Next, we made an in-depth research on the regulatory mechanism of EFNA4/PYGO2 in the development of HCC. Firstly, a significantly increased expression of PYGO2 after transfection with oe-PYGO2 indicated a successful transfection (Figure 6, a and b). Subsequently, Huh7 cells were

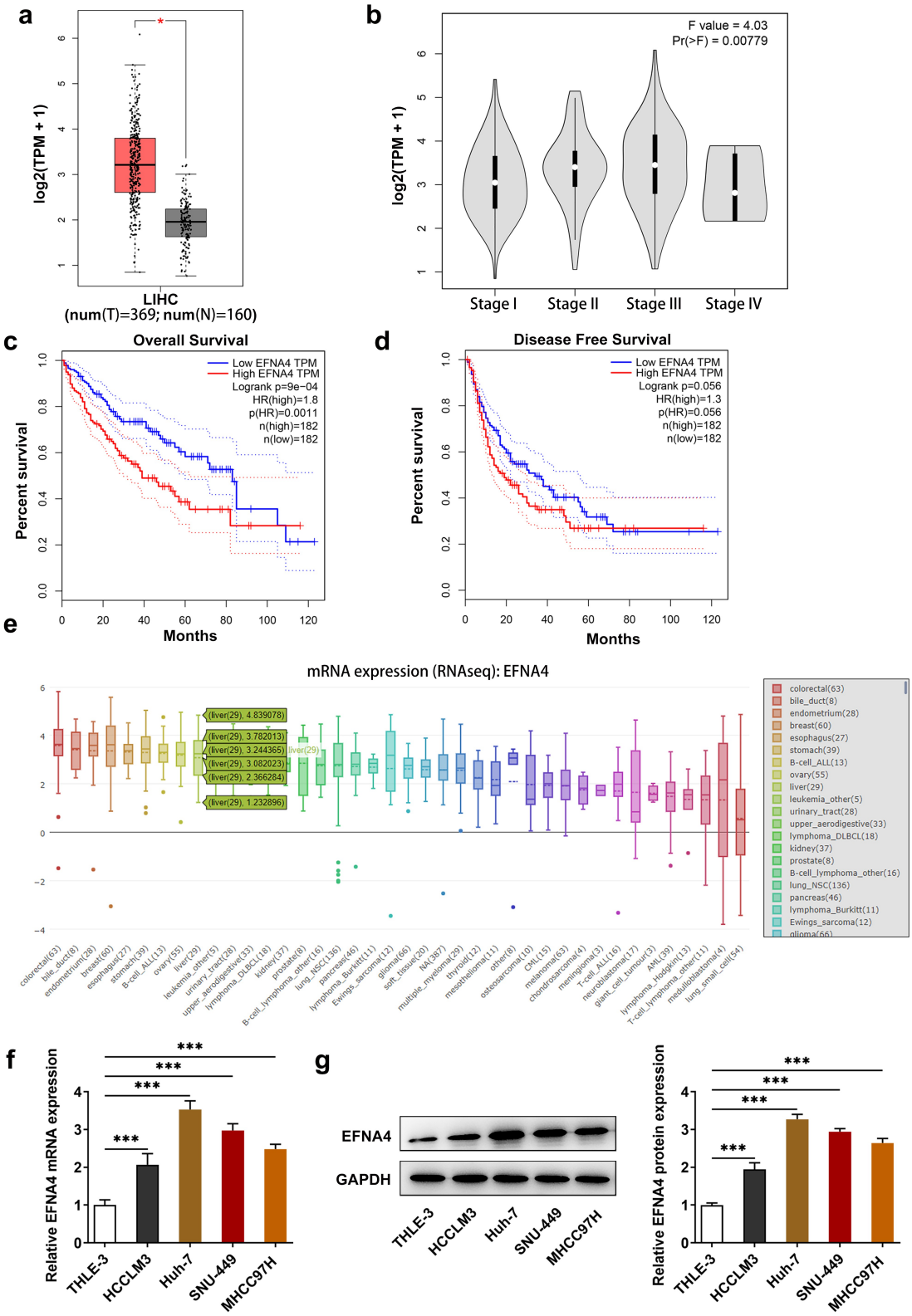

Figure 1. EFNA4 was upregulated in HCC and correlated with poor prognosis GEPIA database were applied to observe the expression level of EFNA4 in tumor samples of HCC patients and normal samples. (B) The correlation between EFNA4 expression and TNM stages in patients. (C) The correlation between EFNA4 expression and overall survival (OS). (D) The correlation between EFNA4 expression and disease free survival (DFS). (e) CCLE database were also employed to evaluate the expression of EFNA4 in different cancer cells. (F) The mRNA level and (G) protein expression of EFNA4 in the immortalized human liver epithelial cell THLE-3 and HCC cell lines (Huh7, MHCC97-H, SNU-449 and HCCLM3). *p < .05, ***p < .001.

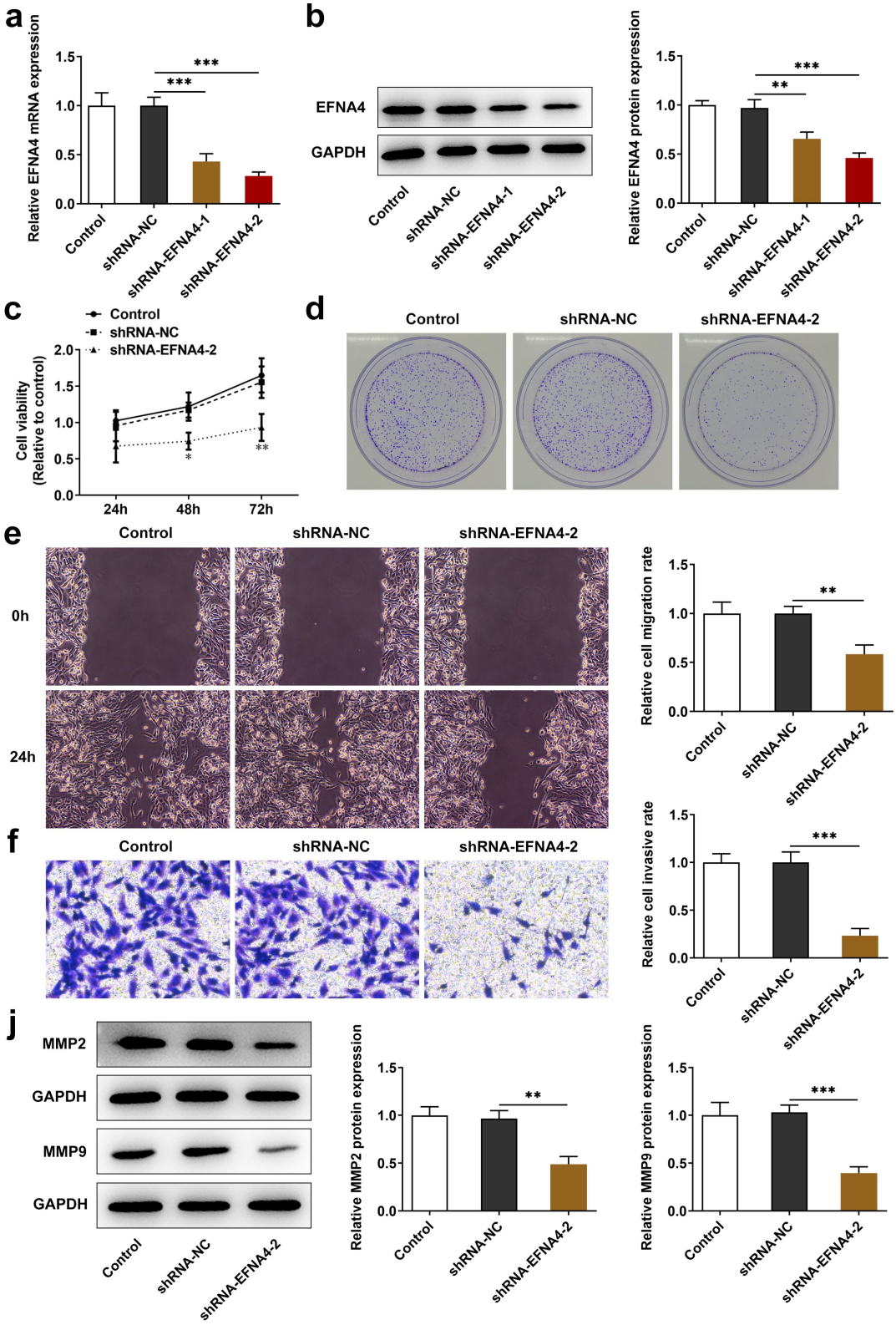

Figure 2. Interference of EFNA4 suppressed Huh7 cell proliferation, migration and invasion Huh7 cells were transfected with shRNA-NC or shRNA-EFNA4-1/2, and (A) the mRNA level and (B) protein expression of EFNA4 were detected using qRT-PCR and western blot, respectively. (C) CCK-8 assay and (D) colony formation assay were conducted to evaluate cell proliferation ability. (E) Wound-healing assay and (F) Transwell assay were performed to assess cell migration ability and invasion ability, respectively. (G) Western blot was performed to detect protein expression of MMP2 and MMP9. **p < .01, ***p < .001.

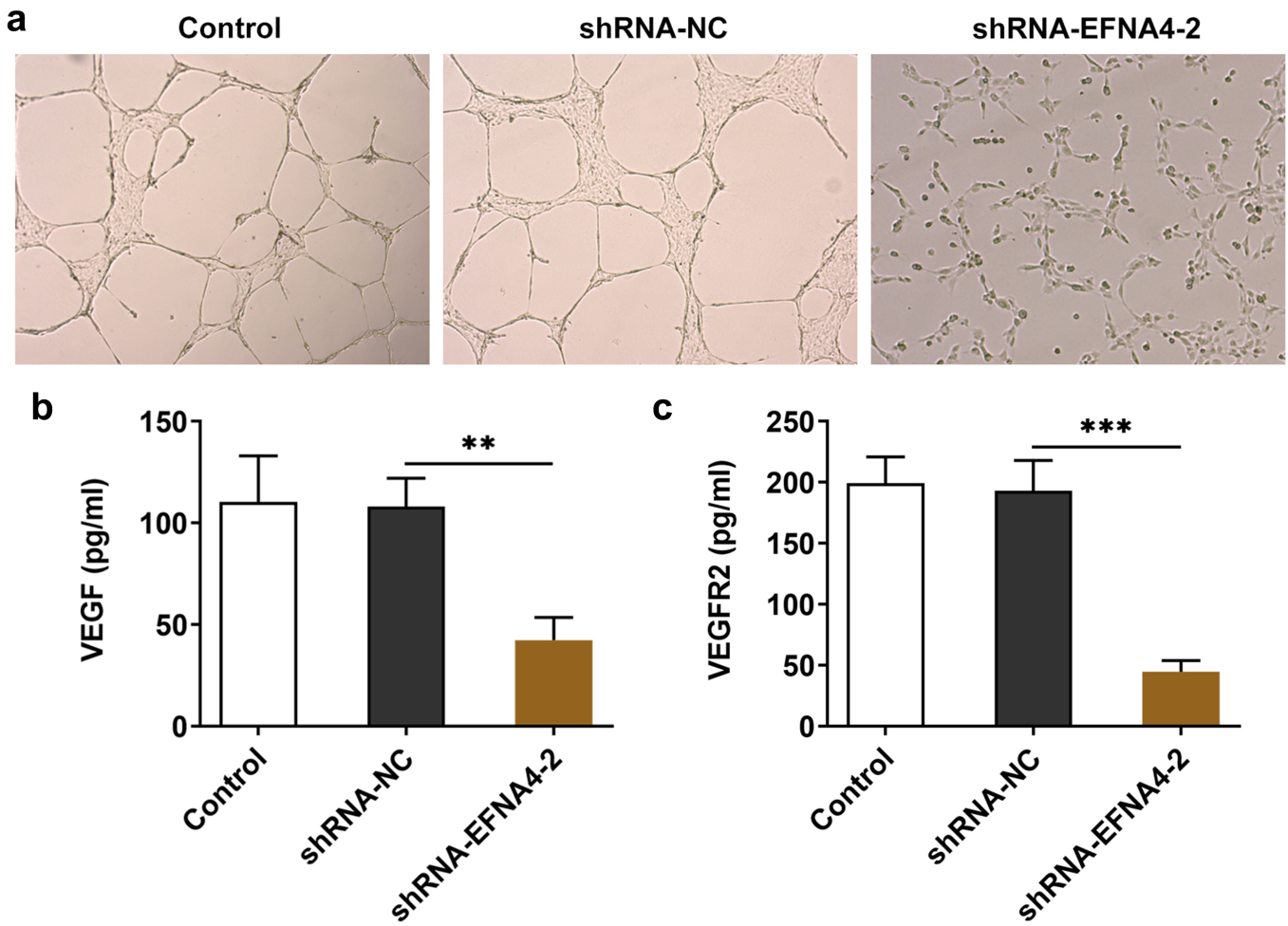

Figure 3. Interference of EFNA4 suppressed angiogenesis in Huh7 cells Huh7 cells were transfected with shRNA-NC or shRNA-EFNA4-2, and the tube formation was observed under a light microscopy. The concentration of (B) VEGF and (C) VEGFR2 in the cell supernatant was detected using ELISA assay. **p < .01, ***p < .001.

transfected with shRNA-EFNA4-2 or co-transfected with shRNA-EFNA4-2 and oe-NC/oe-PYGO2. The analysis from following cellular biological activities in each group through CCK-8, colony formation, wound-healing, Transwell and tube formation assays, as well as western blot and ELISA assay, showed that the inhibitory effects of EFNA4 knockdown on cell viability, cell colonies, migration and invasion, and angiogenesis, accompanied with protein expression of MMP2/9 and concentration of VEGF/VEGFR2, were partly weakened upon simultaneously over-expressing PYGO2 (Figure 6, c and g and Figure 7, a–c). The above results suggested that EFNA4 knockdown might inhibit cell proliferation, invasion and angiogenesis via down-regulating PYGO2.

EFNA4/PYGO2 regulated Wnt/β-catenin signaling in Huh7 cells

To further explore the potential regulatory mechanism, Wnt/β-catenin signaling was introduced. Cyclin D1 and c-Myc are the downstream target genes of Wnt/β-catenin.[14] The data revealed that the protein expression of β-catenin, cyclin D1, and c-myc was down-regulated in Huh7 cells transfected with shRNA-EFNA4-2, which was partly reversed by simultaneous transfection with shRNA-EFNA4-2 and oe-PYGO2 (Figure 8), suggesting that EFNA4 knockdown suppressed Wnt/β-catenin signaling partly by down-regulating PYGO2.

Discussion

HCC is the most common type of liver cancer, accounting for 70%–90%.[15] In the past decades, great improvements on the diagnostic techniques and therapeutic strategies have been achieved; however, due to its unsatisfactory 5-y survival rate, there is an urgent requirement for the identification of novel biomarkers to accurately monitor HCC development and progression. In previous studies, EFNA4 has been shown to act as an oncogene in multiple malignant tumors, and participated into the regulation of tumor cell growth and metastasis.[4,12] However, the current research of EFNA4 in HCC is limited.

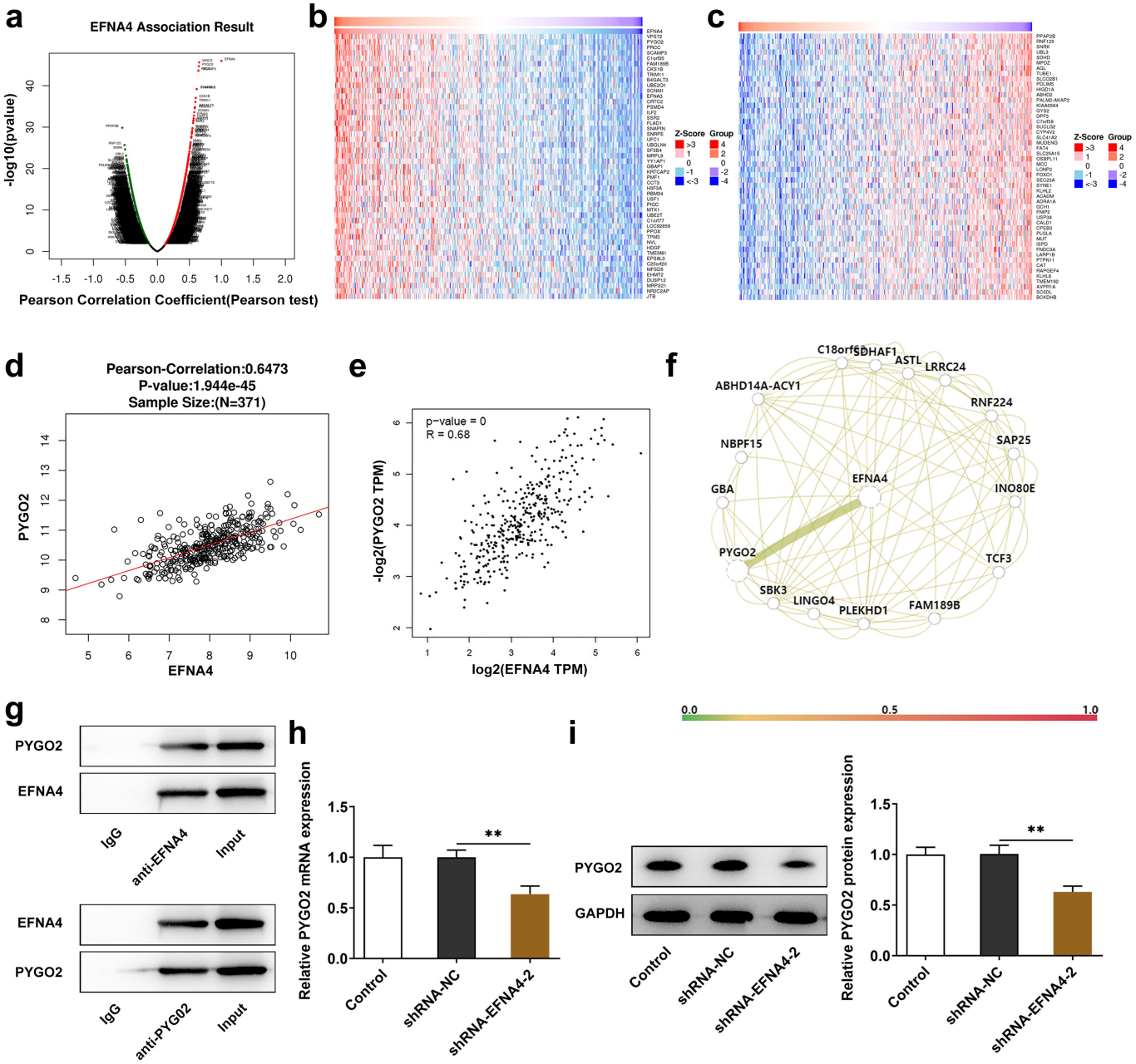

Figure 4. EFNA4 was positively correlated with PYGO2 in HCC (A–C) From LinkedOimcs database, the EFNA4-associated genes were observed. The correlation between EFNA4 and PYGO2 in HCC was analyzed through (D) LinkedOimcs and (E) GEPIA database. (F) Humanbase website predicted the interaction relationship between EFNA4 and PYGO2. (G) Co-IP verified the interaction between EFNA4 and PYGO2. Huh7 cells were transfected with shRNA-NC or shRNA-EFNA4, and the (H) mRNA level and (i) protein expression of PYGO2 was detected, respectively. **p < .01.

In the present study, we made a further research on the specific regulatory role of EFNA4 in HCC and clarified the potential regulatory mechanism underlying its oncogenic activity in HCC. Here, we provided evidence that EFNA4 knockdown not only inhibited cell proliferation, migration and invasion abilities in Huh7 cells, but also hindered angiogenesis. Mechanistically, EFNA4 might exert its oncogenic function by regulating PYGO2-mediated Wnt/β-catenin signaling.

PYGO2 is a critical element of the Wnt/β-catenin transcriptional complex, and can modulate the activity of Wnt/β-catenin signaling through linking PYGO2 PHD domain to β-catenin and activating T-cell factor (TCF)/lymphoid-enhancing factor 1 (LEF) transcription.[16,17] PYGO2 is usually maintained at a low level in normal cells but exhibits an aberrantly high expression level in cancer cells. Abnormal expression of PYGO2 indicated a malignant phenotype in

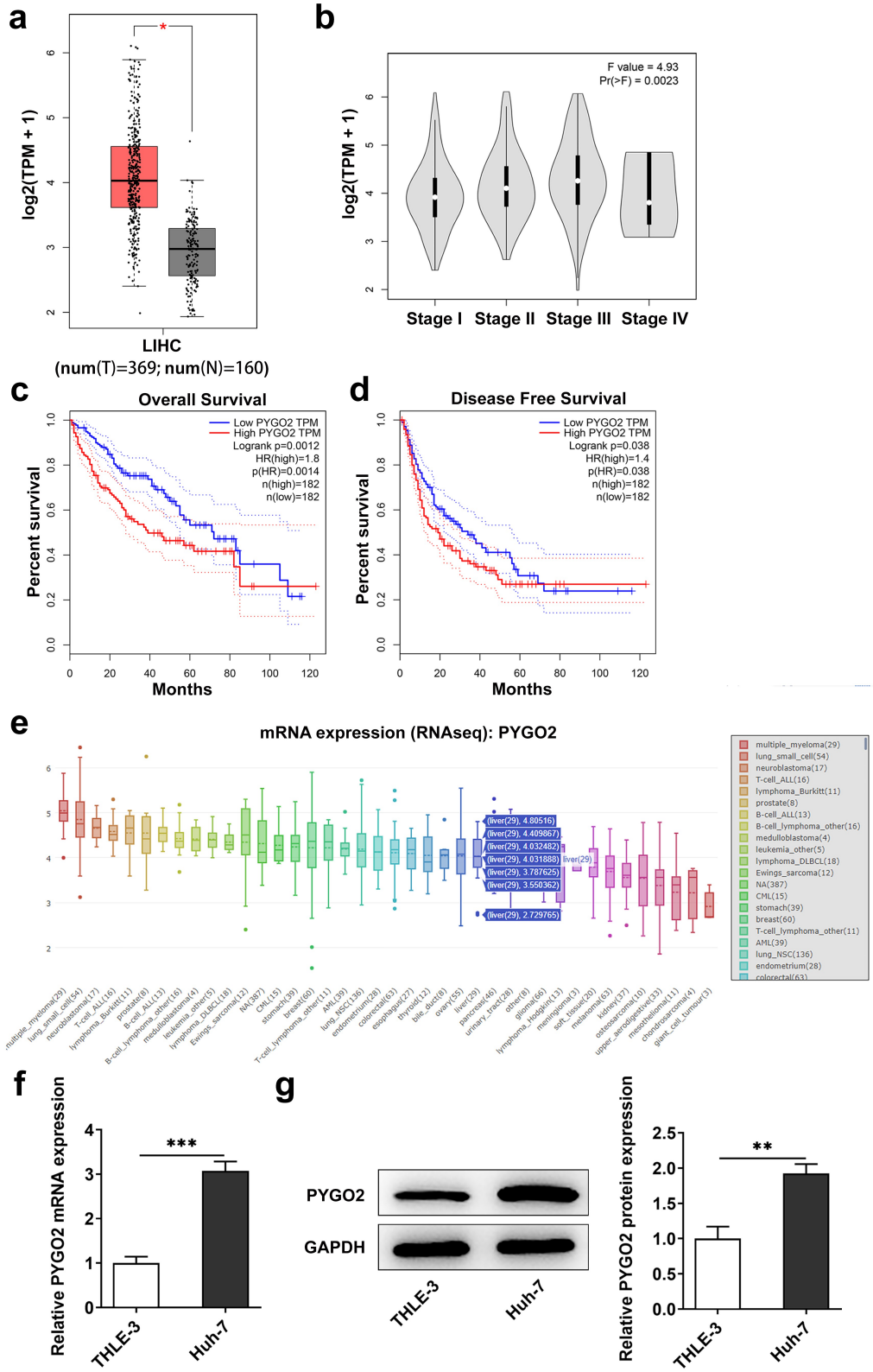

Figure 5. PYGO2 is upregulated in HCC and correlated with poor prognosis (A) GEPIA database were applied to observe the expression level of PYGO2 in tumor samples of HCC patients and normal samples. (B) The correlation between PYGO2 expression and TNM stages in patients. (C) The correlation between PYGO2 expression and overall survival (OS). (D) The correlation between PYGO2 expression and disease free survival (DFS). (E) CCLE database were also employed to evaluate the expression of PYGO2 in different cancer cells. (F) The mRNA level and (G) protein expression of PYGO2 in the immortalized human liver epithelial cell THLE-3 and HCC cell Huh7. *p < .05, **p < .01, ***p < .001.

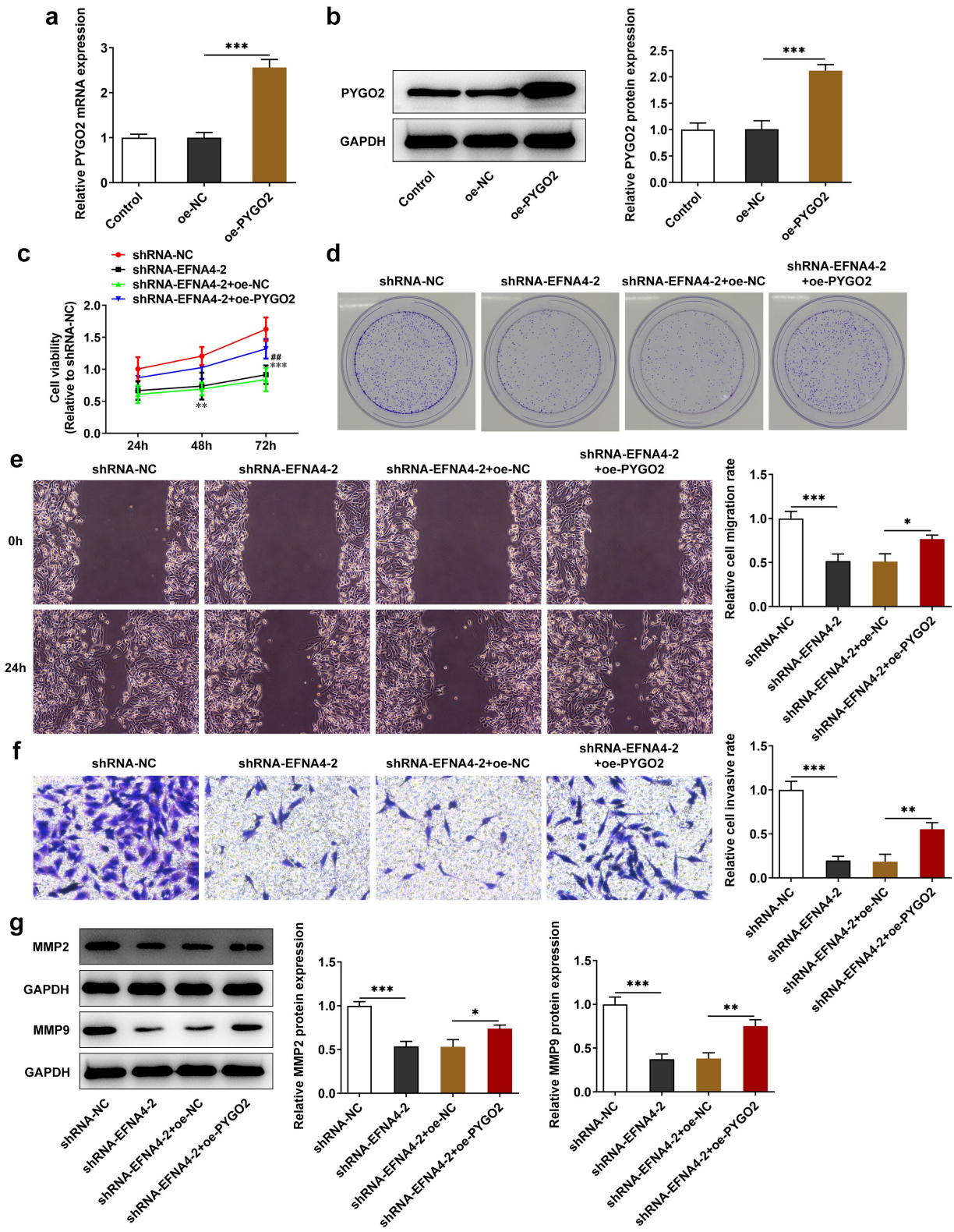

Figure 6. The inhibitory effects of EFNA4 knockdown on Huh7 cell proliferation and invasion were partly abolished by PYGO2 overexpression Huh7 cells were transfected with oe-NC or oe-PYGO2, and the (A) mRNA level and (B) protein expression of PYGO2 were measured. Huh7 cells were transfected with shRNA-EFNA4-2 or co-transfected with shRNA-EFNA4-2 and oe-NC/oe-PYGO2. (C) CCK-8 assay and (D) colony formation assay were conducted to evaluate cell proliferation ability. ***p < .001 vs shRNA-NC; ##p < .01 vs shRNA-EFNA4-2+ oe-NC. (E) Wound-healing assay and (F) Transwell assay were performed to assess cell migration ability and invasion ability, respectively. (G) Western blot was performed to detect protein expression of MMP2 and MMP9. *p < .05, **p < .01, ***p < .001.

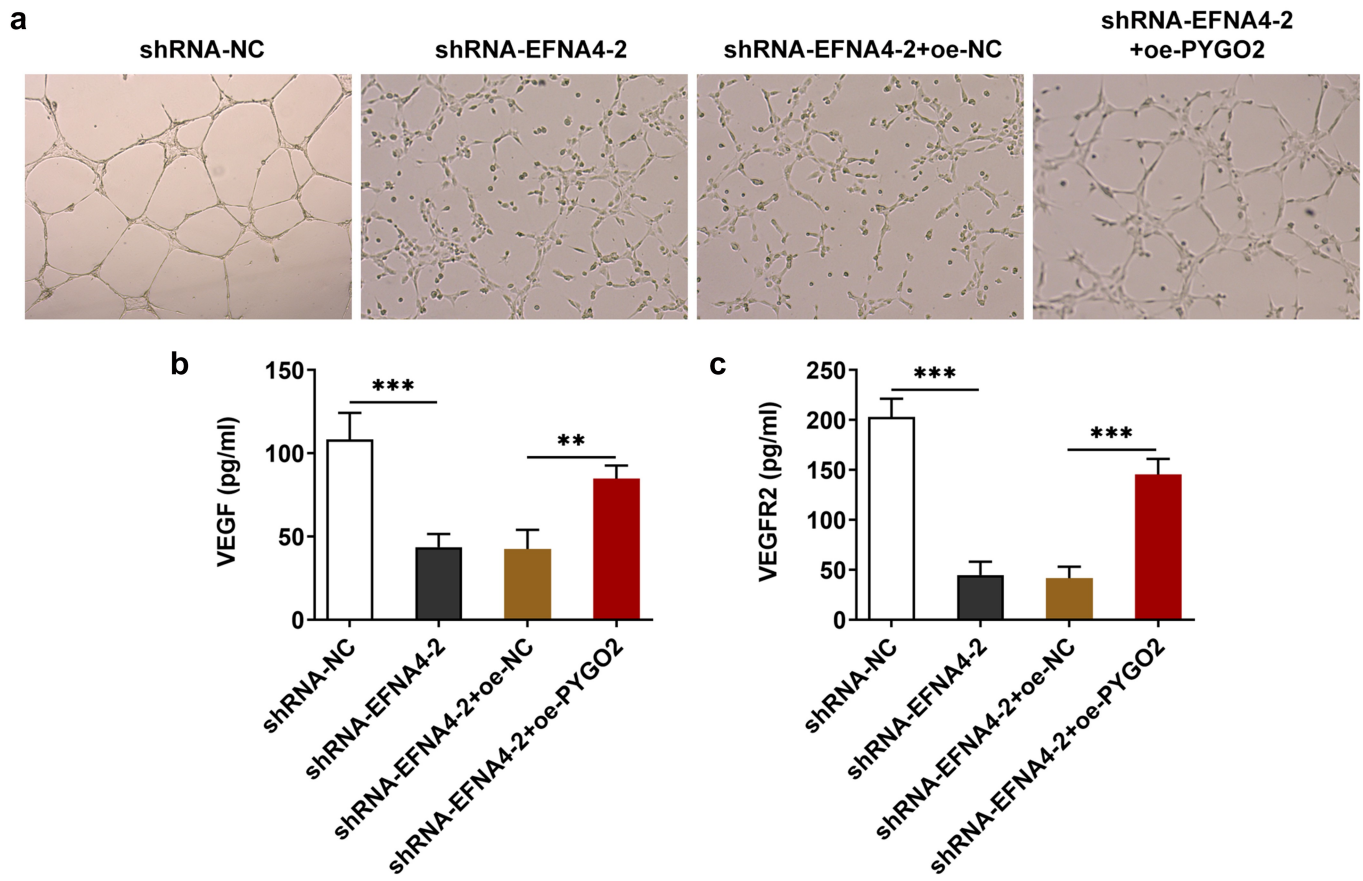

Figure 7. The inhibitory effect of EFNA4 knockdown on angiogenesis in Huh7 cells was partly abolished by PYGO2 overexpression Huh7 cells were transfected with shRNA-EFNA4-2 or co-transfected with shRNA-EFNA4-2 and oe-NC/oe-PYGO2, and the tube formation was observed under a light microscopy. (B) The concentration of VEGF and VEGFR2 in the cell supernatant was detected using ELISA assay. **p < .01, ***p < .001.

advanced lung cancer.[18] PYGO2 was overexpressed in gastric cancer, and acted for monitoring drug resistance by upregulating MDR1.[19] Particularly, PYGO2 was also reported to be overexpressed in HCC, and the high level of PYGO2 was correlated with tumor size, vascular invasion and tumor differentiation of patients with HCC. Thus, PYGO2 was considered as an important predictor of poor outcome in HCC patients and a novel biomarker in HCC. Meanwhile, downregulation of PYGO2 could inhibit HCC cell invasion *in vitro* and metastasis in xenograft tumor models, highlighting that targeting PYGO2 potentially inhibited metastasis of HCC.[13,20,21] In the present study, a potential interaction between EFNA4 and PYGO2 was predicted by Humanbase (https://hb.flatironinstitute.org/), which was further verified using Co-IP assay. In addition, EFNA4 could positively regulate PYGO2 expression, and the results from following rescued experiments showed that simultaneous EFNA4 knockdown and PYGO2 overexpression recovered the phenotypes defect resulted from EFNA4 knockdown, suggesting that EFNA4 might exert its function in Huh7 cells partly by targeting PYGO2. Furthermore, given that PYGO2 is an element of Wnt/β-catenin transcriptional complex, and Wnt/β-catenin pathway is frequently upregulated and has emerged as an alternative target in HCC,[22] PYGO2-mediated Wnt/β-catenin may deeply explain the critical role of EFNA4 during the regulation of HCC development.

However, this study has several limitations. Firstly, we did not investigate the upstream factors, such as transcriptional factors, which influenced EFNA4 expression. Secondly, we only explored the effect of EFNA4/PYGO2 on Wnt/β-catenin signaling, but the critical role of this signaling upon the oncogenic function of EFNA4 was still required to be further investigated. Last but not least, the validation of the present findings in animal models and clinical patients might be helpful to improve the quality.

Conclusion

In conclusion, the present study suggests that EFNA4 may be a reliable oncogene in HCC, and EFNA4 knockdown can repress HCC proliferation, migration, invasion, and angiogenesis, which is partly resulted from PYGO2-

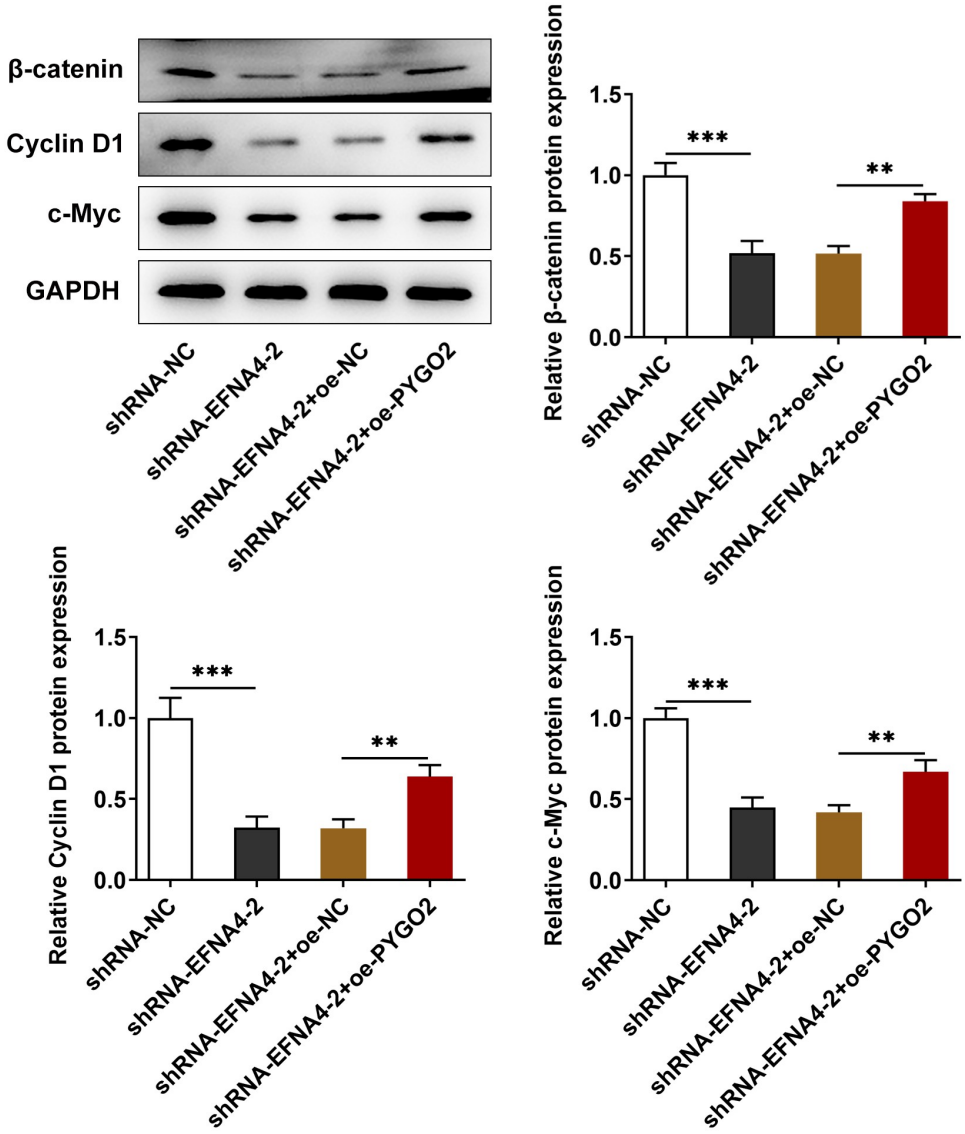

Figure 8. EFNA4/PYGO2 regulated Wnt/β-catenin signaling in Huh7 cells Huh7 cells were transfected with shRNA-EFNA4-2 or co-transfected with shRNA-EFNA4-2 and oe-NC/oe-PYGO2, and the protein expression of β-catenin, cyclin D1, and c-myc was measured by western blot. **p < .01, ***p < .001.

mediated Wnt/β-catenin signaling pathway. Our findings provide a novel insight into the pathological mechanisms of HCC and enrich the potential therapeutic strategies for HCC treatment.

Disclosure statement

The authors declare that they have no competing interests.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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