

The FAK signaling pathway regulates the migration ability of highly metastatic hepatocellular carcinoma cells

Bingyu Zhang

zhangbingyu@cqupt.edu.cn

Chongqing University of Posts and Telecommunications

Jin Li

Chongqing University of Posts and Telecommunications

Yue Zhang

Chongqing University of Posts and Telecommunications

Jiayu Wang

Chongqing University of Posts and Telecommunications

Dui Qin

Chongqing University of Posts and Telecommunications

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Abstract

Hepatocellular carcinoma (HCC) is characterized by high incidence and mortality rates, with its high metastatic potential being the core cause of poor prognosis. This study integrated bioinformatics analysis and cellular experiments to investigate the migration characteristics and molecular mechanisms of HCC cell lines (HCCLM3, Huh7, and Hep3B) with distinct metastatic potentials. Transwell and wound healing assays demonstrated that HCCLM3 cells exhibited significantly stronger migration ability than Huh7 and Hep3B cells. Subsequently, two datasets GSE236039 and GSE203329 were retrieved from the Gene Expression Omnibus (GEO) database and integrated to screen for differentially expressed genes (DEGs) between high- and low-metastatic cell lines. Through Protein-Protein Interaction (PPI) network analysis and clinical prognostic validation from The Cancer Genome Atlas (TCGA) database, 13 key genes closely associated with HCC metastasis were identified. Quantitative real-time PCR (qRT-PCR) validated that 9 genes (PIK3C2B, ITGB5, PXN, ITGB4, SIRPA, CD47, NCK1, CTNNA1, and CDH26) exhibited expression trends consistent with bioinformatics analysis. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis revealed that ITGB4, ITGB5, NCK1, and PXN were significantly enriched in the focal adhesion kinase (FAK) signaling pathway. Western blot further confirmed that the p-FAK/t-FAK ratio was significantly higher in highly metastatic HCCLM3 cells than in poorly metastatic Huh7 cells. Treatment with the FAK inhibitor 1,2,4,5-Benzenetetraamine tetrahydrochloride (Y15) significantly suppressed HCCLM3 migration and downregulated PXN and NCK1 expression. In conclusion, FAK signaling molecules play a critical role in promoting HCC cell metastasis, and FAK along with its upstream and downstream regulatory network molecules may serve as potential targets for predicting metastatic potential and therapeutic intervention.

Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent malignant neoplasms worldwide, with its incidence and mortality remaining persistently elevated for decades, representing a major global public health threat to human populations^[1]. According to the latest data released by the World Health Organization (WHO), in 2022, the number of new liver cancer cases in China ranked fourth among all malignant tumors, while the number of related deaths ranked second, resulting in an extremely heavy disease burden^[2, 3]. HCC is characterized by high malignancy and insidious early symptoms. Most patients have already progressed to the intermediate or advanced stage at diagnosis, missing the optimal window for surgical intervention^[4]. Furthermore, HCC is characterized by extremely high invasiveness and metastatic potential, and its high metastatic propensity is the leading cause of treatment failure and poor prognosis in affected patients^[5]. Therefore, in-depth investigation of the molecular mechanisms underlying HCC metastasis is critical clinical for developing novel effective anti-metastasis therapeutic strategies.

HCC metastasis is a multistep, complex biological process that requires the participation of numerous genes. This metastatic cascade is primarily composed of local invasion, intravasation into blood and

lymphatic vessels, survival within the circulation, extravasation from blood vessels, and subsequent colonization and proliferation in distant organs^[6, 7]. Throughout this process, tumor cells acquire a series of phenotypic and functional alterations, including epithelial-mesenchymal transition (EMT), extracellular matrix (ECM) degradation, and cytoskeleton rearrangement. Previous studies have demonstrated that the activation of EMT-associated transcription factors (e.g., Snail, Slug, and Twist)^[8], the downregulated expression of cell adhesion molecules (e.g., E-cadherin)^[9], and the aberrant activation of multiple signaling pathways (e.g., PI3K/AKT and RAS/MAPK) collectively drive the metastasis progression of HCC^[10, 11]. However, the molecular network underlying HCC metastasis is extraordinarily complex. Studies focusing on a single gene or single pathway cannot fully elucidate the complete landscape of this process, which creates an urgent demand for systematic in-depth analysis at the systems level.

In recent years, the rapid development of bioinformatics has provided powerful tools for investigating the molecular mechanisms underlying tumorigenesis. Through the integration and analysis of datasets retrieved from public databases including the Gene Expression Omnibus (GEO) and The Cancer Genome Atlas (TCGA), genes and key signaling molecules closely correlated with tumorigenesis and cancer progression can be screened efficiently^[12–14]. This big data-based analytical approach enables not only the identification of novel biomarkers, but also provides guidance for subsequent experimental validation and targeted therapy. Nevertheless, the reliability of bioinformatics predictions depends on high-quality input data and rigorous statistical methods. Accordingly, cross-validation via integration with in vitro experiments has become the standard paradigm in this research field, which facilitates the translation of fundamental research discoveries into clinical applications.

In this study, HCC cell lines with different metastatic potentials were employed: the highly metastatic HCCLM3 (LM3) cells, the moderately-to-lowly metastatic Huh7 cells, and the lowly metastatic Hep3B cells. We first identified intergroup differences in the migration capacity of three cell types through cell experiments. Subsequently, bioinformatics approaches were utilized to screen for key genes and signaling pathways that modulate the migration ability of highly metastatic HCC cells, and key signaling molecules governing this phenotype were further validated.

Materials and methods

Cell culture

The human HCC cell lines LM3, Huh7, and Hep3B were purchased from Wuhan Procell Life Science & Technology Co., Ltd. and cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Carlsbad, CA, USA) supplemented with 1% penicillin-streptomycin (Beyotime, Shanghai, China) and 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA). All cultures were maintained at 37°C with 5% CO₂ in a humidified incubator. When cells reach approximately 90% confluence, adherent cells are digested with trypsin (Beyotime, Shanghai, China), after which cell passaging or collection are performed.

Transwell assay

When cells reach approximately 90% confluence, replace the culture medium with serum-free medium and perform starvation treatment for 12 h. After starvation, digest and harvest the cells, then prepare a serum-free cell suspension with a cell density of 5×10^5 cells/mL. Transwell inserts with an 8 μm pore size (Corning,) were placed into 24-well plates. 100 μL of cell suspension was added into each insert, and 600 μL of complete medium was added into the 24-well plate for induction. Subsequently, the multi-well plate containing the Transwell inserts was incubated in a cell culture incubator for 8 h to complete the induction process. Following induction, remove the Transwell chambers, and gently wipe off residual liquid and non-migrated cells from the upper compartment of the chambers using a cotton swab. Fix cells with 4% paraformaldehyde solution for 10 min, then stain with crystal violet solution for 30 min. Finally, acquire images using an inverted microscope (Olympus, Tokyo, Japan), with four random fields of view collected per sample.

Scratch wound healing assay

The cells were seeded into 24-well plates at a density of 1×10^5 cells per well, and were further cultured until they reached 80% confluence, followed by overnight serum starvation. After starvation, a cross-shaped scratch was generated with a 10 μL pipette tip, floating cells in the scratched area were gently washed away with PBS, and the initial images of the cross-scratched area were captured using an inverted microscope (Olympus, Tokyo, Japan). Add 1 mL of complete medium for cell culture, and capture images at 12 h intervals to monitor scratch wound healing until the scratch is completely closed. Finally, perform image analysis via ImageJ software; the calculation formula for the scratch wound healing rate is presented as Eq. (1):

$$\text{Scratch wound healing rate} = \frac{A_0 - A_t}{A_0} \times 100\%$$

1

Let A_0 denote the initial scratch area, and A_t denote the scratch area after a specific healing duration.

CCK-8 assay

To clarify the effect of cell proliferation on cell migration, we evaluated the proliferative capacity of three types of HCC cells using the CCK8 assay (Beyotime, Shanghai, China). Briefly, the cells seeded into 96-well plates at a density of 5×10^3 cells per well. After addition of complete medium, the cells were cultured for 0 h, 8 h, 24 h, and 48 h, respectively. Upon completion of incubation at each time point, 10 μL of CCK-8 reagent was added to each well, and cells were incubated for an additional 2 h. After this incubation step, the absorbance of each sample was measured at a 450 nm wavelength using a microplate reader (Synergy H1, BioTek, Winooski, VT, USA).

Bioinformatics analysis

Two microarray datasets associated with HCC, GSE236039 and GSE203329, were retrieved from the GEO database. These two datasets contain gene expression profile data for highly metastatic LM3 cells and lowly metastatic Huh7 cells, respectively, and only the unprocessed datasets of LM3 and Huh7 cells were retained for downstream analysis. Differentially expressed genes (DEGs) between the two sample groups were screened via GEO2R, with filtering thresholds established as $|\log_2FC| > 1$ and $\text{adj.P.value} < 0.05$. We further performed Gene Ontology (GO) functional enrichment analysis on the DEGs via the DAVID online database. The enrichment significance threshold was set at $P < 0.05$ to screen for significantly enriched GO terms. To further narrow the analysis scope to cell migration, we retained migration-related DEGs annotated under the Biological Process (BP) ontology, and classified them into four groups for subsequent analysis: DEGs unique to GSE236039, DEGs unique to GSE203329, the intersection of DEGs from GSE236039 and GSE203329, and the union of DEGs from GSE236039 and GSE203329.

To further investigate the interaction networks among the proteins encoded by the identified DEGs, migration-associated DEGs were uploaded to the Search Tool for the Retrieval of Interacting Proteins (STRING) database for analysis. Using the STRING database, we constructed a Protein-Protein Interaction (PPI) network based on the proteins encoded by the identified DEGs, and subsequently constructed visual analysis via Cytoscape software. The top 30 DEGs with high connectivity to adjacent genes ($P > 0.7$) were screened as hub genes. Navigate to the Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis module integrated in the DAVID bioinformatics platform. DAVID annotates the input DEGs against the KEGG pathway database. Via KEGG enrichment analysis, we can identify the signaling pathways that are significantly enriched with DEGs, and further elucidate the potential molecular regulatory mechanisms and signal transduction pathways underlying HCC metastasis.

Using the Kaplan–Meier model implemented in the GEPIA platform, this study analyzes the correlation between target gene expression and survival prognosis of HCC patients by combining follow-up data of liver cancer patients from TCGA. After logging into the GEPIA platform, users can input the target gene name, select the HCC dataset and configure the relevant parameters, after which the platform will automatically generate the survival curve and the P-value of the log-rank test. By integrating data from TCGA and the Genotype-Tissue Expression (GTEx) project, and uploading the screened key genes to the GEPIA platform, this tool enables rapid retrieval and normalization of the expression profiles of these genes in HCC tissues and paired adjacent normal tissues, with results visualized via box plots.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cultured cells using an RNA extraction kit (BioTeke, Beijing, China). Following quantification and purity evaluation, 1 μg of the total RNA was reverse-transcribed into cDNA via a reverse transcription kit (MedChemExpress, Shanghai, China). The PCR reaction system was

prepared, and amplification was carried out via a PCR thermocycler (CFX96, Bio-Rad, USA). The primer sequences applied in this experiment are summarized in Table 1. GAPDH was used as the internal reference, and the relative expression level of the target gene was calculated via the $2^{(-\Delta\Delta Ct)}$ method.

Table 1
Primer sequences detected by qRT-PCR

Name	Primer	Primer sequence	Product length
GAPDH	Sense	5'-AAGTTCAACGGCACAGTCAAGG-3'	110bp
	Anti-sense	5'-CGCCAGTAGACTCCACGACATA-3'	
CD2AP	Sense	5'-AGGACGATTGAGAACTGTTTTG-3'	79bp
	Anti-sense	5'-CCAGATGCAGTTTCACTCACA-3'	
CD47	Sense	5'-AGAAGGTGAAACGATCATCGAGC-3'	160bp
	Anti-sense	5'-CTCATCCATACCACCGGATCT-3'	
CDH26	Sense	5'-ACATGGTCAGATGTTGAAGGC-3'	85bp
	Anti-sense	5'-CGAGATCCTTAACTCCCTGCG-3'	
CTNNA1	Sense	5'-GGGGATAAAATTGCGAAGGAGA-3'	144bp
	Anti-sense	5'-GTTGCCTCGCTTCACAGAAGA-3'	
FSCN2	Sense	5'-CCAACCACCGCTACGTCTC-3'	223bp
	Anti-sense	5'-CCACGCCACTCCATCTCAA-3'	
ITGB4	Sense	5'-AGGATGACGACGAGAAGCAGCT-3'	79bp
	Anti-sense	5'-ACCGAGAACTCAGGCTGCTCAA-3'	
ITGB5	Sense	5'-CAGGAGGCTGTGCTATGTTTC-3'	145bp
	Anti-sense	5'-AGGAGGATGCTACCGACCA-3'	
ITGB7	Sense	5'-AGAATGGCGGAATCCTCACCT-3'	113bp
	Anti-sense	5'-TGAAGTTCAGTTGCTTGCACC-3'	
NCK1	Sense	5'-AGAGTGGTGGAAATGCAGGA-3'	193bp
	Anti-sense	5'-TTGATGCCTGGTGACTTTGC-3'	
PIK3C2B	Sense	5'-AGCCCAGTGACATCAACACTT-3'	186bp
	Anti-sense	5'-CCGCATCATAGGTCGATTTCAA-3'	
PXN	Sense	5'-CTGCTGGAAGTGAACGCTGTA-3'	123bp
	Anti-sense	5'-GGGGCTGTTAGTCTCTGGGA-3'	
RHOF	Sense	5'-CCCCATCGGTGTTGAGAAAG-3'	80bp
	Anti-sense	5'-GGCCGTGTCGTAGAGGTTC-3'	
SIRPA	Sense	5'-GCCCTCTACCTCGTCCGAAT-3'	193bp

Name	Primer	Primer sequence	Product length
	Anti-sense	5'-CATACTCCGTGTGGTTGTTGG-3'	

Western blot

Cells were seeded into 6-well plates at a density of 5×10^5 cells per well. When the cell confluence reached 80% confluence, the culture medium was replaced with serum-free medium, and cells were subjected to 12 h of serum starvation. After 8 h of continuous serum-free culture, the culture medium was removed, and 500 μ L of protein lysis buffer (Beyotime, Shanghai, China) was added. Following sufficient cell lysis, samples were harvested, and protein quantification was performed (Beyotime, Shanghai, China). Sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) consisting of an 8% separating gel and a 5% stacking gel were prepared for electrophoretic separation. After protein sample loading, electrophoresis was first carried out at a constant voltage of 60 V, maintained until bromophenol blue migrated completely into the separating gel. The voltage was then adjusted to 110 V, and electrophoresis was continued until bromophenol blue reached the base of the gel. Protein transfer was performed at a constant current of 200 mA. Before use, the PVDF membrane requires activation via methanol. Upon completion of membrane transfer, the PVDF membrane was blocked with 5% skimmed milk blocking buffer at room temperature for 1 h. After three washes with TBST, the membrane was incubated overnight at 4°C in primary antibody solutions against FAK (Abcam, Cambridge UK), p-FAK (Abcam, Cambridge UK) or GAPDH (ZSGB-Bio, Beijing, China). Following incubation with the primary antibody, the membrane was washed three times with TBST, with each wash lasting 10 min. Subsequently, the secondary antibody solution (MultiSciences, Hangzhou, China) was added, followed by 1 h of incubation at room temperature. After completion of the secondary antibody incubation, the PVDF membrane was washed three times with TBST for 10 min each. Finally, chemiluminescent detection was conducted with an enhanced chemiluminescence (ECL) chromogenic substrate (Baoguang Biology, Chongqing, China). Resulting immunoreactive bands were imaged using a gel imaging system (Clinx, Shanghai, China), and the band gray values were quantified via ImageJ software.

FAK inhibition assay

Cells were seeded into 6-well plates at a density of 5×10^5 cells per well. When cell confluence reached 80%, the culture medium was replaced with serum-free medium to induce 12 h of starvation. After starvation, cells were treated for 8 h with serum-free medium containing different concentrations of Y15 (0, 1, 5, 10, and 50 μ M), respectively. Protein samples were harvested, and the expression levels of p-FAK and t-FAK were detected via Western blot to determine the optimal inhibitory concentration. For subsequent transwell assay, wound healing assay and qRT-PCR assay, cells were pre-treated with the FAK inhibitor Y15 for 30 min, and the inhibitor was maintained in the culture system throughout the entire experimental procedure.

Statistical analysis

All the data and results presented in this study were verified via at least three independent replicates. Data are expressed as the mean $\pm$ standard deviation. Intergroup statistical differences were assessed using Student's t-test with Bonferroni correction for multiple comparisons, and differences with $P < 0.05$ were considered statistically significant. All statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA).

Results

Identification of migration abilities of three HCC cell lines

To clarify the differences in migration capacity among the three HCC cell lines, we performed scratch wound healing assay and Transwell assay. As shown in Figs. 1A–C, under the culture condition containing 10% FBS, the scratch in the LM3 cells was almost completely closed within 48 h. In contrast, Huh7 and Hep3B cells exhibited relatively reduced wound healing capacity, with the scratched wound area failing to reach complete closure. Moreover, no significant difference in wound healing capacity was observed between Huh7 and Hep3B cells. Under serum-free culture conditions, scratch wound healing of the three HCC cell lines showed a consistent trend. The results of the Transwell assay are shown in Figs. 2A and 2B. When induced by 10% FBS, the number of migrated LM3 cells were significantly higher than that of Huh7 and Hep3B cells.

To evaluate the effect of cell proliferation on cell migration capacity, we detected the proliferation ability of the three HCC cell lines via the CCK-8 assay. The results showed that within a short incubation period matching the migration induction duration (8 h), the proliferation fold ratios of LM3, Huh7 and Hep3B cells were 3.01, 2.62 and 1.34, respectively; after an extended incubation period corresponding to the final wound healing time (48 h), the proliferation fold ratios of LM3, Huh7 and Hep3B cells reached 12.85, 5.83 and 3.87, respectively (Fig. 2C). These results indicated that no significant difference in proliferation capacity was observed between LM3 and Huh7 cells after a short incubation period, and the proliferation capacity of both cell lines was significantly higher than that of Hep3B cells. However, as incubation time extended, a significant difference in proliferative capacity emerged between LM3 and Huh7 cells: the proliferation capacity of LM3 cells was significantly higher than that of Huh7 cells, which in turn was significantly higher than that of Hep3B cells. In the Transwell assay, the relative migration capacity ratio of the three cell lines (LM3:Huh7:Hep3B) was 10.30:2.84:1.20. Comparative analysis of proliferation and migration data indicated that although LM3 cells exhibit a moderate proliferative advantage over Huh7 and Hep3B cells, their advantage in migration capacity is substantially more pronounced. While Huh7 cells have enhanced migration capacity compared with Hep3B cells, their proliferation capacity is concurrently increased as well. This finding indicates that the greater number of migrated Huh7 cells compared with Hep3B cells is most likely attributable to the larger total cell counts of Huh7 cells. The scratch wound healing assay can reflect this finding more clearly. The LM3 cells possessed stronger

wound healing capacity than both the Huh7 and Hep3B cells, whereas no significant difference in wound healing capacity was observed between Huh7 and Hep3B cells.

Mechanistic investigation via bioinformatics approaches

DEGs were screened from the GSE236039 and GSE203329 datasets. Specifically, 6384 DEGs were identified from the GSE236039 dataset, and 8263 DEGs were identified from the GSE203329 dataset (Fig. 3A). GO functional enrichment analysis was conducted independently on the two sets of DEGs. Since this study primarily focuses on the biological behaviors of cell migration, only the enrichment results of BP are presented. As shown in Fig. 3B, a large proportion of DEGs from both datasets are enriched in cell migration. DEGs associated with cell migration, identified from two datasets, were categorized into four sets for PPI network construction: GSE236039 dataset-derived DEGs, GSE203329 dataset-derived DEGs, the intersection of the two DEG lists, and the union of the two DEG lists. The top 30 genes ranked by significance score across each of the four scenarios were identified as hub genes (Fig. 3C).

To further validate the biological significance and clinical value of the identified hub genes, we conducted survival prognostic analysis using transcriptomic clinical data from TCGA database. The results revealed that the expression levels of a subset of these hub genes are significantly correlated with the survival prognosis of patients with HCC. Among these, individual analysis of GSE236039 identified 9 genes: PLCG1, NCK1, CD47, ITGB5, CDH26, CD2AP, ITGB7, LAMB1, and ARHGAP35 (Supplementary Fig. 1). Individual analysis of GSE203329 contained 6 genes: PXN, ITGB4, CTNNA1, CDH26, BCAR1, and NCK1 (Supplementary Fig. 2). Intersection analysis of GSE236039 and GSE203329 yielded 10 genes: CDH26, TYRO3, RHOF, SIRPA, CD24, FSCN2, CD47, MST1R, NCK1, and PIK3C2B (Supplementary Fig. 3). Finally, union analysis of GSE236039 and GSE203329 identified only 2 genes: NCK1 and PLCG1 (Supplementary Fig. 4).

Subsequent tissue-level expression analysis was performed on all survival-associated hub genes. The results showed that only CTNNA1 and ITGB4 exhibit differential expression between HCC tissues and adjacent normal tissues (Fig. 4). Given the diversity and heterogeneity of tissue-level analytical results, we retained genes with consistent expression trends identified via GEO database analysis for downstream analysis. The retained genes are CD2AP, ITGB4, PXN, CD47, ITGB5, RHOF, CDH26, ITGB7, SIRPA, CTNNA1, NCK1, FSCN2, and PIK3C2B.

In vitro validation of hub genes and confirmation of the FAK signaling pathway

To verify the reliability of the bioinformatics analysis results, we detected the mRNA expression levels of the 13 aforementioned hub genes in LM3 and Huh7 cells via qRT-PCR. The results are shown in Fig. 5A. The expression trends of PIK3C2B, ITGB5, PXN, ITGB4, SIRPA, CD47, NCK1, CTNNA1, and CDH26 in LM3 cells were consistent with the results obtained from database analysis, while the expression of RHOF, FSCN2, ITGB7, and CD2AP exhibited no significant difference or an opposite trend. To further identify the

core signaling pathways associated with these key genes exhibiting consistent expression trends, we performed KEGG enrichment analysis, and found that most of these genes, namely ITGB4, ITGB5, NCK1, and PXN, are associated with FAK signaling molecules (Fig. 5B). Western Blot assays results showed that in LM3 cells, although the expression level of p-FAK was comparable to that in Huh7 cells, the expression level of t-FAK was significantly lower in LM3 cells than in Huh7 cells. As a result, the p-FAK/t-FAK ratio was significantly higher in LM3 cells than in Huh7 cells (Fig. 5C), indicating that FAK is sustained in a highly phosphorylated activated state in LM3 cells.

Inhibition of FAK signaling mediates transcriptional regulation of LM3 cell migration and its downstream key molecules

To verify the role of the FAK signaling in the migration of LM3 cells, we performed intervention using the FAK-specific inhibitor Y15. First, we detected changes in FAK protein expression following treatment with different concentrations of Y15 via Western blot. Upon treatment with 10 μ M Y15, p-FAK protein expression was significantly down-regulated (Fig. 6A), indicating that Y15 at this concentration exerts a robust inhibitory effect on FAK signaling. The effect of FAK signaling inhibition on the migration ability of LM3 cells was further evaluated via wound healing assay and Transwell assay. The results demonstrated that Y15 significantly inhibits the migration of LM3 cells (Fig. 6B-E). To verify the association between the FAK signaling and the ITGB4, ITGB5, NCK1, and PXN genes, qRT-PCR was performed on Y15-treated cells. The results showed that Y15 treatment did not exert a significant effect on the mRNA expression levels of ITGB4 and ITGB5, whereas the mRNA expression of PXN and NCK1 was significantly inhibited (Fig. 6F). This observation was consistent with the findings from bioinformatics analysis (Fig. 5B): specifically, as upstream signaling molecules of FAK, ITGB4 and ITGB5 were not affected by FAK inhibition, while PXN and NCK1, as downstream signaling molecules of FAK, were significantly inhibited following treatment with FAK inhibitors.

Discussion

This study elucidates the central role of FAK signaling in regulating the migration of HCC cells with high metastatic potential by integrating bioinformatics prediction and in vitro experiments. The results revealed that LM3 cells exhibit significantly stronger migratory capacity than Huh7 and Hep3B cells. Through comprehensive bioinformatics screening, we screened 13 genes that are closely associated with the high metastatic capacity of LM3 cells. Subsequently, qRT-PCR verified that the expression profiles of PIK3C2B, ITGB5, PXN, ITGB4, SIRPA, CD47, NCK1, CTNNA1, and CDH26 in the LM3 cells are consistent with the analytical results obtained from the GEO database. Through KEGG pathway enrichment analysis, we found that ITGB4, ITGB5, NCK1, and PXN genes are significantly enriched in FAK-related pathways. Western blot analysis revealed that the p-FAK/t-FAK ration was significantly higher in LM3 cells compared with Huh7 cells. Pharmacological inhibition of FAK activity significantly attenuated the migration and wound healing capacity of LM3 cells. Furthermore, the expression of the downstream effector molecules PXN and NCK1 was positively regulated by the FAK signaling. These

findings provide insights into the molecular mechanism underlying HCC metastasis, and underscore the clinical value of the FAK signaling network as a potential therapeutic target for this malignancy.

Based on the integrated results of Transwell assay, wound healing assay and CCK-8 assay, this study confirmed that LM3 cells exhibit stronger migration capacity compared with Huh7 and Hep3B cells. However, no statistically significant difference in migratory capacity was observed between Huh7 and Hep3B cells. This finding contradicts the widely accepted consensus that Huh7 cells exhibit higher migratory ability than Hep3B cells^[15–17]. This inconsistency may arise from inherent biological differences among the three cell lines. LM3 cell line is a monoclonal cell line derived from the highly metastatic HCC cell line MHCC97-H. It exhibits prominent EMT characteristics and upregulated expression of mesenchymal markers, which endows it with enhanced cytoskeletal remodeling and migratory ability^[18, 19]. Although Huh7 cells have been reported to exhibit moderate metastatic potential in numerous studies, their differentiation status and metastatic phenotype vary to a certain degree under distinct laboratory conditions. In particular, their migration capacity is relatively sensitive to changes in cell density and serum concentration^[20, 21]. Hep3B cells retain a relatively typical epithelial phenotype, exhibiting high expression of E-cadherin and low expression levels of mesenchymal markers, and their low migration characteristics remain stable under most experimental conditions^[22]. Overall, a greater number of variables need to be taken into consideration when evaluating the migration capability of tumor cells.

Using bioinformatics approaches, we performed in-depth data mining on two gene expression datasets, GSE236039 and GSE203329, and identified key signaling molecules associated with migration regulation in highly metastatic LM3 cells. GO functional enrichment analysis revealed that the DEGs are primarily enriched in biological processes including cell migration, cell adhesion and angiogenesis, which is highly consistent with the high metastatic biological characteristics of LM3 cells. To further focus on the biological process of cell migration, we retained genes associated with this process, constructed a PPI network using the STRING database, and applied the Degree algorithm implemented in Cytoscape software to screen the top 30 hub genes ranked by interaction score. These hub genes are presumed to act as core nodes of the regulatory network and play key roles in the migration process of HCC cells. Notably, for the integrated analysis of multiple datasets, as researchers with limited programming expertise, we employed online tools to conduct separate analyses on two datasets generated via the same high-throughput RNA sequencing technology, and subsequently obtained processed data by extracting intersections and unions of the two datasets. Our analysis reveals that, when controlling for identical cell sources and identical sequencing protocols, the analytical results of datasets generated by distinct research groups exhibit significant discrepancies. Furthermore, both intersection-based and union-based approaches present inherent limitations when applied to the integrative analysis of multiple datasets. Specifically, intersection-based method tends to focus on core genes that are ubiquitously present in all datasets. Although this strategy simplifies the identification of widely recognized key genes, it frequently excludes biologically meaningful information unique to individual context-specific datasets. Conversely, the union approach is designed to include as many relevant genes as possible to

achieve comprehensiveness. However, due to its high breadth of inclusion, this method frequently incorporates a substantial number of irrelevant genes, which increases data noise and impairs the accuracy of downstream analysis. Given that both cell types investigated in this study are established cell lines, and the two datasets were collected via consistent experimental protocols, we finally performed survival prognostic analysis on the top 30 hub genes associated with migration behavior that were obtained across four experimental scenarios, which further narrowed the detection scope.

Based on the results of survival prognosis analysis, tissue-level gene expression analysis was conducted. Since only CTNNA1 and ITGB4 exhibited significant differences at the tissue level, we selected hub genes whose tissue-level expression trends were consistent with the results of GEO database analysis for qRT-PCR validation. The results revealed that the expression trends of most genes (PIK3C2B, ITGB5, PXN, ITGB4, SIRPA, CD47, NCK1, CTNNA1, and CDH26) were consistent with the outcomes of our preliminary data analysis, while the expression trends of four genes (RHOF, FSCN2, ITGB7, and CD2AP) did not align with the results of bioinformatics analysis. This inconsistency may be attributed to the following reasons: first, the quantitative accuracy of RNA-seq data is influenced by sequencing depth, read length, reference genome annotation version, and alignment algorithm. Meanwhile, differing sequencing platforms, library construction strategies, and normalization processes employed across independent datasets can also introduce batch effects, leading to systematic deviations in quantified gene expression levels. Second, as a targeted quantitative method, qRT-PCR depends on specific primer design and consistent amplification efficiency. Inherent differences exist between qRT-PCR and RNA-seq in detection sensitivity and the resolution capability for low-abundance transcripts or alternative splicing isoforms, which may lead to discrepancies in the expression trends of partial genes between the two technologies. Finally, the sequencing data deposited in public databases are derived from cell culture samples originating from multiple laboratories and processed across different batches. Subtle variations in in vitro culture conditions, including serum concentration, cell density, and culture passage number, can alter cellular transcriptomic profiles, leading to incomplete consistency between sequencing data and the qRT - PCR results obtained under defined experimental conditions. Collectively, bioinformatics approaches can still yield critical insights for our mechanistic investigations.

KEGG pathway enrichment analysis revealed that most of the identified genes, including ITGB4, ITGB5, NCK1, and PXN, were significantly enriched in the FAK signaling pathway. Western blot assays further showed that the p-FAK/t-FAK ratio was significantly higher in LM3 cells than in Huh7 cells, indicating that FAK signaling is constitutively hyperactivated in LM3 cells. The transition from t-FAK to p-FAK is a critical step in FAK activation^[23, 24]. The enzymatic activity of FAK is activated by inducing a conformational change of the protein, which subsequently recruits downstream effector molecules and initiates signaling cascades mediated by the PI3K/Akt, Ras/MAPK, and Rho family GTPases (e.g., Rac1, Cdc42, and RhoA)^[25–27]. These signaling pathways modulate cytoskeleton remodeling, cell morphological changes and migration directionality, thereby promoting the enhancement of metastatic capacity in HCC. Furthermore, the FAK-related signaling pathway is closely implicated in the remodeling of the tumor

microenvironment^[28]. Specifically, in HCC cells with high metastatic potential, where FAK activation can further promote distant metastasis by enhancing the EMT process.

To confirm the role of FAK signaling in the migration of LM3 cells, we performed intervention using the FAK-specific inhibitor Y15. Results from both the wound healing assay and Transwell assay verified that inhibition of FAK signaling suppressed the migration capacity of LM3 cells. Furthermore, qRT-PCR assays demonstrated that Y15 treatment did not significantly affect the expression of ITGB4 and ITGB5, but significantly inhibited the expression of PXN and NCK1, indicating that the expression of PXN and NCK1 is positively regulated by the FAK signaling pathway. Existing studies have confirmed that, as critical effector molecules downstream of FAK, both PXN and NCK1 participate cytoskeletal remodeling and signal transduction. PXN is a core protein in focal adhesions, and reduced functional activity of PXN can impair the dynamic reorganization capacity of the cytoskeletal, which in turn attenuates the cell migration ability^[29, 30]. As an adaptor protein, NCK1 bridges the FAK signaling to multiple downstream signaling cascades, including the Ras/MAPK pathway^[31]. Some studies have demonstrated that downregulation of NCK1 can subsequently inhibit the transduction of signals associated with cell migration^[32]. As members of the integrin family, ITGB4 and ITGB5 are primarily involved in cell-matrix adhesion^[33, 34]. In the KEGG enrichment analysis, both ITGB4 and ITGB5 are located upstream of the FAK signaling pathway, and therefore their transcription levels are not affected by FAK inhibitors.

Conclusion

By combining bioinformatics analysis with experimental validation, this study elucidates the core regulatory role of FAK signaling molecules in HCC metastasis, and demonstrates that the activation level of FAK signaling is positively correlated with the migration ability of LM3 cells. These findings not only deepen the current understanding of the molecular mechanism underlying HCC metastasis, but also offer methodological references and a theoretical basis for HCC metastasis risk assessment and the development of therapeutic strategies targeting the FAK signaling pathway.

Declarations

Consent for publication

Not applicable.

Conflict of interest

The authors declare that they have no conflict of interest.

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Author Contribution

BZ designed the experiment and prepared the manuscript; JL performed the experiment and prepared the manuscript; YZ performed the experiment; XW performed the experiment; JW analyzed the data; DQ helped to performing the research. All authors read and approved the final manuscript.

Data availability

The data that are available from the corresponding author upon reasonable request.

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Figures

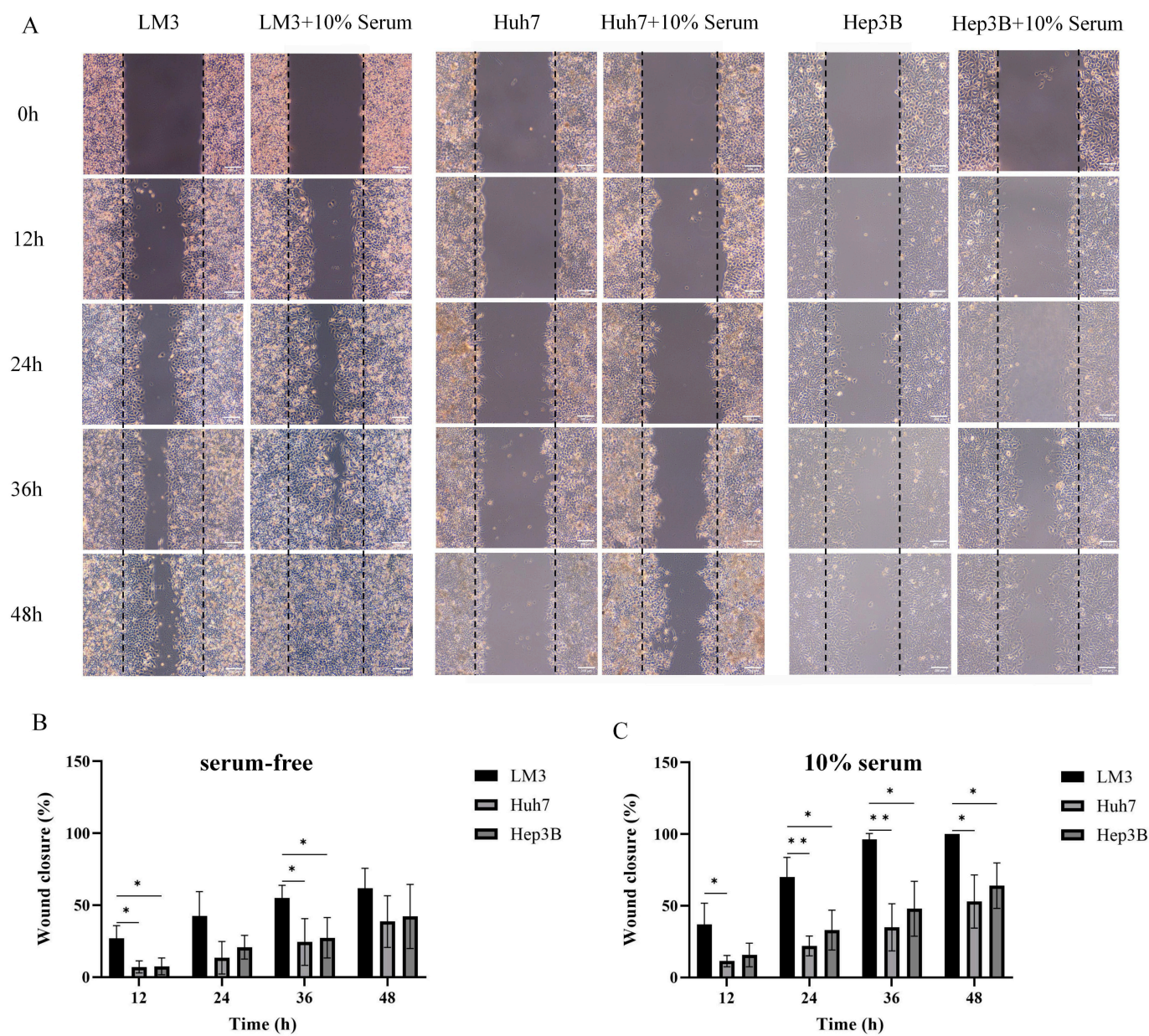


Figure 1

Evaluation of healing capacity of three HCC cell lines. **A** Comparison of healing ability among the three HCC cell lines, scale bar = 200 μ m; **B** Statistical comparison of healing ability under serum-free conditions; **C** Statistical comparison of serum-induced healing ability; $n = 3$, means $\pm$ SD, * $p < 0.05$, ** $p < 0.01$.

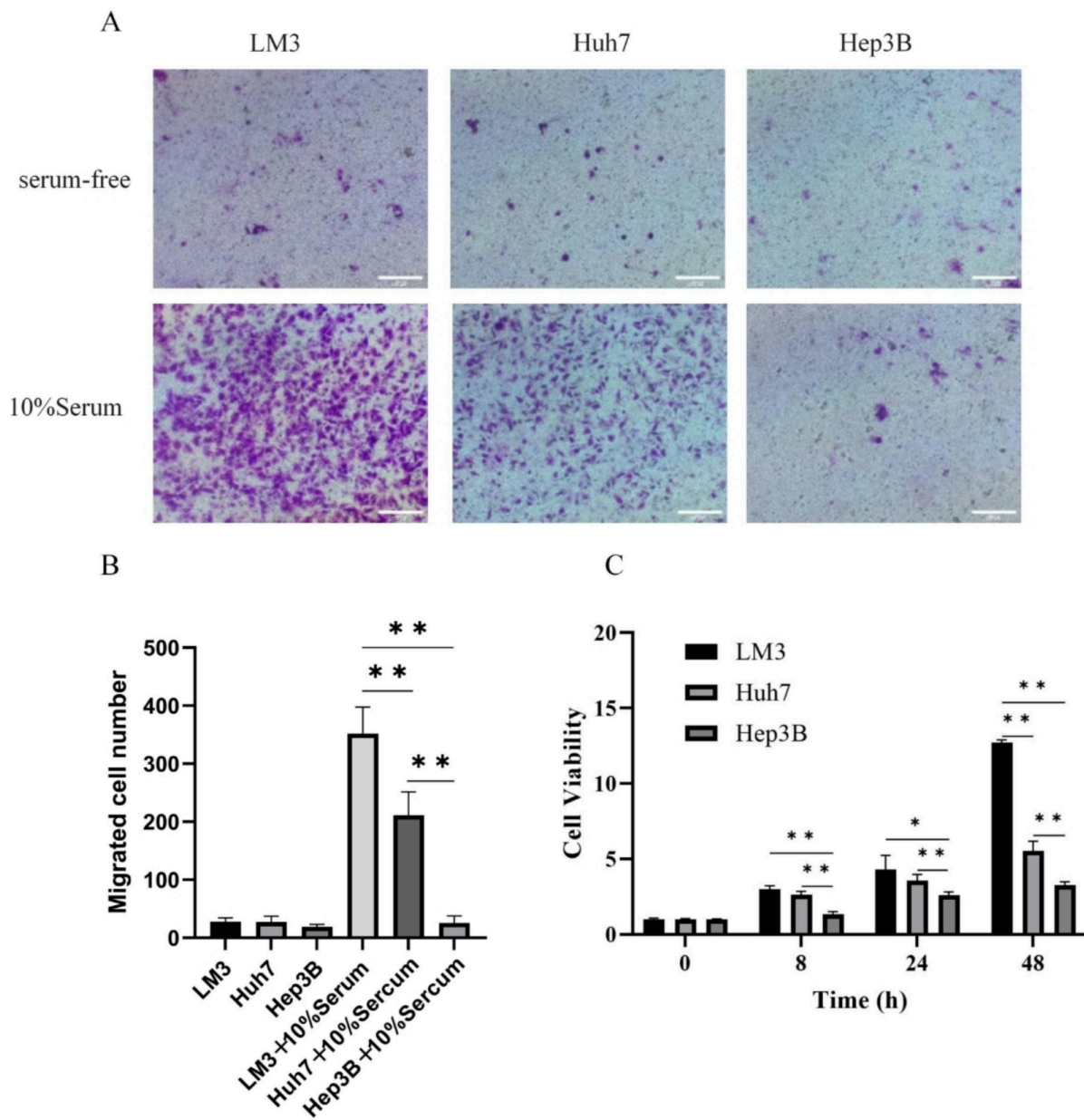


Figure 2

Assessment of migration and proliferation capacity of three HCC cell lines. **A** Comparison of migration ability among the three HCC cell lines, scale bar = 200 μ m; **B** Statistical analysis of migration ability; **C** Cell viability assay of the three HCC cell lines; $n = 3$, means $\pm$ SD, * $p < 0.05$, ** $p < 0.01$.

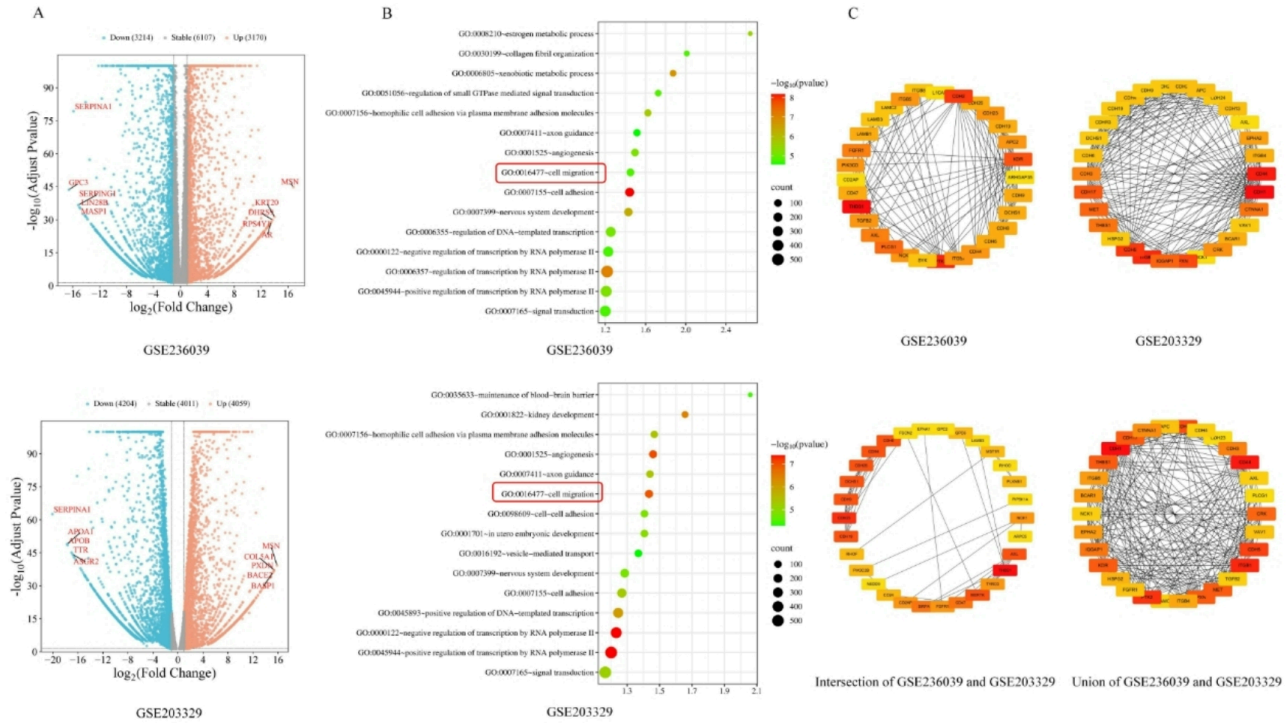


Figure 3

Analysis of the GSE236039 and GSE203329 datasets. **A** Results of DEGs analysis; **B** Results of GO enrichment analysis; **C** Construction of PPI networks of migration-related DEGs under four experimental conditions (GSE236039, GSE203329, the intersection of GSE236039 and GSE203329, and the union of GSE236039 and GSE203329), where the top 30 genes were identified as hub genes.

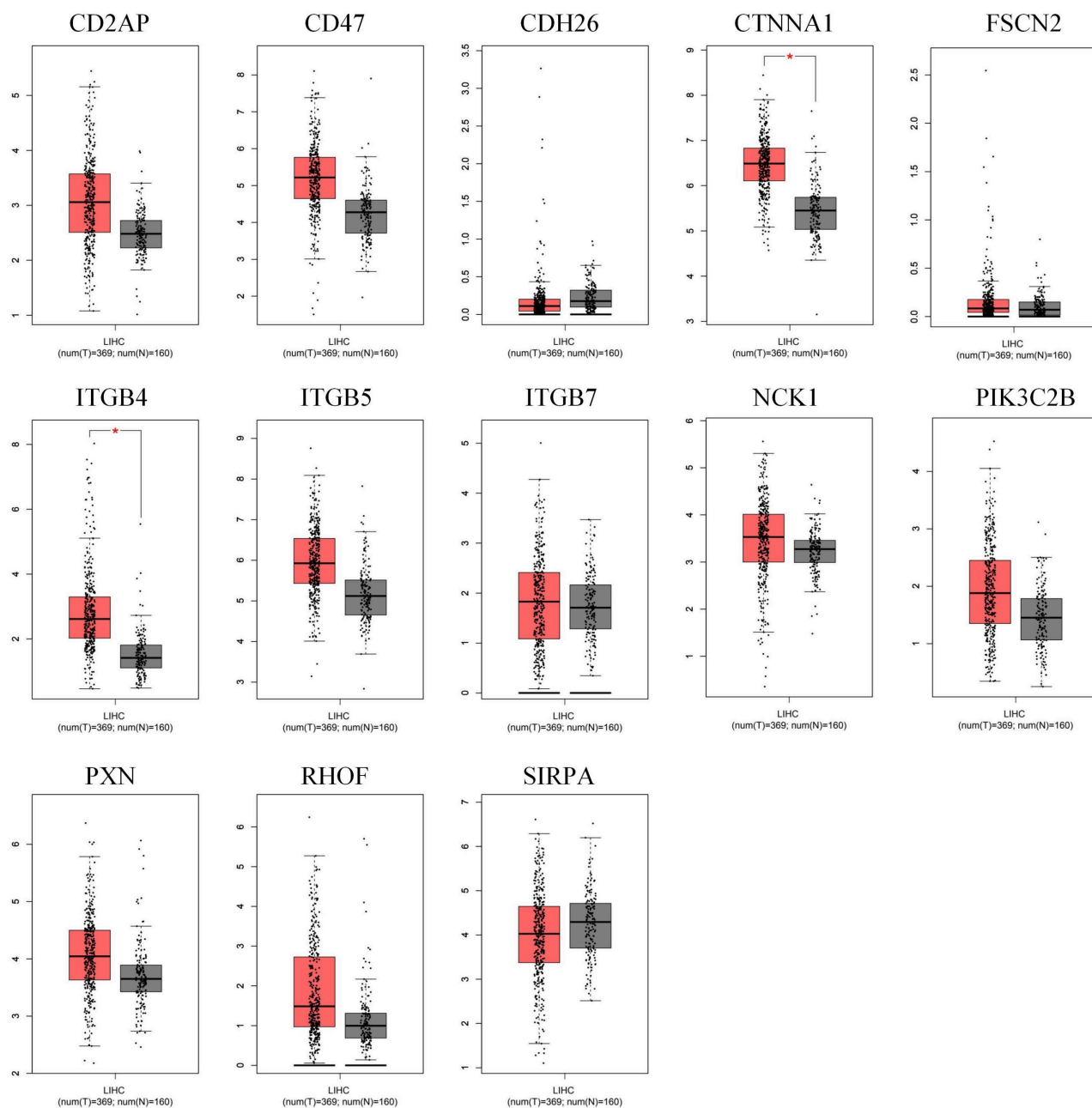


Figure 4

Expression analysis of relevant hub genes in HCC and normal tissues ($p < 0.05$)

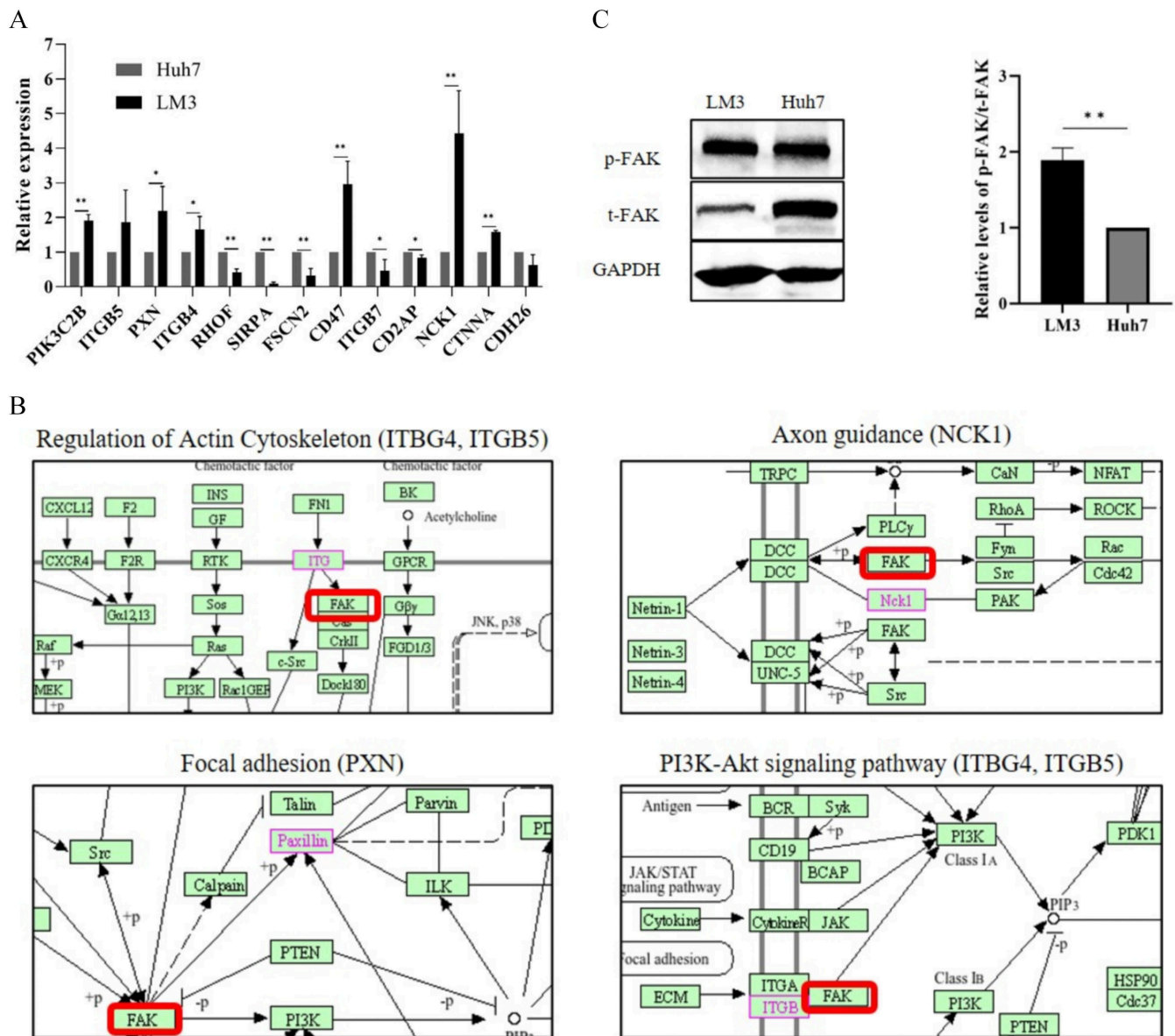


Figure 5

Validation of the expression of key genes and their associated signaling pathways. **A** Validation of mRNA expression of hub genes by qRT-PCR; **B** KEGG enrichment analysis; **C** FAK protein expression in LM3 and Huh7 cells; $n=3$, means $\pm$ SD, * $p<0.05$, ** $p<0.01$.

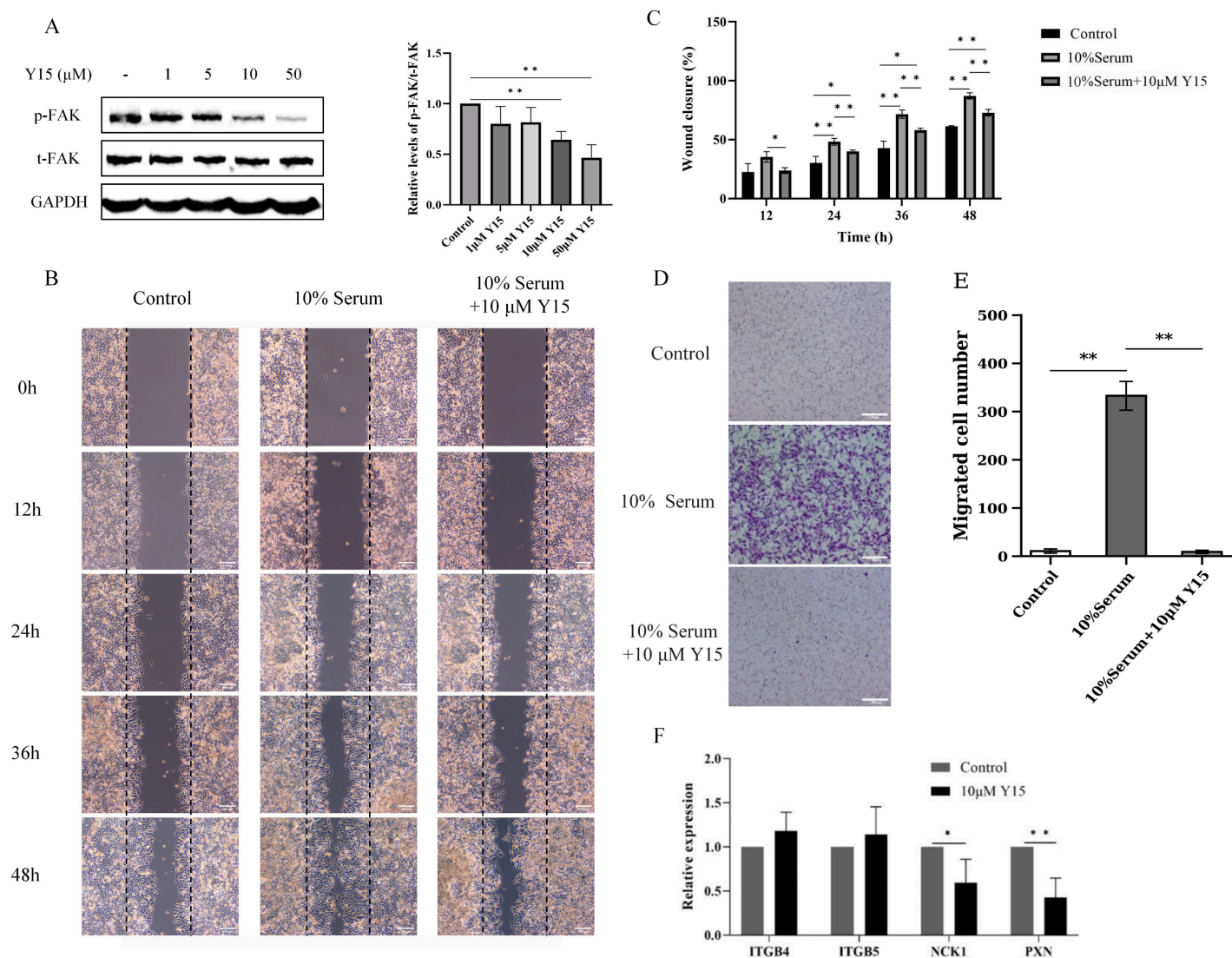


Figure 6

Effects of FAK signaling on the migration of LM3 cells. **A** Effect of the FAK inhibitor Y15 on FAK protein expression; **B** Effect of Y15 on the wound healing capacity of LM3 cells, scale bar = 200 μm; **C** Quantitative analysis of wound healing capacity in Y15-treated LM3 cells; **D** Effect of Y15 on the migration of LM3 cells, scale bar = 200 μm; **E** Quantitative analysis of migration capacity in Y15-treated LM3 cells; **F** Effect of Y15 on the mRNA expression of ITGB4, ITGB5, NCK1, and PXN; n = 3, means ± SD, * $p < 0.05$, ** $p < 0.01$

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