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ORIGINAL ARTICLE

HNF4α is possibly the missing link between epithelial–mesenchymal transition and Warburg effect during hepatocarcinogenesis

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Abstract

Hepatocellular carcinoma (HCC) is a heterogeneous, late-diagnosed, and highly recurrent malignancy that often affects the whole body's metabolism. Finding certain theranostic molecules that can address current concerns simultaneously is one of the priorities in HCC management. In this study, performing protein–protein interaction network analysis proposed hepatocyte nuclear factor 4 alpha (HNF4α) as a hub protein, associating epithelial–mesenchymal transition (EMT) to reprogrammed cancer metabolism, formerly known as the Warburg effect. Both phenomena improved the compensation of cancerous cells in competitive conditions. Mounting evidence has demonstrated that HNF4α is commonly downregulated and serves as a tumor suppressor in the HCC. Enhancing the HNF4α mRNA translation through a specific synthetic antisense long non-coding RNA, profoundly affects both EMT and onco-metabolic modules in HCC cells. HNF4α overexpression decreased featured mesenchymal transcription factors and improved hepatocytic function, decelerated

Abbreviations: ^{18}FDG, fluorodeoxyglucose; ALB, late functional genes; *APOA1*, apolipoprotein A-I; *APOB*, apolipoprotein B; EMT, epithelial–mesenchymal transition; *GLUT1*, glucose transporter 1; *GLUT2*, glucose transporter 2; HCC, hepatocellular carcinoma; HK2, hexokinase 2; HNF4α, hepatocyte nuclear factor 4 alpha; HNF4α AS lncRNA, HNF4α antisense long non-coding RNA; JAMs, junctional adhesion molecules; *LDHA*, lactate dehydrogenase A; MET, mesenchymal–epithelial transition; MFI, mean fluorescence intensity; *MMP14*, matrix metallopeptidase 14; PAS, Periodic acid-Schiff; *PPARG*, peroxisome proliferator-activated receptor gamma; PPI, protein–protein interaction; *SOAT1*, sterol *O*-acyltransferase 1; TJP1, tight junction protein 1; *VIM*, vimentin.

glycolysis, accelerated gluconeogenesis, and improved dysregulated cholesterol metabolism. Moreover, HNF4α overexpression inhibited the migration, invasion, and proliferation of HCC cells and decreased metastasis rate and tumor growth in xenografted nude mice. Our findings suggest a central regulatory role for HNF4α through its broad access to a wide variety of gene promoters involved in EMT and the Warburg effect in human hepatocytes. This essential impact indicates that HNF4α may be a potential target for HCC treatment.

KEYWORDS
aerobic glycolysis, epithelial–mesenchymal transition, hepatocellular carcinoma, HNF4α overexpression, Warburg effect

1 | INTRODUCTION

Hepatocyte nuclear factor 4 alpha is a member of the steroid hormone nuclear receptors and a ligand-dependent transcription factor with 12 annotated isoforms under the control of two distinct promoters and alternative splicing. The proximal promoter, P1, can generate α1 to α6 isoforms, which are involved in regulating cell differentiation and hepatic functions. In contrast, the distal promoter, P2, produces HNF4α7 to 12 transcripts, which are associated with cell proliferation and cancer progression.[1] During hepatocyte differentiation, P2 epigenetically switches to P1 promoter-derived isoforms, a change that occurs in reverse during hepatocarcinogenesis; when, in addition to an overall stage-dependent reduction in HNF4α expression, P1 isoforms expression is inhibited, and P2 isoforms are re-expressed.[2]

HNF4α, through docking to 42% of protein-coding genes in hepatocytes and locating at the center of a complex transcriptional network, is crucial for normal liver development and necessary for the maintenance of hepatocyte phenotype and functions. HNF4α disruption is directly linked to lipid and carbohydrate metabolism, detoxification of xenobiotics, regulation of transcription factors network, cell–cell junctions, and EMT.[1,3]

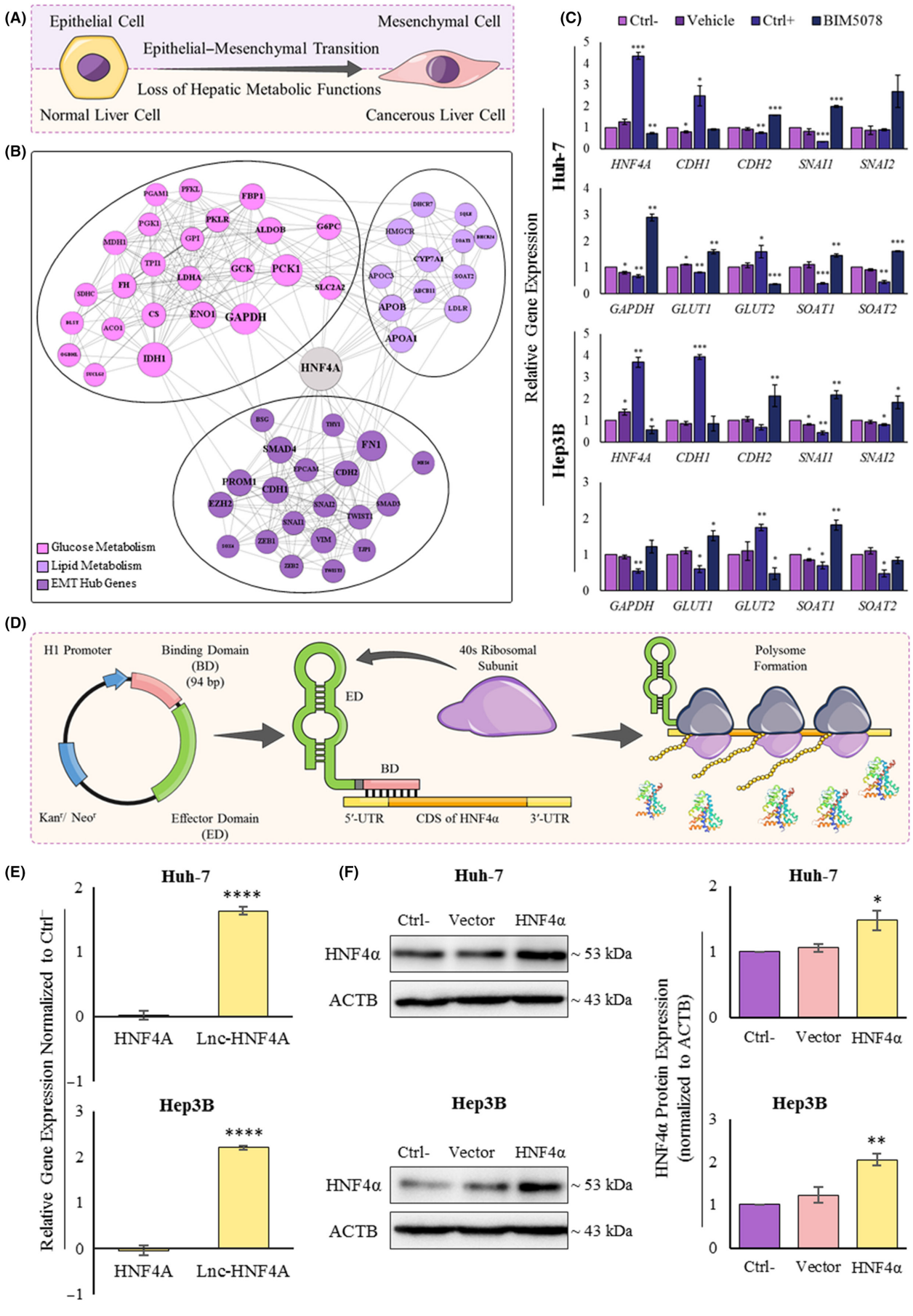
Epithelial–mesenchymal transition is a dynamic and stepwise shift in which epithelial cells lose their polarity and polygonal shapes and decrease cell–cell and cell–ECM interactions, turning into mesenchymal cells characterized by a spindle shape and the ability to migrate and invade. In the upstream of these changes, there are several common families of transcription factors, including SNAIL, TWIST, and ZEB.[4,5]

SNAIL1 and SNAIL2 suppress epithelial genes such as *CDH1*, *OCLN*, and *MUC1* and upregulate mesenchymal genes, including *CDH2* and *ZEB1*, through direct interaction with the promoter of these genes and indirectly by histone hypermethylation and deacetylation.[6] In addition, they are able to bind directly to the HNF4α P1 promoter and reduce α1–6 isoforms. Reciprocally, HNF4α has also been shown to be involved in EMT suppression by direct inhibition of SNAIL1 and SNAIL2 and upregulation of E-cadherin and gap junction proteins such as connexin 6, 22, and tight junction proteins such as occludin, claudin, TJP1, and JAMs.[5,7]

In addition to invasion and migration, reprogrammed metabolism is another critical hallmark of cancer progression. The best characterized metabolic phenotype of cancer cells is the Warburg effect (aerobic glycolysis); it is a common phenomenon in proliferating or developing cells, in which the rate of glucose uptake and lactate secretion dramatically increases, even in the presence of oxygen and fully functioning mitochondria.[8] Apparently, aerobic glycolysis gives tumor cells a selective advantage by supplying more ATP and limiting glucose availability in a multicellular competitive microenvironment.[9] Notably, the Warburg effect in liver cancer takes on different aspects and meanings, because glucose in hepatocytes plays additional roles than in other cells. The consequences of the Warburg effect affect not only cancerous cells in the liver, but act throughout the entire system.[10]

In this study, we tried to find a link between the alterations in energy management and EMT using a network-based approach in HCC cells (Figure 1A). In addition, we revealed that using SINEUP® technology and increasing the expression of HNF4α protein in the

FIGURE 1 HNF4α is a central regulator for EMT and hepatocytic metabolism. (A) Schematic representation of the transformation of a normal epithelial cell into a cancerous mesenchymal cell and loss of specific morphology and physiology during hepatocarcinogenesis. (B) Protein–protein interaction network of hepatocyte main metabolic and EMT-involved proteins was obtained from the STRING database and refined by Gephi software. (C) Quantifying mRNA expression levels of the main genes involved in EMT and metabolism were assessed in Huh-7 and Hep3B cell lines by qRT-PCR. (D) Schematic representation of the SINEUP technology mechanism of action in upregulating protein translation and enhancing the desired protein expression. (E) Quantified mRNA expression levels of *HNF4A* and *Lnc-HNF4A* after transfection of Huh-7 and Hep3B cells by HNF4α AS lncRNA. (F) Western blot analysis showed that transfection of Huh-7 and Hep3B cells by HNF4α AS lncRNA increased HNF4α protein levels normalized to ACTB and measured using ImageJ 1.52a software. Data are presented as the mean ± SD for three independent experiments. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ compared with the ctrl^{-} group.


(A)
Epithelial Cell
Mesenchymal Cell
Epithelial–Mesenchymal Transition
Loss of Hepatic Metabolic Functions
Normal Liver Cell
Cancerous Liver Cell
(B)
HNF4A
Glucose Metabolism
Lipid Metabolism
EMT Hub Genes
(C)
Ctrl-
Vehicle
Ctrl+
BIM5078
Huh-7
Hep3B
Relative Gene Expression
HNF4A
CDH1
CDH2
SNAI1
SNAI2
GAPDH
GLUT1
GLUT2
SOAT1
SOAT2
(D)
H1 Promoter
Binding Domain (BD) (94 bp)
Kanr/ Neor
Effector Domain (ED)
ED
BD
40s Ribosomal Subunit
5′-UTR
CDS of HNF4α
3′-UTR
Polysome Formation
(E)
Huh-7
Hep3B
Relative Gene Expression Normalized to Ctrl-
HNF4A
Lnc-HNF4A
(F)
Huh-7
Hep3B
Ctrl-
Vector
HNF4α
ACTB
~ 53 kDa
~ 43 kDa
HNF4α Protein Expression (normalized to ACTB)

physiological range, the status of both modules of metabolism and the cellular behavior in HCC cells are principally restored to normal conditions.

2 | RESULTS

2.1 | HNF4α is a central regulator for EMT and hepatocytic metabolism

To investigate the associations and find the hub genes involved in the Warburg effect and EMT, a PPI network of proteins involved in the metabolism of glucose, lipids, and EMT was built using the STRING database. Next, the data were refined, and network centrality parameters such as closeness, betweenness, and eigenvector were calculated using Gephi-0.9.2 software. The results showed that HNF4α, with the highest degree, closeness centrality (0.622), betweenness (263.83), and an eigenvector of 0.815 could be the most important central hub protein in this PPI network (Figure 1B). These results were consistent with previous studies, indicating that HNF4α is a crucial transcription factor in hepatocyte development and function.[11–13]

To confirm the regulatory role and evaluate the effects of gain and loss of function of *HNF4A* on the main genes involved in EMT and the Warburg effect, we evaluated their expression levels after 48 h of treatment in Huh-7 and Hep3B hepatic cells using qRT-PCR in four groups (Figure 1C): untreated (ctrl$^-$), transfection reagent treated (vehicle), transfected with plasmids carrying the *HNF4A* gene (ctrl$^+$), and BIM5078 (treated with the small molecule BIM5078 as an inhibitor of HNF4A).

Transfecting the cells with an *HNF4α*-expressing plasmid significantly upregulated the *HNF4α* mRNA levels in ctrl$^+$ cells compared with the ctrl$^-$ group in both Huh-7 and Hep3B cell lines. The mRNA levels of *CDH1* in both cell lines were significantly upregulated in ctrl$^+$ cells, while *CDH2* and *SNAI1* in Huh-7 cells and, *SNAI1* and *SNAI2* levels in Hep3B were decreased compared with their ctrl$^-$ groups. In contrast, *CDH2* and *SNAI1* in both cell lines, and *SNAI2* in Hep3B were significantly increased in cells treated with BIM5078 compared with ctrl$^-$ cells (Figure 1C). Additionally, we evaluated the critical molecules involved in hepatocytes metabolism, and it was shown that, compared with the ctrl$^-$ group *GAPDH*, *GLUT1*, *SOAT1*, and *SOAT2* were significantly decreased in the ctrl$^+$ group, whereas *GLUT2* was increased. Expectedly, all evaluated genes in Huh-7 and *GLUT1*, *GLUT2* and *SOAT1* in Hep3B have shown significant opposite results in the BIM5078 group compared with the ctrl$^-$ cells (Figure 1C; Table S1).

After preliminary validation of the effect of gain and loss of function of the *HNF4A*, SINEUP® technology was used to increase the expression of HNF4α via expression of the HNF4α AS lncRNA in the following experiments (Figure 1D). Initially, the 48 h transient expression of the SINEUP® construct was examined to test its function. After confirming the construct, the Huh-7 and Hep3B cells were stably transfected with the SINEUP®-carrying plasmid (HNF4α group) and the SINEUP® vector (vector group) without the binding domain fragment.

A 48 h expression of HNF4α AS lncRNA or SINEUP®-carrying plasmid in both Huh-7 and Hep3B cell lines had no significant effect on the *HNF4A* mRNA expression (Figure 1E), while protein expression increased 1.47 ± 0.15-fold in Huh-7 and 2.05 ± 0.14-fold in Hep3B cells (Figure 1F). It should be emphasized that the anti-HNF4α antibody (ab41898) used here recognizes P1-HNF4α isoforms.[14]

2.2 | Induction of HNF4α, induced mesenchymal–epithelial transition

Notably, HNF4α AS lncRNA stable transfection, in contrast with transient expression, increased *HNF4A* mRNA expression by creating a positive feedback loop (Figure 2A). To accurately investigate the effects of increased HNF4α on the expression of major transcription factors in the EMT process, cytoskeletal and junction proteins, ALB and AFP, the mRNA levels were evaluated using qRT-PCR.

The expression levels of *SNAI1*, *SNAI2*, *TWIST1*, and *PPARG* were significantly reduced in both HNF4α AS lncRNA stably expressing Huh-7 and Hep3B cell lines. The "cadherin switch," which is a hallmark of the EMT–MET process, was evaluated at the mRNA level, and the results showed an increase in *CDH1* and a decrease in *CDH2* expression (Figure 2A; Table S1).

In both HNF4α AS lncRNA-expressing HCC cell lines the expression levels of *VIM* as a mesenchymal cell marker and *MMP14* as a matrix metalloproteinase were also decreased; increased expression of both and their association with increased cell invasion in cancer cells has been well established. In addition, *AFP* expression as a hallmark of HCC cells decreased, and, conversely, *ALB* as an indicator of liver cell maturity and functionality increased (Figure 2A).

The expression levels of stemness and mesenchymal markers, CD133, CD90, and CD24, which are often elevated in HCC cells, and their expression is associated with a poor prognosis,[15–17] were also evaluated. *CD133* and *CD24* levels in both Huh-7 and Hep3B cell lines were significantly decreased in the HNF4α group compared with the ctrl$^-$ cells. However, the decreased expression of *CD90* was not statistically significant (Figure 2B).

We then carried out some investigations at the protein level, using western blot, ELISA, immunofluorescence, and immunophenotyping. Based on the qRT-PCR results, we evaluated CD133 and CD24 mesenchymal markers at the protein level using flow cytometry (Figure S2). The evaluated MFI of CD133 in the HNF4α-induced group of both HCC cell lines was reduced significantly. However, a significant decrease in CD24 MFI was observed only in the Huh-7 HNF4α group.

The results of the examinations at the mRNA level were consistent with those arising from our western blot analysis, and the "cadherin switch" reversion was also observed at the protein level

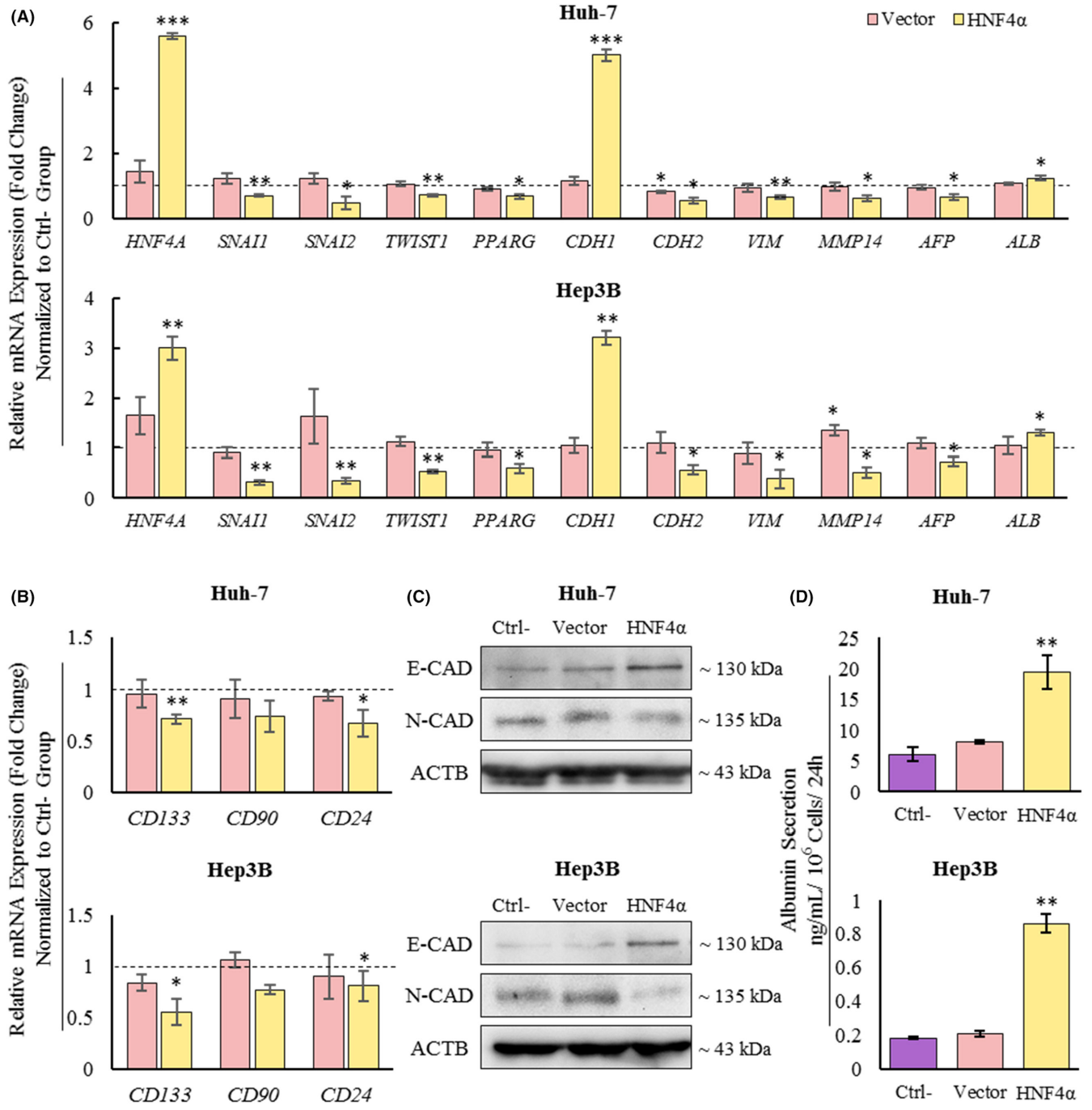


FIGURE 2 Induction of HNF4α induced mesenchymal–epithelial transition. (A) The HNF4α-related regulation of genes involved in the EMT process in Huh-7 and Hep3B cell lines, normalized to the ctrl$^-$ group. (B) The mRNA expression levels of mesenchymal markers in Huh-7 and Hep3B cells, normalized to the ctrl$^-$ group. (C) Western blot analysis showed that overexpression of HNF4α increased E-CAD expression and decreased N-CAD expression in Huh-7 and Hep3B cells compared with the ctrl$^-$ group. (D) Increased albumin secretion in the HNF4α group (HNF4α AS lncRNA-expressing group) was assayed using ELISA. Data are presented as the mean ± SD for three independent experiments. $^*p \le 0.05$, $^{**}p \le 0.01$, $^{***}p \le 0.001$.

(Figure 2C). Western blot analysis showed increased E-cadherin and decreased N-cadherin in HNF4α AS lncRNA-expressing cells.

The increased *ALB* expression at the mRNA level was consistent with the results of the ELISA test, which represented secretory ALB (Figure 2D) and immunofluorescence staining as well (Figure 3).

2.3 | Induction of HNF4α, induced mesenchymal–epithelial transition and hepatocyte late functional proteins

Immunofluorescence staining was performed to evaluate the expression changes at the protein level in the main genes involved in

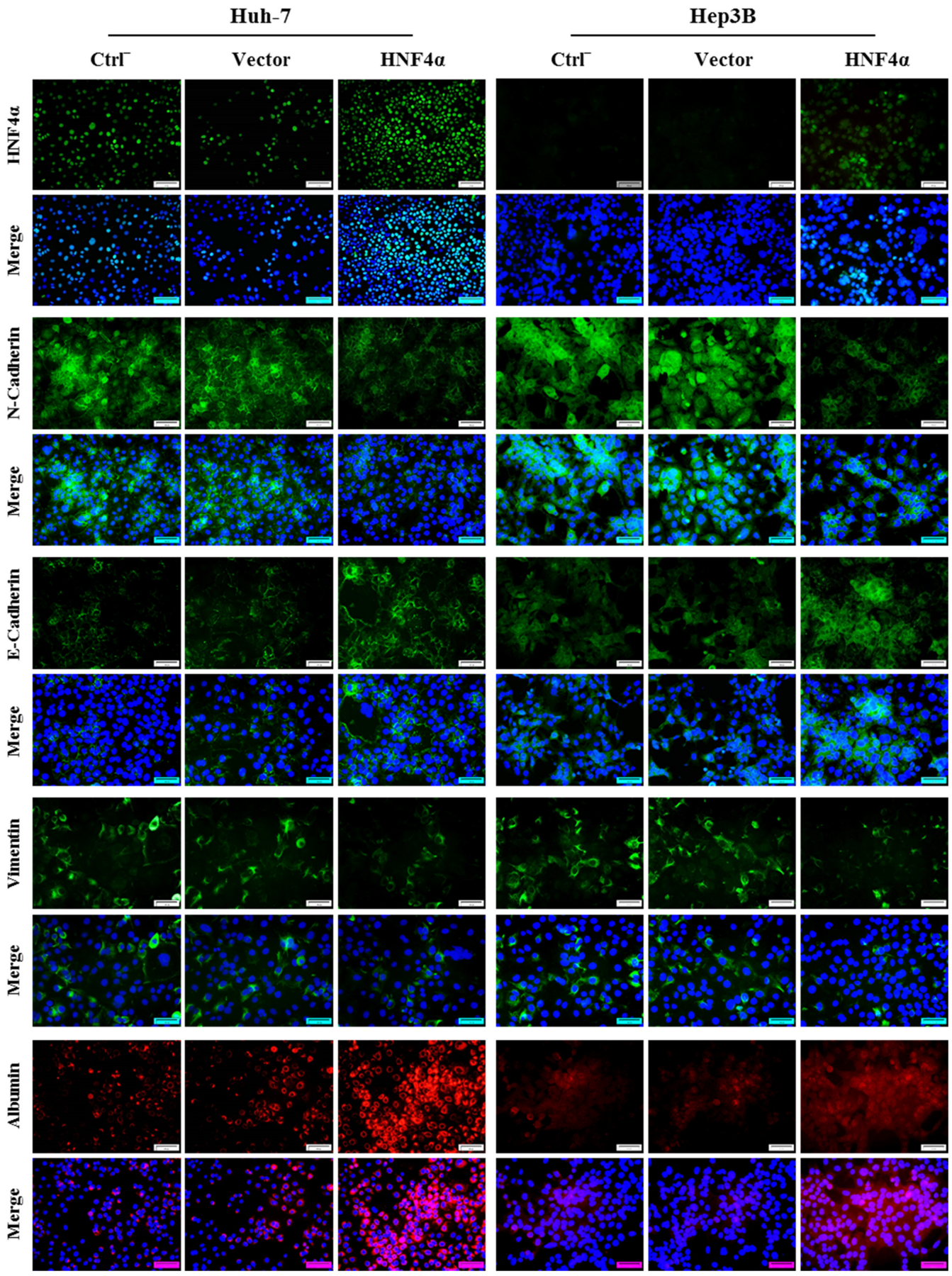


FIGURE 3 Induction of HNF4α induced mesenchymal–epithelial transition and hepatocyte late functional proteins. Immunofluorescence staining of the main proteins involved in the EMT–MET processes and hepatocyte maturity, counterstained with DAPI (scale bars, 100 μm).

EMT. The results showed an increase in the expression of HNF4α, E-cadherin, and ALB as well as a decrease in the expression of N-cadherin and vimentin in the HNF4α AS lncRNA-expressing group compared with the ctrl⁻ and vector groups (Figure 3), which confirmed the results of the qRT-PCR analysis. Collectively, these results indicated that HNF4α might function as an EMT regulator in HCC cells (Table S2).

2.4 | HNF4α induction resulted in cell-cycle arrest and reduced migration capacity of the HCC cell lines

HNF4α induction markedly attenuated the proliferation ability of HCC cells (Figure S3A), and increased the doubling time from 28.6±0.8 h in ctrl⁻ cells to 38.5±1.1 h in the Hep3B HNF4α-overexpressing group, and from 37.4±0.7 h to 40.8±0.8 h in Huh-7 cells. We next aimed to define whether HNF4α regulates the cell-cycle progression of HCC cells. Through analyzing cell-cycle distribution using FACS we found that the overexpression of HNF4α significantly reduced the percentage of both cell lines in the S phase with G0/G1-phase arrest in Huh-7 cells and G2/M arrest in Hep3B cells compared with the ctrl⁻ group (Figure 4A).

To investigate whether increased HNF4α expression, in addition to reducing proliferation rate, may affect the migration ability of the HCC cells, we used a wound-healing assay (Figure 4B). Cells were first mitomycinated to eliminate the effect of proliferation on the migration rate. The results showed that the percentage of wound closure and the migration rate were significantly reduced in the HNF4α AS lncRNA-expressing group compared with the ctrl⁻ groups, in both Huh-7 and Hep3B cell lines (Figure 4C).

2.5 | HNF4α induction decreased migration, invasion, and colony-formation capacities in both cell lines

To further study the effects of HNF4α on Huh-7 and Hep3B cell migration and invasion capacity, we performed Transwell® migration and invasion assays. Overexpression of HNF4α significantly inhibited cell migration and invasion rates (Figure 5A,B). Concordant with these results, colony-formation assays showed that overexpression of HNF4α impaired cell growth and the colony-forming ability of the Huh-7 and Hep3B cells compared with the vector and ctrl⁻ groups (Figure 5C). As shown in Figure 5D, the quantified areas of colonies and plating efficiency percentage of HNF4α groups in both cell lines were decreased significantly. The results of the colony-formation assay were in accordance with and supported the results of the qRT-PCR test for stemness and mesenchymal markers (*CD133*, *CD90*, and *CD24*). Moreover, in both cell lines, microscopic images of colonies showed more meroclones in ctrl⁻ and vector groups compared with the HNF4α group in which paraclones were more. Higher magnification on the edges of colonies clearly indicated more cellular projections with spindle-shaped cells in ctrl⁻ and vector groups compared with the HNF4α-overexpressing group, in which polygonal cells were observed (Figure S3B). In line with these results, platting the spheres in culture plates showed that in the ctrl⁻ and vector groups the cells were detached from the peripheral zone and migrated more in comparison with the HNF4α AS lncRNA-expressing cells (Figure S3C). This phenomenon reflects the summation of faster cell proliferation rate and more migration ability.

2.6 | Induction of HNF4α, induced normal hepatocytic metabolism and reduced the Warburg effect

As our above results showed that HNF4α markedly affected HCC cell growth, EMT, migration, and invasion ability, we sought to determine whether HNF4α regulated the expression of "energy management" genes, including those involved in glucose and lipid metabolism in HCC cells. Overexpression of HNF4α via HNF4α AS lncRNA significantly decreased the expression of *GLUT1* and conversely increased *GLUT2* in both cell lines, a phenomenon that is called "GLUT switching" in its reverse form, and observed routinely in HCC cells to increase glucose uptake (Figure 6A). These results were consistent with those obtained from our western blot analysis (Figure 6B), and "GLUT switching" was also observed at the protein level. Moreover, in HCC, glucokinase or hexokinase 4 (GCK) is replaced by HK2, a higher affinity isoform of hexokinases. Overexpression of HNF4α increased the expression of *GCK*, and it decreased the *HK2* in both cell lines. *GAPDH* and *LDHA* were also decreased, and two gluconeogenesis rate-limiting enzymes, *PCK1* (phosphoenolpyruvate carboxykinase 1) and *G6PC* (glucose 6-phosphatase), were increased (Figure 6A). For lipid metabolism, *SOAT1* and *SOAT2*, which are routinely upregulated in HCC cells, were decreased in the HNF4α group compared with the ctrl⁻ cells. Additionally, HNF4α, via direct interaction with *APOA1* and *APOB* promoters, significantly increased their expression in Huh-7 cells. In Hep3B cells, although their expression levels were increased on average, changes were not statistically significant. CYP7A1 is the first rate-limiting enzyme in the bile acid synthesis pathway and has an important role in cholesterol metabolism. It is generally considered a maturity and functionality indicator of hepatocytes and decreased in HCC cells. *CYP7A1* was significantly increased in HNF4α AS lncRNA-expressing Hep3B cells, but its increase was not significant in Huh-7 cells (Figure 6A).

To further demonstrate that the signature of the Warburg effect has faded and the functionality of hepatocytes in terms of glycogen storage was increased, we stained cells with PAS. As indicated in Figure 6C, the amount of stored glycogen (bright magenta) in HNF4α AS lncRNA-expressing cells is increased compared with the vector and ctrl⁻ groups in both Huh-7 and Hep3B cell lines. Moreover, to

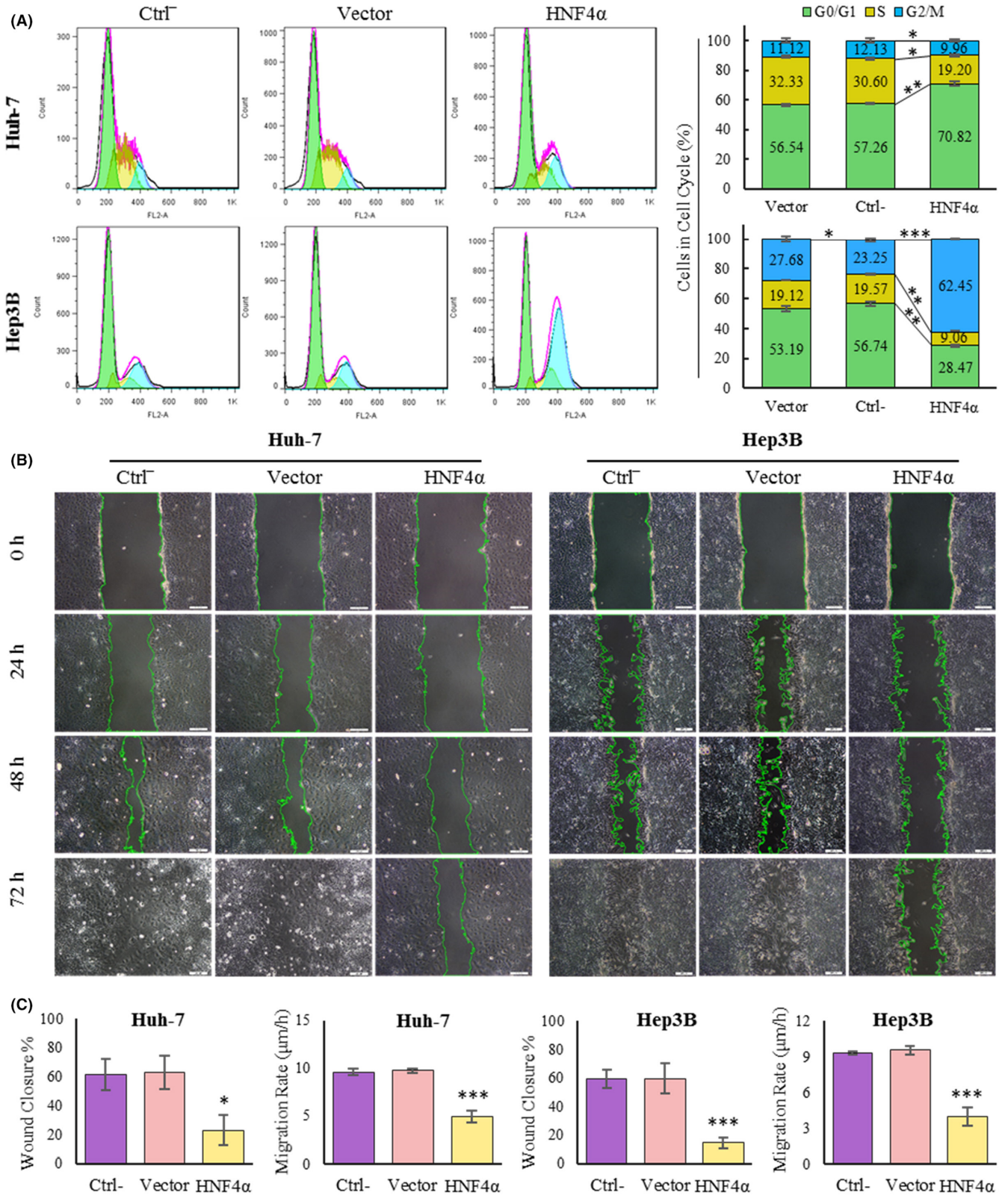


FIGURE 4 HNF4α induction resulted in cell-cycle arrest and reduced migration capacity of the HCC cell lines. (A) Overexpression of HNF4α induced G0/G1 arrest in Huh-7 and G2/M arrest in Hep3B cells. (B, C) The wound-healing assay indicated a decrease in the percentage of wound closure and migration rate in the HNF4α-overexpressing group compared with the ctrl⁻ group. The scratches area was quantified and the distance between the two edges was measured using ImageJ 1.52a software (scale bars, 200 μm). Data are presented as mean ± SD for three independent experiments. $^{*}p \le 0.05$, $^{**}p \le 0.01$, $^{***}p \le 0.001$.

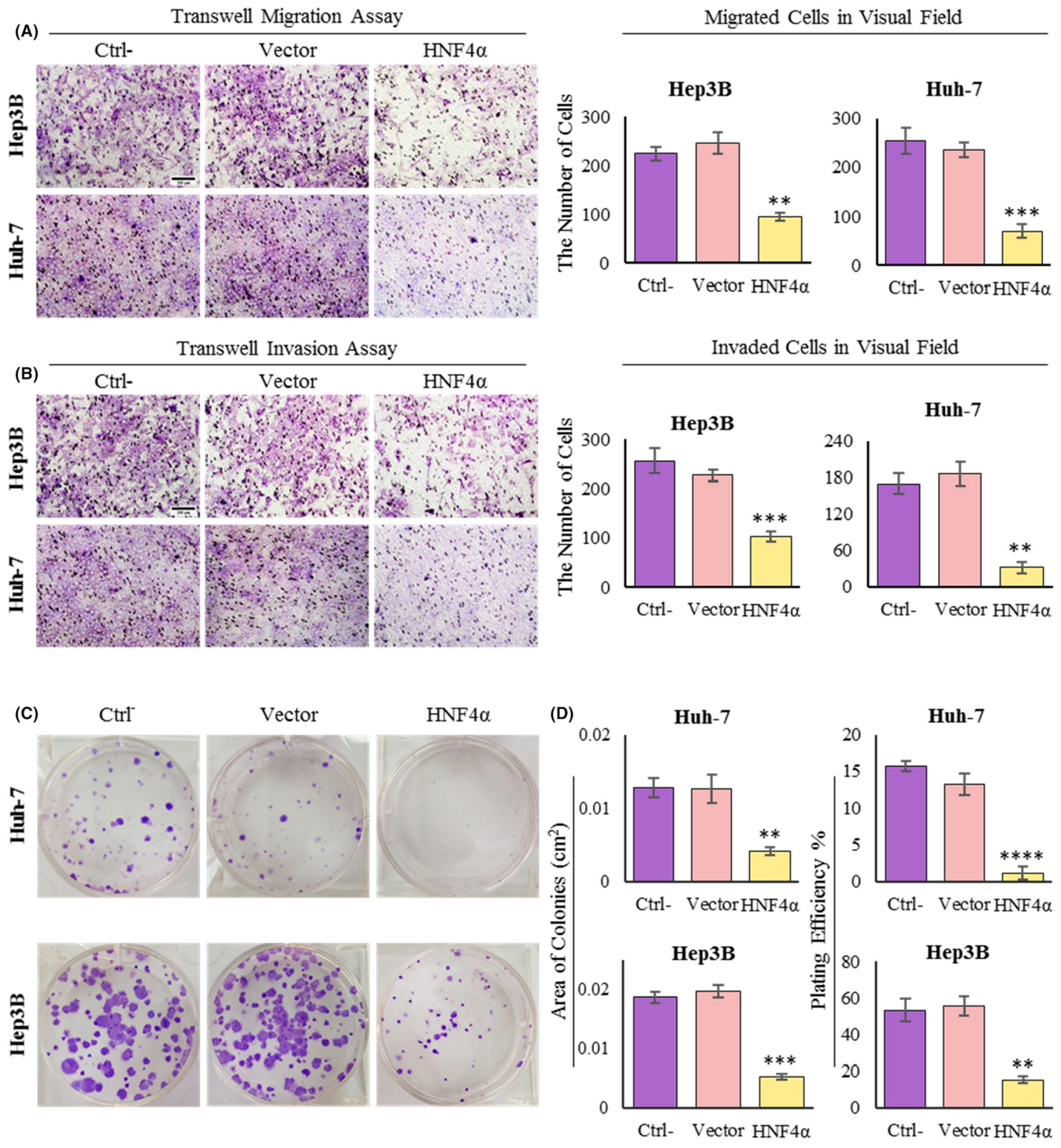


FIGURE 5 HNF4α induction decreased migration, invasion, and colony-formation capacities in both cell lines. (A, B) Transwell migration and invasion assays. The numbers of migrated and invaded cells were counted from five randomly selected fields under a phase-contrast microscope (scale bars, 200 μm). (C) The colony-formation assay indicated alterations in the proliferative ability of cells after treatment. (D) The average area of colonies and the percentage of plating efficiency were decreased in HNF4α-overexpressing Huh-7 and Hep3B cells compared with the ctrl⁻ group. Data are presented as the mean ± SD for three independent experiments. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

confirm the results of the gene expression alterations, the concentration of produced lactate was assayed in the cell culture medium. As expected, by decreasing the glycolysis rate as well as decreasing the expression of *LDHA*, the amount of produced lactate decreased (Figure 6D).

2.7 | Induction of HNF4α, induced metabolism-related hepatocytic proteins

Immunofluorescence staining was performed to confirm the alterations in the gene expression profile following HNF4α induction

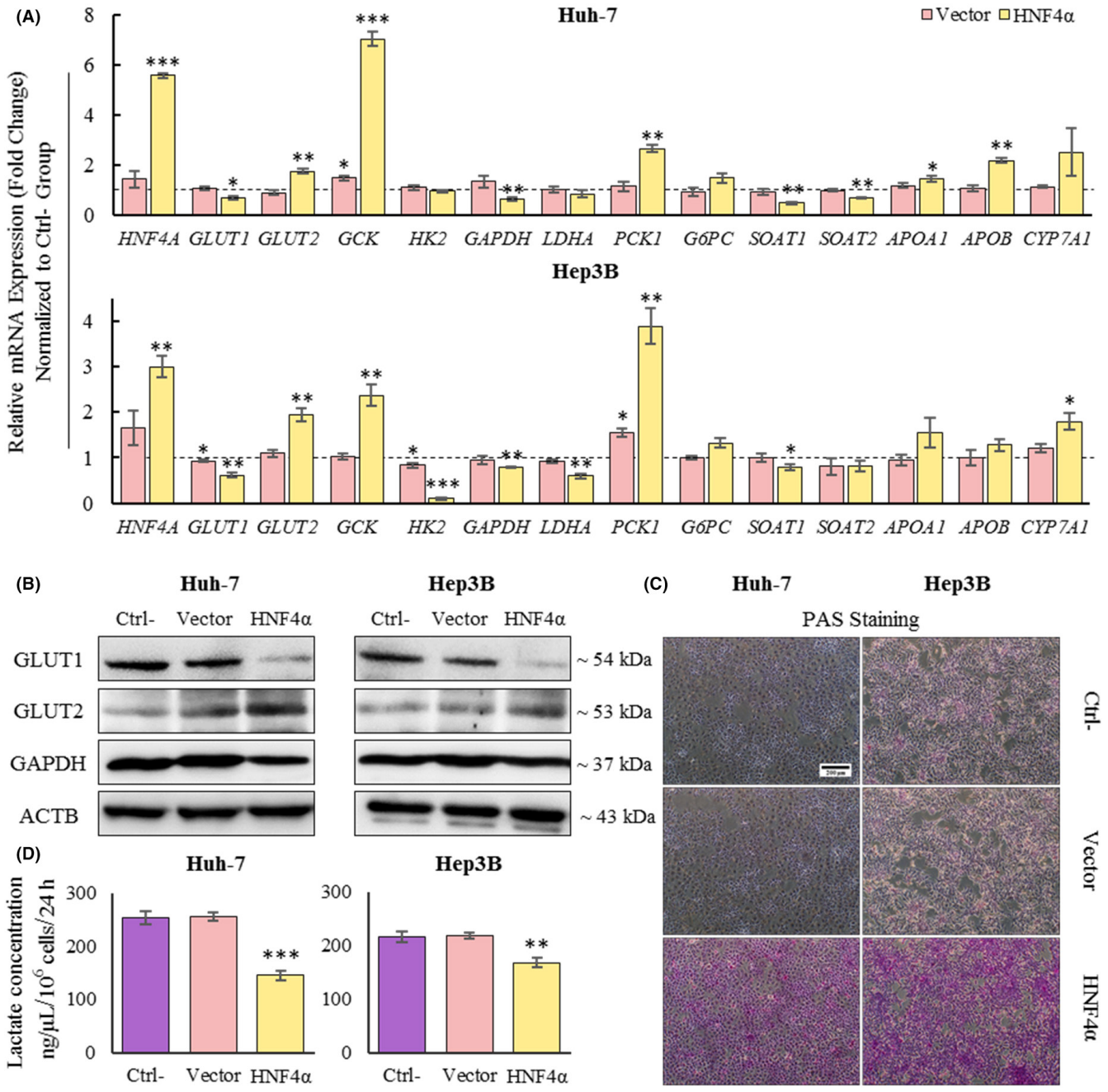


FIGURE 6 Induction of HNF4α induced normal hepatocytic metabolism and reduced the Warburg effect. (A) The HNF4α-related regulation of genes involved in glucose and lipid metabolism in Huh-7 and Hep3B cell lines, normalized to the ctrl$^-$ group. (B) Western blot analysis indicated that overexpression of HNF4α increased GLUT2 expression and decreased GLUT1 and GAPDH expression in Huh-7 and Hep3B cells. (C) Periodic acid-Schiff (PAS) staining showed the glycogen storage (bright magenta) increase in HNF4α-overexpressing Huh-7 and Hep3B cells. (D) Overexpression of HNF4α decreased lactate production in Huh-7 and Hep3B cell media. Data are presented as the mean ± SD for three independent experiments. $^*p \le 0.05$, $^{**}p \le 0.01$, $^{***}p \le 0.001$ compared with the ctrl$^-$ group.

at the protein production level. The results approved the "GLUT switching" reversion, in which, by increasing the expression of HNF4α, the GLUT1 expression was reduced, whereas the GLUT2 elevated. Moreover, the results indicated a decrease in the expression of GAPDH as well as an increase in the expression of CYP7A1 in the HNF4α group compared with the ctrl$^-$ and vector cells (Figure 7). Together, these results indicated that HNF4α functions as a metabolism regulator in HCC cells.

2.8 | HNF4α induction decreased HCC xenograft growth and metastasis ability of HCC cells in vivo

To investigate whether HNF4α induction could suppress the growth rate of a xenograft in a BALB/c nude mice model, HCC cells were implanted subcutaneously. Mice were monitored and the volume of tumors was measured every 3 days. Results indicated that the growth rate of tumors in the HNF4α group was significantly

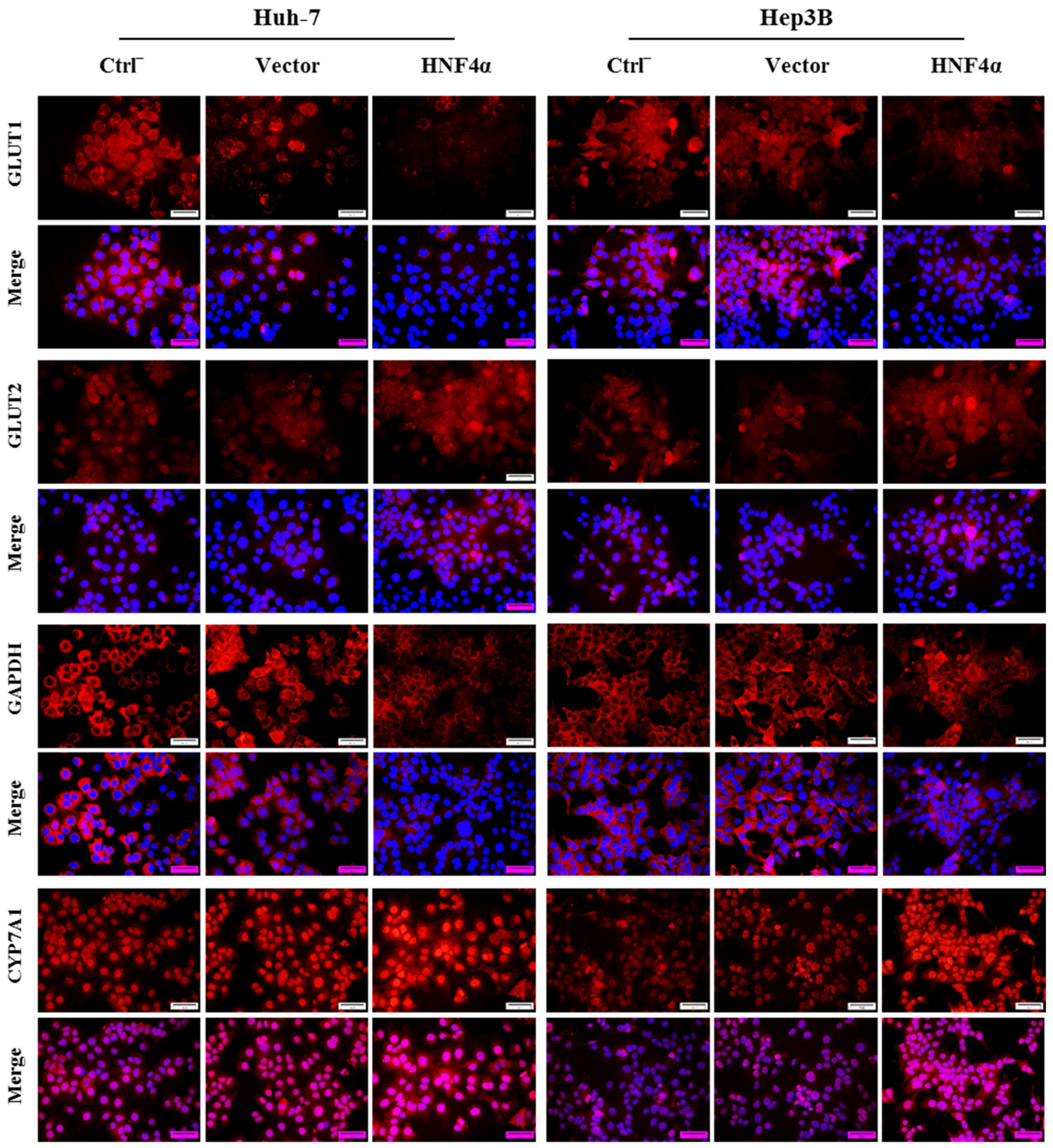
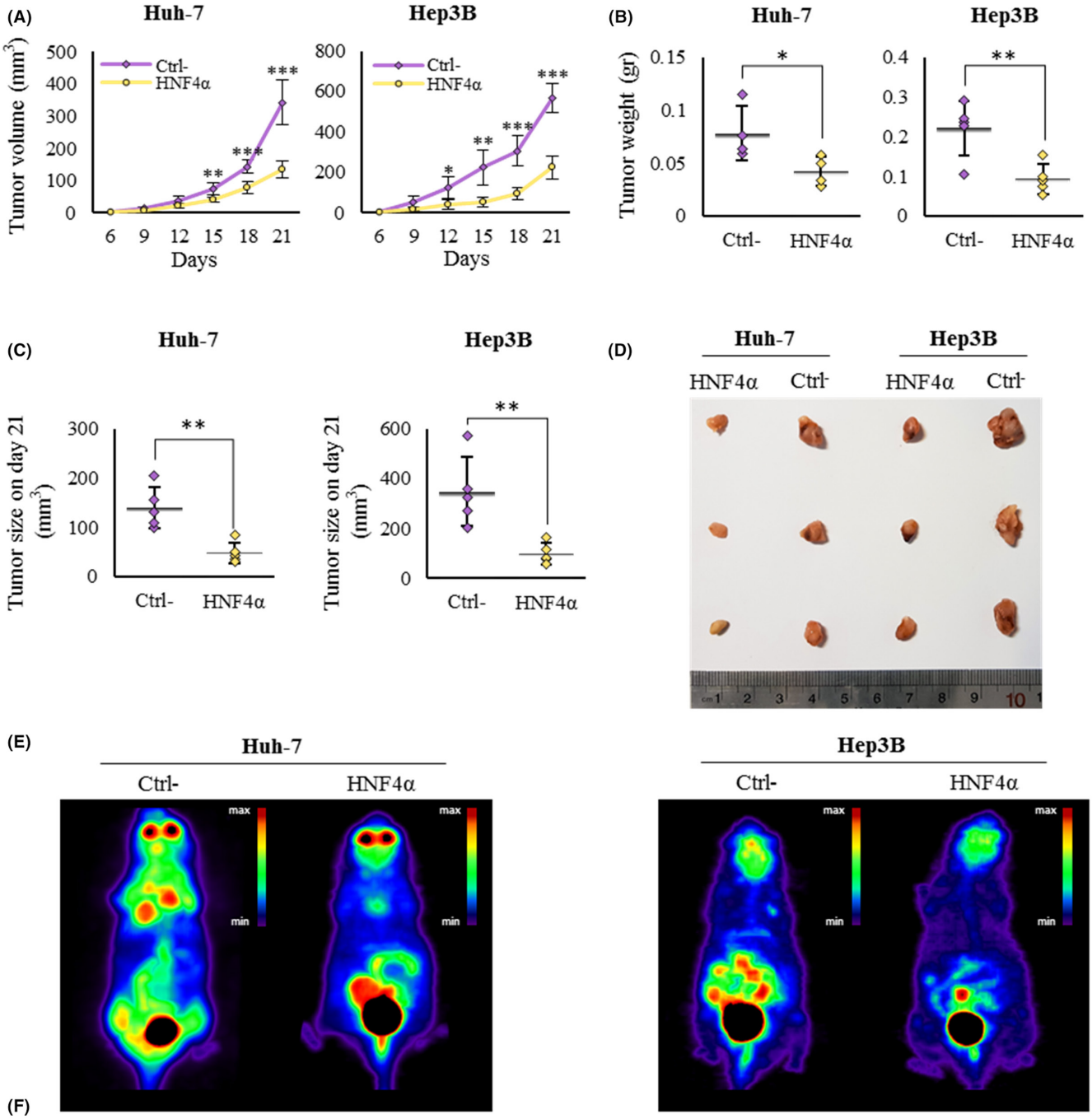


FIGURE 7 Induction of HNF4α induced metabolism-related hepatocytic proteins. Immunofluorescence staining of the main proteins involved in glucose and lipid metabolism counterstained with DAPI (scale bars, 100 μm).

lower than in the ctrl⁻ group (Figure 8A). After 3 weeks, mice were euthanized and tumors were collected. Compared with ctrl⁻ mice, the weight and size of tumors in the mice who received HNF4α AS lncRNA-expressing Huh-7 or Hep3B cells were significantly lower (Figure 8B–D).

Additionally, we examined the effect of HNF4α induction on the metastatic potential of the tumor cells using a liver xenograft tumor model in nude mice (Figure S4). A positron emission tomography (PET) scan was performed on mice after 3 weeks. As shown in Figure 8E,F, compared with the ctrl⁻ group, the amount of the ^{18}FDG uptake, in the liver and other organs such as the brain, intestine, lung, and stomach decreased, which indicated a smaller number of metastatic cells in the mice that received HNF4α AS lncRNA-expressing Huh-7 and Hep3B cells.



(F)

Photon / mm³	Liver	Brain	Intestine	Lung	Stomach
Huh-7 Ctrl-	442	2085	2629	1664	394
Huh-7 HNF4α	226	1035	1992	17	7
Hep3B Ctrl-	549	733	859	28	401
Hep3B HNF4α	107	713	419	21	82

FIGURE 8 HNF4α induction decreased HCC xenograft growth and metastasis ability of HCC cells in vivo. (A, B) Overexpression of HNF4α reduced tumor volume and weight compared with the untreated cells ($n = 5$ per group). Tumor volume was calculated based on the following equation: volume (mm^3) = length (mm) × width^2 (mm^2) × 0.5. (C) HNFα induction significantly resulted in a decrease in the final tumor size on day 21 (measured after tumor removal). (D) Photographs of the tumors in different groups. (E) In images of positron emission tomography (PET) scan, the more the color shifted from blue to red, the more the radioactive substrate was absorbed, indicating the presence of more cells with a higher metabolism. (F) The amounts of the tumor radionuclide uptake in different organs in each mouse (photon/mm^3). Data are presented as the mean ± SD for three independent experiments. $^*p \le 0.05$, $^{**}p \le 0.01$, $^{***}p \le 0.001$ compared with the ctrl$^-$ group.

3 | DISCUSSION

Hepatocellular carcinoma (HCC) is a heterogeneous malignancy with limited treatment options due to underlying predisposing diseases such as fibrosis and cirrhosis.[18] Due to the importance of the liver in regulating body metabolism, any impairment of the liver physiology as the consequence of HCC affects the entire system.[19] Therefore, finding a hub molecule that simultaneously regulates different metabolic and signaling pathways could be promising in developing new therapeutic modalities.

In this study, we first implemented a network-based analysis, and found that HNF4α is the central hub protein that links the metabolic alterations to the phenotypic changes of hepatocytes in the HCC. Then, we proved this concept by transient assessment of gain/loss of function tests in Huh-7 and Hep3B cells and with four experimental groups, using a forced HNF4α expressed vector representing a gain of function, and BIM5078, a small molecule that is an HNF4α inhibitor representing the loss of function.

Moreover, for further investigation, we increased the stable expression of HNF4α protein levels using a novel molecular technology named SINEUP® that specifically increases the translational rates of the desired proteins in a physiological range. The experiments were continued in both Huh-7 and Hep3B cells with three determined groups: untreated cells, empty-SINEUP® vector stably expressing cells, and HNF4α AS lncRNA or SINEUP® stably expressing cells.

Previous studies have shown that HNF4α is the most abundant transcription factor in hepatocytes, and is downregulated in a stage-dependent manner during hepatocarcinogenesis; its reduction is associated with EMT progression and poor prognosis.[20–22] We knocked up the HNF4α protein levels within the physiological range (Huh-7: 1.4 ± 0.1, Hep3B: 2 ± 0.1) using SINEUP technology, in accordance with other studies that utilized SINEUP.[23–27] We revealed that stable increased expression of HNF4α protein in Huh-7 and Hep3B cells generally decreased the expression of mesenchymal genes (including *SNAI1*, *SNAI2*, *TWIST1*, *CDH2*, *VIM*, and *MMP14*) and conversely increased the expression of epithelial (*CDH1*) and hepatocytic late functional genes (*ALB*). This activated and reversed the "cadherin switch," which is a MET characteristic. It is also well demonstrated that HNF4α induction arrests the cells in the G0/G1 and G2/M phases. HNF4α increases the expression of the *CDKN1A* gene (p21 protein) by antagonizing c-*Myc* activation, and increased *CDKN1A* expression is known as the most important factor in G2/M arrest.[28] Moreover, miR-194/192 contributes to the activation of *CDKN1A*. It is indicated that HNF4α directly upregulates the *miR-194/192* gene through a binding site in the *miR-194/192* promoter region.[29] In addition, a negative reciprocal regulatory axis exists between cyclin D1 and HNF4α. It seems that HNF4α downregulates cyclin D1 and arrests cell-cycle progression at the G1 phase.[30] As the basic expression levels of p21 and cyclin D1 are different in various cells, they show different manifestations after induction of *HNF4A* expression. Furthermore, given the decreasing impact of the HNF4α overexpression on *SNAI*, *TWIST*, *CDH2*, *VIM*, and *MMP14* and increasing *CDH1*, we showed that in vitro migration and invasion rates and in vivo intra- and extrahepatic metastasis rates decreased in the HNF4α AS lncRNA-expressing group, consistent with the previous studies in renal cell carcinoma, and gastric and colon cancers.[31–33]

Conversely, the "increased energy and building blocks demand" for survival, proliferation, and metastasis, as a differentiating factor between healthy and cancer cells, has been one of the most important issues in targeted cancer therapy. The ^{18}F-FDG-PET scan indicated that 50%–70% of the ATP required by cancer cells is supplied by glycolysis, even in the presence of sufficient oxygen.[34] The choice of aerobic glycolysis by cancer cells implies that energy supplementation speed takes priority over its efficiency. By putting this strategy at the forefront, cancer cells reprogram their metabolic enzymes and transporter proteins to accelerate building blocks providing glucose uptake, glycolysis, and metabolic end-product elimination.[35]

In the first stages of glucose metabolism in HCC cells, GLUT2, which has the lowest affinity for glucose and highest expression in hepatocytes, switches to GLUT1, a high-affinity glucose transporter, and avidly increases glucose uptake.[36,37] In our study, we found that HNF4α upregulation restored GLUT balance and modulated glucose uptake.

In the next step, there is a similar shift from GCK, a low-affinity hexokinase, to the HK2 in HCC cells, which are the first rate-limiting enzymes in glycolysis and have a significant effect on its acceleration.[38] Interestingly, HNF4α induction reversed the GCK to HK2 switch and restored its balance to the normal status.

Growing evidence has shown that *GAPDH*, which is routinely considered as the reference gene or protein for expression quantification, is dysregulated in many cancer types, including lung,[39,40] renal,[41] breast,[42] gastric,[43] and liver cancer.[44,45] Therefore, in this study, we used *HPRT1* or β-actin (ACTB) as the housekeeping gene/protein and showed that increasing HNF4α expression downregulated *GAPDH* expression in HCC cells. Upregulation of the *LDHA* expression in HCC and its association with tumor progression and metastasis, as well as decreased oxygen dependency have been

demonstrated.[46,47] We found that *LDHA* expression and extracellular lactate production decreased following the overexpression of HNF4α in both HCC cell lines.

Increased rates of gluconeogenesis have been reported in several types of cancer, including colon,[48] lung,[49] and melanoma,[50] and attributed to the increased anabolic metabolism required for extra cell proliferation.[47] Markedly, the expression levels of rate-limiting enzymes and so the overall rate of gluconeogenesis is reduced in HCC. Even enforced PCK1 expression induces apoptosis by causing tricarboxylic acid (TCA) cycle cataplerosis and high reactive oxygen species (ROS) levels.[51] In our study, we revealed that *PCK1* was upregulated as a direct target of P1-*HNF4α*, consistent with previous studies into alcoholic hepatitis.[52]

Previous studies have suggested that altered lipid metabolism, particularly cholesterol metabolism, plays an important role in the development and progression of the HCC.[53,54] In addition, several reports have identified HNF4α as one of the master regulators of lipid and cholesterol homeostasis. They indicated that *HNF4A* knockdown in Huh-7 cells leads to the downregulation of *GATA4*, *APOC3*, *APOA1*, *APOB*, and *FOXO1*, and that the downregulation of these genes may promote cancer development.[55–57] Whereas the most essential proteins in the cholesterol–bile acid metabolic pathway (CYP7A1, APOA1, and APOB) are downregulated, key enzymes in cholesterol metabolism (SOAT1, SOAT2, etc.) are upregulated in tumors.[3,58,59] As CYP7A1, APOA1, and APOB are the direct targets of the HNF4α, an increase in their expression can be expected following the increased expression of HNF4α.

Until recently, attempts to increase *HNF4A* expression to treat various metabolic and genetic disorders of the liver have been investigated in different ways. However, the role of HNF4α in bridging different functional and metabolic aspects in hepatocytes has remained relatively unknown. In the metabolic network controlled by HNF4α, a systematic integrative analysis by Baciu et al. identified *HNF4α* as a central gene in the pathogenesis of non-alcoholic steatohepatitis.[60] Here, Lee et al. revealed that HNF4α controls liver fat storage, and they used two potent HNF4α agonists derived from alverine and benfluorex to activate HNF4α and thereby reduced the steatosis in diet-induced obese mice.[61] Furthermore, Yang et al. demonstrated that increasing HNF4α expression through mRNA delivery in a mouse model could effectively improve fibrosis and cirrhosis.[13] In an interesting study, Haque et al. pointed out the linker role of the HNF4α. They indicated that inhibition of HNF4α via an siRNA leads to 748 differentially expressed genes, in which the most downregulated genes were involved in lipid and cholesterol metabolism and were implicated in HCC.[55] HNF4α is a tumor suppressor factor, Cheng et al. demonstrated that the combination of *HNF1A*, *HNF4A*, and *FOXA3* synergistically reprograms HCC cells to hepatocyte-like cells, in which the malignant phenotype is reduced, and hepatocyte-specific characteristics retrieved.[62] Moreover, Miri-Lavasani et al. reported that the treatment of HCC cells using conjugated linoleic acid, as a natural ligand of HNF4α, decreased EMT marker level, migration, and proliferation capacities.[63]

Taken together, this study provided a comprehensive and integrated insight into the importance of HNF4α protein in linking several key modules involved in HCC and proposed it as a potential therapeutic target. We revealed that overexpression of HNF4α protein by a synthetic AS lncRNA in the physiological range could simultaneously modulate aberrant changes in metabolism and cellular behavior. However, further study is needed to elucidate the molecular mechanism of HNF4α induction in MET and cell metabolism. Targeting cancer cells through HNF4α and attenuating cancerous phenotypes may provide a promising avenue from which to develop effective therapies for HCC.

AUTHOR CONTRIBUTIONS

B.S. and Z.H. performed the assays in vitro. B.S. performed the experiments in vivo. B.S. and M.V. analyzed the data. B.S., A.P., and M.V. interpreted the data and wrote the manuscript. B.S., M.V., B.N., and M.T. designed this study. M.V., B.N., A.P., AAES, E.M., and M.T. revised the paper. M.V. and B.N. financially supported the project. M.V. and E.M. carried out the final approval. All authors have read and approved the final manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICS STATEMENT

Animal Studies: All animal experiments were performed under the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 80–23, revised 1996) and approved by the Research and Ethics Committee of Royan Institute (Approval No. IR.ACECR.ROYAN.REC.1397.262).

Approval of the research protocol by an Institutional Reviewer Board: All animal experiments were approved by the Research and Ethics Committee of Royan Institute (Approval No. IR.ACECR.ROYAN.REC.1397.262).

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Informed Consent: N/A.

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