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Silencing of OGDHL promotes liver cancer metastasis by enhancing hypoxia inducible factor 1 α protein stability

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Abstract

Hepatocellular carcinoma (HCC) is one of the most common malignant diseases associated with a high rate of mortality. Frequent intrahepatic spread, extrahepatic metastasis, and tumor invasiveness are the main factors responsible for the poor prognosis of patients with HCC. Hypoxia-inducible factor 1 (HIF-1) has been verified to play a critical role in the metastasis of HCC. HIFs are also known to be modulated by small molecular metabolites, thus highlighting the need to understand the complexity of their cellular regulation in tumor metastasis. In this study, lower expression levels of oxoglutarate dehydrogenase-like (OGDHL) were strongly correlated with aggressive clinicopathologic characteristics, such as metastasis and invasion in three independent cohorts featuring a total of 281 postoperative HCC patients. The aberrant expression of OGDHL reduced cell invasiveness and migration in vitro and HCC metastasis in vivo, whereas the silencing of OGDHL promoted these processes in HCC cells. The pro-metastatic role of OGDHL downregulation is most likely attributed to its upregulation of HIF-1α transactivation activity and the protein stabilization by promoting the accumulation of L-2-HG to prevent the activity of HIF-1α prolyl hydroxylases, which subsequently causes an epithelial–mesenchymal transition process in HCC cells. These results demonstrate that OGDHL is a dominant factor that modulates the metastasis of HCC.

KEYWORDS

HIF-1α, liver cancer, metastasis, OGDHL, tricarboxylic acid cycle

Weiqi Dai, Yueyue Li, Weijie Sun and Meng Ji contributed equally to this work.

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1 | INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant diseases and the fourth leading cause of death from cancer worldwide.[1] Most patients with this malignant disease are diagnosed at an advanced stage due to its rapid progression. Frequent intrahepatic spread, extrahepatic metastasis, and tumor invasiveness are the key factors responsible for the poor prognosis of patients with HCC.[2] The molecular events underlying HCC invasiveness and metastasis are largely unknown, and novel causative genes and molecular pathways are fundamental to improving the treatment and prognosis of HCC.

Epithelial–mesenchymal transition (EMT) is implicated in phenotypic changes in which epithelial cells lose cell–cell polarity and adhesion and then acquire a mesenchymal phenotype with migratory potential. The development of EMT is characterized by certain molecular hallmarks, including the reduction of epithelial markers (e.g., E-cadherin) and the upregulation of mesenchymal markers (e.g., vimentin).[3] EMT is related to the metastatic process of various cancers, including HCC.[4]

One of the most important triggers of EMT in tumors is the activity of hypoxia-inducible factor 1 (HIF-1), which is enhanced through hypoxia-independent or hypoxia-dependent pathways.[5,6] The biological activity of HIF-1 mostly relies on HIF-1α expression, which is strictly modulated by factor-inhibiting HIF-1 (FIH-1) and the prolyl hydroxylase domain enzymes (PHDs), respectively.[7] HIF-1α is hydroxylated by PHDs at two conserved proline residues, P564 and P402, within the N-terminal activation domain (N-TAD). This hydroxylation signal drives the subsequent proteasomal degradation of HIF-1α via recruitment of the E3 ubiquitin ligase pVHL.[8] Meanwhile, a specific asparaginyl (N803) of HIF-1α, the C-terminal transactivation domain (C-TAD), is hydroxylated by FIH-1, leading to the inactivation of its transactivating activity.[9] Consequently, the HIF-1α protein is stabilized because of the inhibition of these hydroxylases, translocates to the nucleus, and then binds to its heterodimeric partner, HIF-1β.[10] The resulting heterodimeric complex (HIF-1) adheres to the hypoxia-responsive element to promote the transcription of a set of downstream targets, including vital regulators for EMT.[11,12]

Clinical and basic research has demonstrated that HIF-1α protein expression is positively correlated with the poor outcomes of both distant tumor metastasis and tumor recurrence, thus identifying the value of HIF-1α as a target for tumor therapy, along with its upstream activators.[6] The role of oxoglutarate dehydrogenase-like (OGDHL), one of the rate-limiting components of the OGDHC, has been investigated in several tumors. Some research groups have previously determined that OGDHL level was suppressed by cancer-specific promoter methylation in cervix, lung, esophagus, pancreas, breast, and colon cancers. Downregulation of OGDHL was demonstrated to contribute to the upregulation of 2-OG by reducing OGDHC activity.[13] Alternatively, previous research has demonstrated that PHDs, members of the 2-oxoglutarate (2-OG) dioxygenases, are highly sensitive to the changing levels of intermediates in the tricarboxylic acid (TCA) cycle, such as 2-hydroxyglutarate (2-HG), a chiral compound derived from 2-OG.[14,15] Therefore, HIF-1α protein is likely to be modulated by small molecular metabolites, which are still largely obscure.

In our previous research, we identified the value of exploiting OGDHL as an independent prognostic factor for HCC diagnosis and treatment.[13] In our present study, the downregulation of OGDHL was associated with metastasis and invasion in patients with HCC. The aberrant expression of OGDHL inhibited HCC cell migration and invasiveness as well as metastasis of nude mice. Furthermore, inverse correlations of the expression of OGDHL with HIF-1α and EMT were further validated in two independent HCC cohorts (cohorts 1 and 2). Mechanistic studies further identified that the downregulation of OGDHL led to an upregulation in HIF-1α transactivation and HIF-1α protein stability as a component of the OGDH complex (OGDHC) to promote EMT in HCC cells. The silencing of OGDHL stabilized HIF1α by promoting the formation of (L)-enantiomer of 2-hydroxyglutarate (L-2-HG) from 2-OG. Therefore, the aims of this study were to identify the pro-metastatic mechanisms underlying the upregulation of HIF-1α protein stabilization in response to OGDHL downregulation in HCC cells and elucidate the role of L-2-HG in preventing the activity of HIF-1α prolyl hydroxylases, which subsequently causes an EMT process in HCC cells. Thus, we are the first to demonstrate that OGDHL is a dominant factor that regulates the metastasis and invasiveness of HCC.

2 | MATERIALS AND METHODS

2.1 | Clinical samples and tissue microarray

We extracted EMT-related mRNAs of 225 HCC specimens (cohort 1) from the National Center for Biotechnology Information Gene Expression Omnibus Data Platform (NCBI-GEO) under accession number GSE14520. Paired primary-metastatic HCC specimens from cohort 2 patients (n = 24), who underwent curative hepatic resection at Shidong Hospital, were enrolled for HCC tissue microarray analysis (TMA). In addition, another TMA (cohort 3, n = 56) was randomly selected by a computer and featured 21 primary HCCs, 11 metastatic HCCs, 9 chronic liver cirrhosis tissues, 10 normal liver tissues, and 5 liver hemangioma specimens; these specimens were used to assess the clinical relevance of OGDHL in the metastasis of patients with HCC. The experiment protocol was conducted according to the Declaration of Helsinki with the approval of the Shidong Hospital Research Ethics Committee, and informed written consent was obtained from all recruited patients.

2.2 | Histology

Clinical samples were fixed in 4% paraformaldehyde. Rabbit monoclonal antibodies against OGDHL-HIF-1α-vimentin-E-cadherin diluted at 1/200 was used to perform immunohistochemistry (IHC) staining. Dako Autostainer was implied to visualize antigen

distribution and expression on each slide. Two experienced pathologists independently analyzed all IHC staining. For quantification, blinded semi-quantitative scoring was implied and quantified as 0 (0%), 1 (1–5%), 2 (6–25%), 3 (26–75%) or 4 (76–100%) staining according to the percentage of positively stained cells. The intensity score of staining was classified as 0 to 2 (0, negative; 1, moderate; 2, strong staining). A score ranging from 0 to 8 was assigned by multiplying the staining extent by the staining intensity. The final score of ≤4 was defined as low expression and >4 as high expression.

2.3 | Mammalian cell lines and reagents

Hepatocellular carcinoma cell lines MHCC-97H, HCC-LM3, MHCC-97L, HepG2, Huh-7, PLC, SMMC-7721, Bel-7402, and Hep3B were obtained from the cell bank of the Chinese Academy of Sciences (Shanghai, China). Short tandem repeat (STR) profiling of HCC cells was verified at GenePharma (Shanghai, China). All cell lines were maintained under standard conditions in high glucose DMEM (Thermo Fisher) with 10% FBS. MG132 (SML1135), $CoCl_2$ (232696), DM-2OG (D3695), D-2-HG (H8378), and L-2-HG (90790) were obtained from Sigma.

2.4 | Stable transfectant

Short hairpin RNAs (shRNA) targeting the OGDHL (shOGDHL-A:5′-TTAGGTGTGA AGATAATCAGC-3′; shOGDHL-B:5′-ATCTC CTCCATGTAACTGGAG-3′), the DLD sequence (shDLD-A:5′-AT TCTGTTTGAATAAGTGGGC-3′; shDLD-B:5′-ATACCCTCTTCTTT CAACTGC-3′), and the DLST sequence (shDLST-A:5′-ATCACTGCA TTTACAACAGGC-3′; shDLST-B:5′-AATGGCAAGTTCATTCTTTCG-3′) were obtained by siRNA Ambion Target Finder software. A scrambled sequence was designed as a control. HEK293T was used for virus packaging after the co-transfection of pWPXL-OGDHL, pWPXL-HIF-1α, pWPXL-FIH-1 or pWPXL-L-2-HG dehydrogenase (L-2-HGDH) with packaging plasmid psPAX2 (Addgene) and envelope plasmid pMD2.G (Addgene) or the co-transfection of pGreenPuro-shRNA with envelope plasmid pVSV-G (System Biosciences) and packaging plasmid pPACK-REV, pPACK-GAG (System Biosciences) by Lipofectamine 3000 (Invitrogen). After 48 h of transfection, the lentivirus-containing supernatant was isolated, and infections were conducted by culturing cells in medium containing the lentiviral supernatant with 0.5 μg/mL of puromycin (Wisent) for 48 h. The efficiency of transfection was examined by western blot analysis.

2.5 | Plasmids and luciferase assays

The pGL3 luciferase vector (Promega) was used to prepare plasmids containing luciferase-reporters for the HIF-1α 5′UTR and the HIF-1α promoter. In brief, a 284-bp fragment of the HIF-1α 5′UTR (+1 to +284) and the fragment of HIF-1α promoter (−570 to +34) were generated by PCR and inserted between HindIII and NcoI sites and also between the BglII and HindIII sites of the pGL3 promoter vector, respectively (Figure S7A,B). Moreover, we cloned SV40p-ODD-Luc into the corresponding fragments of pEF-myc-cyto to construct pEF-SV40p-ODD-Luc plasmid.[16,17] Alternatively, the following plasmids were obtained by GenePharma (Shanghai, China): HIF-1αTAD-P564A-Gal4 DNA binding domain (Gal4 DBD) and HIF-1αTAD P564A and N803A-Gal4 DBD in the pcDNA3 vector. HCC cells (1×10^4 cells per well) with the indicated treatments were maintained overnight in a 24-well plate. One day after HCC cells were transfected with the indicated plasmids, they were mixed in 100 μL of lysis buffer (Promega). Then, the luciferase reporter activity was tested according to the manufacturer's instructions.

2.6 | RNA isolation and quantitative RT-PCR

RNAs were isolated from cells by TRIzol reagent (Invitrogen). qRT-PCR experiments were implied by a real-time PCR kit on a real-time PCR system. β-actin was picked as a control for normalizing mRNA. The comparative Ct method ($2^{-\Delta\Delta Ct}$) was implied to analyze the data, and primer sequences are provided in Supplemental Table 1.

2.7 | Immunoblotting

Cells were lysed in an SDS lysis buffer for 30 min before heating for 10 min at 100°C. Proteins were probed with a range of primary antibodies: anti-OGDHL (Proteintech), anti-HIF-1α, anti-DLD (Proteintech), anti-DLST (Abcam), anti-E-cadherin (Abcam); anti-vimentin (Proteintech); anti-hydroxy-HIF-1α (Pro564; Cell Signaling); and anti-PHD2 and anti-β-actin (Proteintech). Primary antibodies were examined through anti-mouse or anti-rabbit secondary antibodies (GE Healthcare Bioscience) and developed by the ECL-PLUS system (GE Healthcare Bioscience).

2.8 | Quantification of 2-OG and 2-HG

Hepatocellular carcinoma cells were maintained in a six-well plate (1×10^6 cells/well) in high glucose DMEM 24 h prior to harvesting. Intracellular metabolites were then extracted after adding extraction buffer (20% H_2O, 100 ng/mL HEPES, 50% methanol, 30% acetonitrile) and incubating the plates over cold methanol for 20 min. After the cells were moved to a thermomixer and shaken for 15 min at 350 *g*, the cellular supernatants were stored at −80°C and analyzed via a liquid chromatography-mass spectrometry (LC/MS; Thermo Scientific), as described previously.[18]

2.9 | Fluorometric 2-OG assays

Hepatocellular carcinoma cells were treated and incubated into a six-well plate (1×10^6 cells/well) for 24 h. Then, cell lysates were collected from 500 µL of NP-40 buffer. Then, the cells were deproteinized with a Deproteinizing Sample Preparation Kit and preformed to detect 2-OG concentrations using the 2-OG Colorimetric/Fluorometric Assay Kit (BioVision).

2.10 | Migration and invasiveness assays

For wound healing assays, HCC-LM3, MHCC-97H, SMMC-7721, or Bel-7402 cells were seeded in a six-well plate (5×10^5 cells/well). A scratch wound was then produced by scratching the surface of confluent cell layers with a 200-µL pipette tip. At 0 and 24 h post scratching, the distance of the scratches was tested with an inverted microscope (Olympus).

For transwell invasion assays, the upper chambers (8.0 µm, Corning Costar Corp, Corning, NY) were first pre-coated with 100 µL of 1 mg/mL of Matrigel (BD biosciences) for 2 h at 37°C. Next, after starvation in serum-free medium for 24 h, HCC-LM3, MHCC-97H, SMMC-7721 or Bel-7402 (5×10^4) were seeded to the upper chambers. The lower chamber contained culture medium with 10% FBS (500 µL). After cells were incubated for 24 h, the invaded cells were stained with 0.5% crystal violet and visualized using an inverted microscope (Olympus). The cell invasion index was calculated by normalizing the number of invasive cells to the number of seeded cells in the upper chamber.

2.11 | Animal experiments and ethics statement

This research was approved by the Animal Care and Use Committee of Shidong Hospital. Serum-free medium with HCC-LM3/EV cells (1×10^7) or HCC-LM3/OGDHL cells (1×10^7) was subcutaneously seeded into the upper flank region of BALB/C nu/nu mice. A few days later, 1.0 mm^3 of subcutaneous tumor tissue was inoculated into the livers of other mice, as described previously.[13] Male BALB/C nu/nu mice (4-week-old) were randomly allocated into groups (six mice per group) before inoculation. After 8 weeks, the mice were killed by cervical dislocation and the number of metastatic tumors was determined by double-blinded evaluation.

2.12 | Statistical analyses

Statistical analysis was performed with SPSS 17.0 software. Pearson's χ^2-test or Fisher's exact test were implied to compare qualitative variables. For quantitative variables, Student's *t*-test was analyzed in two groups while ANOVA was implied to compare more than three groups of samples. For survival analysis, *p*-values were determined by the two-sided log-rank test. A *p*-value <0.05 was regarded as statistically significant for all tests.

3 | RESULTS

3.1 | Low levels of oxoglutarate dehydrogenase-like protein expression were related to the metastasis and invasion of primary hepatocellular carcinoma

In our previous study, silencing of OGDHL in primary HCC tissues was closely related to aggressive clinicopathologic characteristics and was an independent factor for an adverse prognosis.[13] We prospectively investigated whether OGDHL plays an essential role in the migration and invasiveness of HCC cells. We investigated the relative expression levels of OGDHL from 24 patients with extrahepatic metastasis (cohort 2) and identified that levels of OGDHL were significantly reduced in tumors at metastatic sites (Figure 1A,B; $p < 0.001$). We further verified the inhibitory effect of OGDHL in HCC metastasis by implying a TMA (cohort 3) featuring a total of 56 cores with 10 normal liver tissues, 9 chronic liver cirrhosis tissues, 5 liver hemangioma specimens, 21 HCC tissues and 11 tissue cores from metastatic HCC (IHC scores: Normal livers>Primary HCCs, $p < 0.01$; Primary HCCs>Metastatic HCCs, $p < 0.001$; Figure 1C–E). Surprisingly, as a kind of benign tumor, OGDHL levels in hemangioma were as low as those in metastatic HCCs.

3.2 | The aberrant expression of oxoglutarate dehydrogenase-like reduced the migration and invasiveness in hepatocellular carcinoma cells

Next, the levels of OGDHL in different HCC cells were tested. OGDHL expression was significantly attenuated in MHCC-97H and HCC-LM3 compared to Bel-7402 and SMCC-7721 cells (Figure 2A). To further study the functional effects of OGDHL in the development of HCC cells, we generated OGDHL overexpression constructs in HCC-LM3 and MHCC-97H cells, as well as shRNA-OGDHL plasmids (sh-A, sh-B) in Bel-7402 and SMMC-7721 cell lines (Figure 2B and Figure S7C). Compared with the control cells, the exogenous overexpression of OGDHL in HCC-LM3 cells led to an obvious reduction in both cell migration (Figure 2C–E) and invasiveness (Figure 2F–H), whereas the endogenous knockdown of OGDHL (OGDHL-shA and OGDHL-shB) markedly stimulated the invasion and migration of Bel-7402 cells in vitro (Figure S1). Moreover, overexpression of OGDHL suppressed cell migration and invasiveness in MHCC-97H cells (Figure S8A,B,D), whereas knockdown of OGDHL induced cell migration and invasiveness in SMMC-7721 cells (Figure S8A,C,E). Taken together, these results suggested that downregulation of OGDHL had pro-metastatic functions in hepatoma cells.

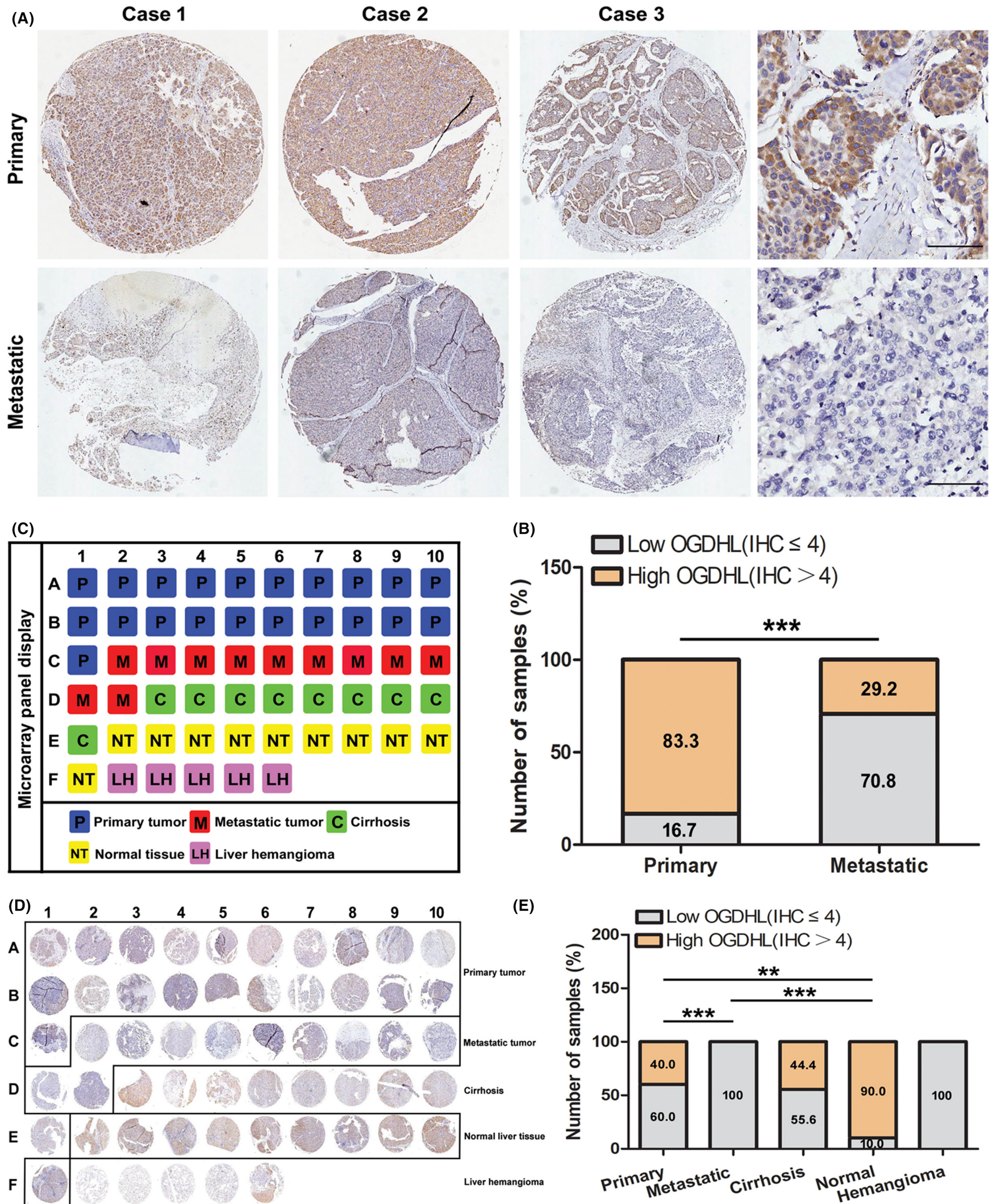

FIGURE 1 The depletion of oxoglutarate dehydrogenase-like (OGDHL) in hepatocellular carcinoma (HCC) is related to tumor metastasis. (A and B) Comparison of OGDHL expression in paired primary-metastatic HCCs from 24 HCC cases with extrahepatic metastasis ($p < 0.001$, Scale bar = 50 μm). (C and D) immunohistochemistry (IHC) for OGDHL on a tissue microarray analysis (TMA) slide containing 10 normal liver tissues, 21 primary HCCs, and 11 metastatic HCCs. (E) Quantification of IHC scores. ** indicated $p < 0.01$; *** indicated $p < 0.001$

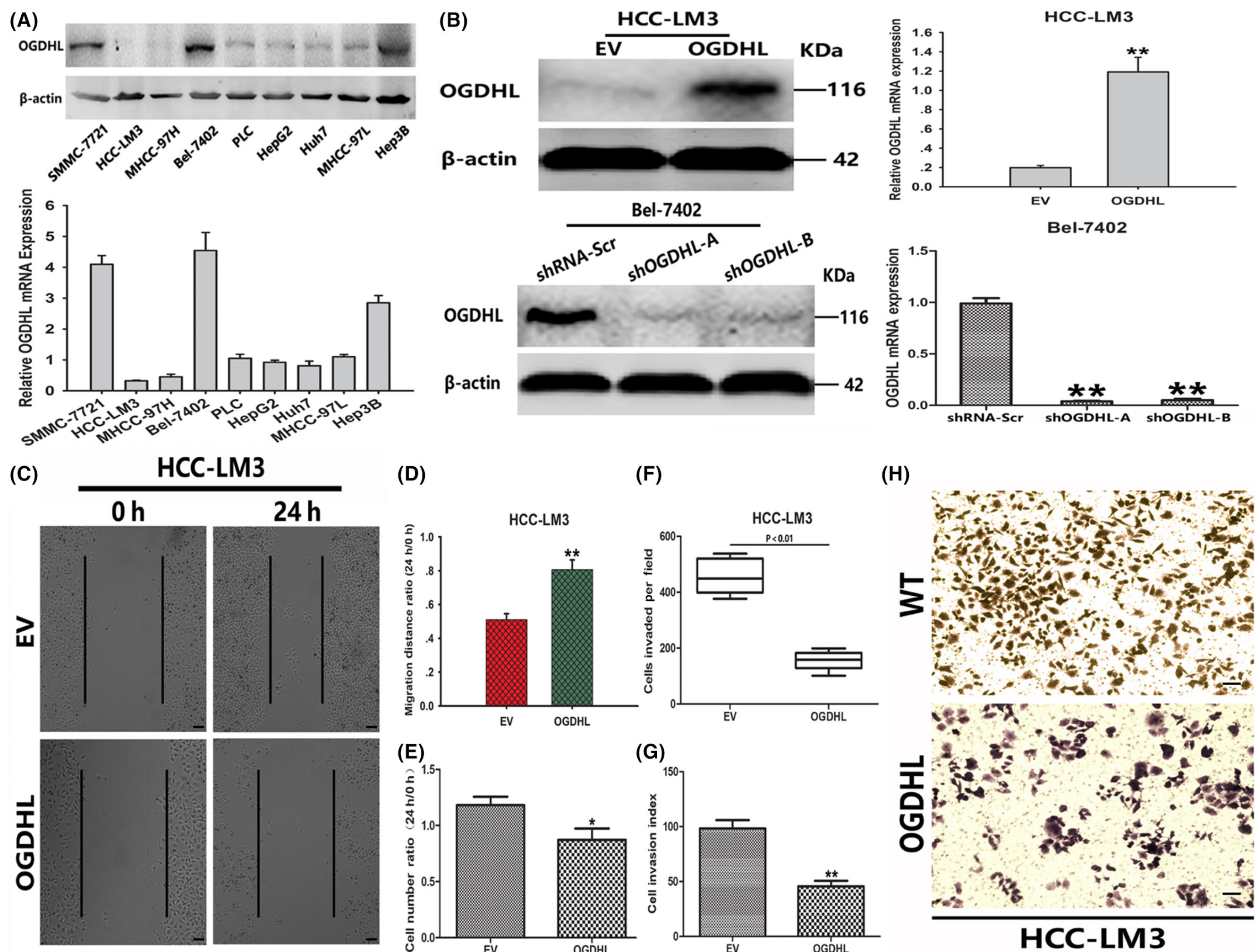

FIGURE 2 The upregulation of oxoglutarate dehydrogenase-like (OGDHL) inhibited the invasiveness and cell migration in hepatocellular carcinoma (HCC) cells. (A) The protein expressions of OGDHL in various HCC cells. (B) Overexpression of OGDHL mRNA and protein in HCC-LM3 cells (upper figure) and the knockdown of OGDHL mRNA and protein expression in Bel-7402 cells by shOGDHL-A and shOGDHL-B. Scr, scrambled shRNA (lower figure). (C) Wound-healing assays of HCC-LM3 cells were performed three times and representative figures are shown (50×). Scale bar, 50 μm. (D and E) Migration distance ratio (D) and cell number ratio (E) in wound-healing assays are displayed. (F-H) The number of invasive cells (F) and the cell invasion index (G) were tested in transwell assays. Transwell assays were carried out twice in triplicate wells (H). Representative images are shown (100×). Scale bar, 50 μm (means ± SD, *$p < 0.05$; **$p < 0.01$ *vs* control group)

3.3 | The depletion of oxoglutarate dehydrogenase-like led to the accumulation of HIF-1α under aerobic conditions

A previous report showed that shOGDHL groups exhibited a significant inhibition of OGDHC activity[13] and found that the disruption of any OGDHC component stabilized HIF-1α in aerobic conditions.[18] Therefore, we first investigated whether OGDHL might influence HIF-1α expression in HCC cells.

The silencing of endogenous OGDHL in Bel-7402 cells, which had no effect on the mRNA expression of other subunits of OGDHC, such as OGDH (E1), DLST (E2), and DLD (E3) (Figure S2A), contributed to the significant upregulation of HIF-1α expression (Figure 3A). Meanwhile, the induction of HIF-1α protein expression in HCC-LM3 cells was significantly attenuated when cells were treated by the lentivirus-mediated rescue of OGDHL expression (Figure 3B,C). We also identified that the modulatory roles of OGDHL on HIF-1α protein expression were evident not only in Bel-7402 and HCC-LM3 cells but also in SMMC-7721 and MHCC-97H cells (Figure S2B,C), thus suggesting that OGDHL regulated HIF-1α protein expression via a common mechanism in HCC cells. Next, to identify whether the silencing of OGDHL was related to a HIF-1 transcriptional response, we found that the forced knockdown or overexpression of OGDHL led to the obvious upregulation and reduction of the mRNA expression levels of different HIF-1 target genes, respectively (Figure 3D–I and Figure S3). Moreover, consistent with previous reports,[18] the silencing of either the DLST (E2) or DLD (E3) subunits of OGDHC increased the expression levels of endogenous HIF-1α, as with OGDHL depletion (Figure S2D). These data showed that OGDHL

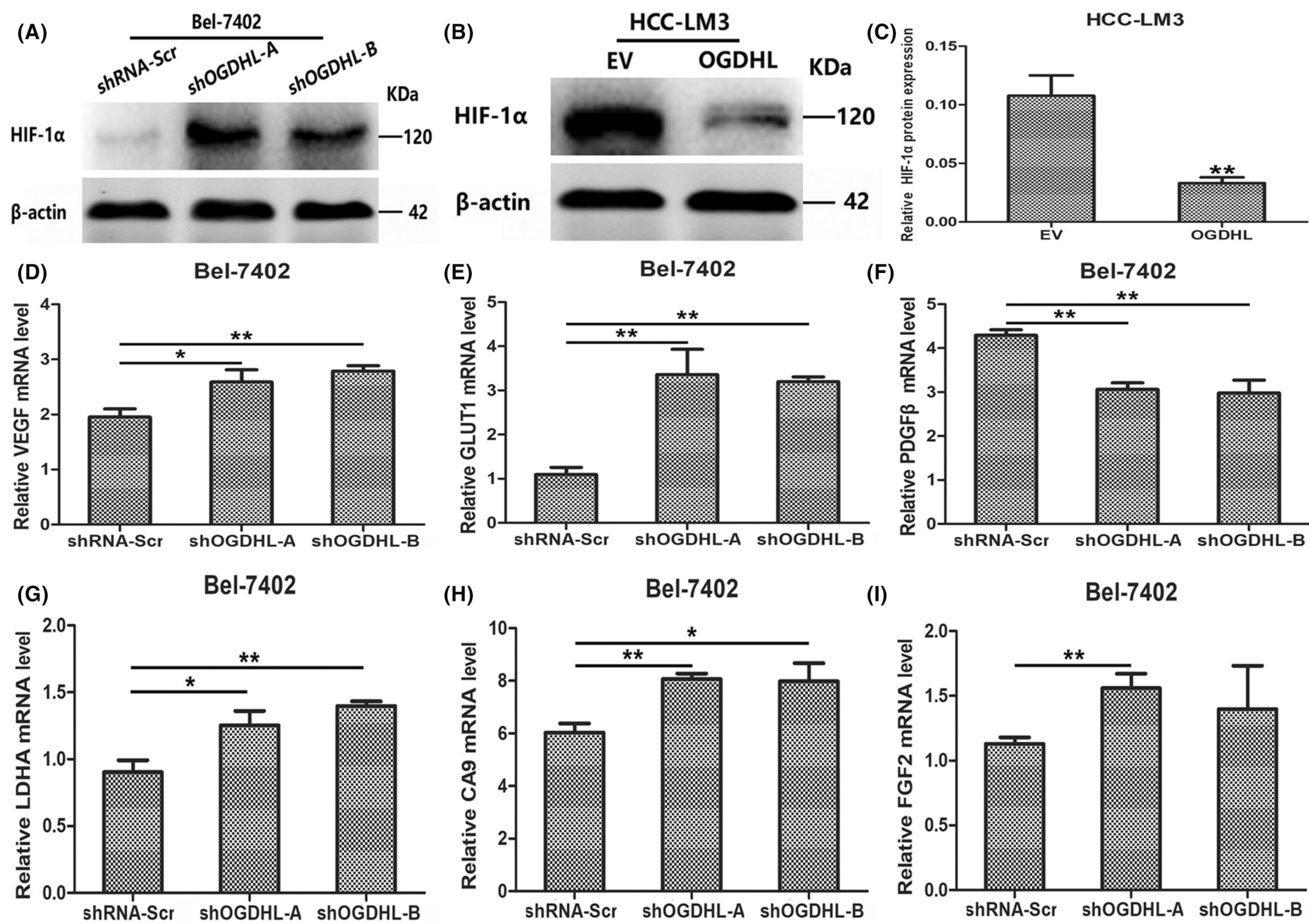

FIGURE 3 The depletion of oxoglutarate dehydrogenase-like (OGDHL) led to the accumulation of hypoxia-inducible factor 1 (HIF)-1α under aerobic exposure. (A–C) OGDHL-shRNA in Bel-7402 (A) and Lenti-OGDHL in HCC-LM3 (B and C). HIF-1α levels were tested by immunoblotting. β-Actin was implied as a normalized control. (D–I) Bel-7402 cells were transfected with shRNA-Scr, shOGDHL-A, or shOGDHL-B, and subjected to quantitative RT-PCR to quantify the mRNA expression of representative HIF-1 downstream genes, vascular endothelial growth factor (VEGF; D), glucose transporter 1 (GLUT1; E), platelet-derived growth factor β (PDGF-β; F), lactate dehydrogenase A (LDHA; G), carbonic anhydrase 9 (CA9; H), and fibroblast growth factor 2 (FGF2; I). All experiments (except for A) were repeated in triplicate (means ± SD, *$p < 0.05$; **$p < 0.01$)

functions, at least in part, as a component of OGDHC to upregulate the levels of HIF-1α.

3.4 | The downregulation of oxoglutarate dehydrogenase-like promoted epithelial–mesenchymal transition response in a hypoxia-inducible factor 1α-dependent manner

HIF-1α is thought to trigger EMT programming, which contributes to tumor metastasis and invasiveness. Next, we tried to investigate the mechanisms responsible for how OGDHL can regulate the migration of HCC cells. First, we found that OGDHL could influence markers for EMT in HCC cells. The level of E-cadherin was reduced in shRNA-OGDHL cells and increased in OGDHL-overexpressing cells; this was reversely related to the changes of vimentin expression (Figure 4A,B). Notably, the exogenous expression of HIF-1α attenuated the roles of OGDHL overexpression in HCC-LM3 cells (Figure 4C). Next, we further investigated whether OGDHL might cause the direct alteration of cell morphology and behavior. shRNA-OGDHL clones in Bel-7402 and SMMC-7721 cells appeared to have more of a mesenchymal spindle-like morphology than the epithelial-like morphology that was characteristic of the control Bel-7402-Scr and SMMC-7721-Scr cells (Figure 4D and Figure S8F). Otherwise, the morphology of the stable OGDHL-overexpressing cells changed significantly into the epithelial type from the original HCC-LM3 and MHCC-97H mesenchymal-like type (Figure 4E and Figure S8F). To confirm the effect of OGDHL in HIF-1α expression and EMT, we implied a cohort of 225 HCC cases (cohort 1) from the NCBI-GEO under accession number GSE14520. *OGDHL* and *CDH1* exhibited a strong and positive correlation. Furthermore, we detected an inverse correlation between *CDH1* and *HIF1A* expression and positive correlations between *HIF1A* and both *TWIST1* and *VIM* expression levels (Figure S4A). In addition, the IHC detection of HIF-1α in 14 high-OGDHL HCC specimens and 10 low-OGDHL HCC specimens

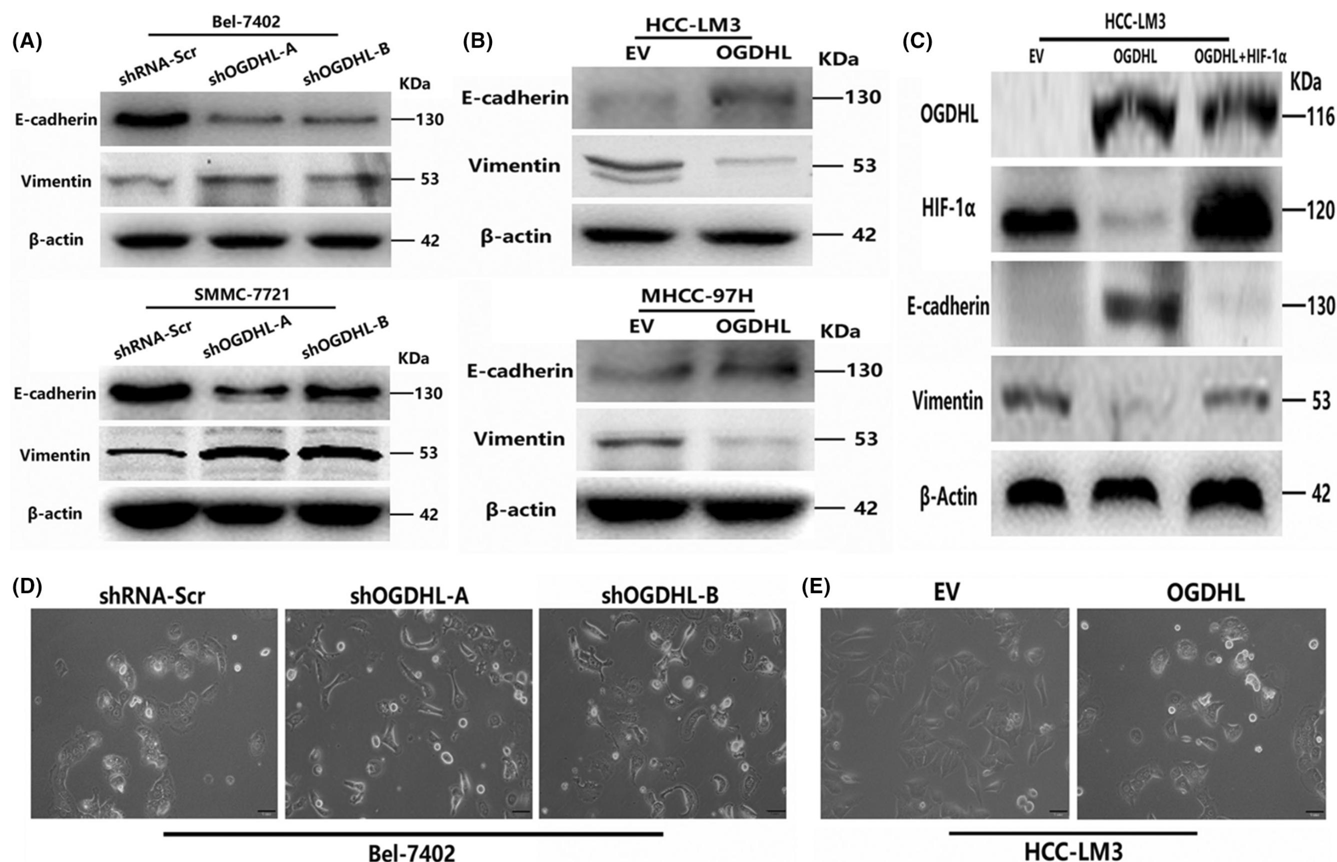

FIGURE 4 The knockdown of oxoglutarate dehydrogenase-like (OGDHL) expression increased hypoxia-inducible factor 1 (HIF)-1α protein expression and promoted the epithelial–mesenchymal transition (EMT) process. (A) OGDHL downregulation increased vimentin levels and reduced E-cadherin levels. (B) The overexpression of OGDHL in MHCC-97H and HCC-LM3 cells reduced vimentin levels and increased E-cadherin. (C) HIF-1α counteracted OGDHL overexpression and E-cadherin as well as vimentin were tested by immunoblotting. (D and E). Representative morphological changes were visualized using a phase-contrast microscope (400×) in Bel-7402 (D) and HCC-LM3 cells (E), respectively. Scale bar, 20 μm

from cohort 2 suggested that lower OGDHL protein levels in HCCs were associated with higher HIF-1α protein expressions (Figure S4B,C). Overall, these data suggested that OGDHL could regulate the cellular EMT process in HCC and that HIF-1α played a key role in this conversion.

3.5 | The silencing of oxoglutarate dehydrogenase-like enhanced the stability and transactivating function of hypoxia-inducible factor 1α

To investigate how the downregulation of OGDHL increased the expression of HIF-1α protein, we performed luciferase assays with different types of reporter genes.

First, we tested whether OGDHL affected HIF-1α mRNA expression. Interestingly, through qRT-PCR and a luciferase reporter assay, OGDHL did not affect the mRNA expression (Figure 5A) or the promoter activity of HIF-1α (Figure 5B; Figure S5A,B).

Then we generated the SV40p-ODD-Luc reporter, in which an ODD domain of HIF-1α was fused into a plasmid containing the luciferase reporter gene and the SV40 promoter (Figure S5C). As the stabilization of the fusion protein was modulated by the same mechanism as the HIF-1α protein, the stability of HIF-1α was able to be tested indirectly by luciferase activity. The results suggested that the knockdown and overexpression of OGDHL markedly up-regulated and decreased the activity of the reporter, respectively (Figure 5C; Figure S5D). In addition, the overexpression of OGDHL significantly reduced the half-life time of luciferase activity after oxygen deprivation (Figure 5D). The data revealed that the stabilization of the HIF-1α was affected by OGDHL. Alternatively, the upregulation of OGDHL contributed to the reduced stability of the HIF-1α following oxygen deprivation (Figure 5E).

Next, we explored the function of OGDHL in the translation initiation activity of HIF-1α. We used an HIF-1α-5′UTR-luc construct, in which an internal ribosome entry site of the HIF-1α 5′UTR region enhanced the translation initiation of the luciferase reporter[19] and found that OGDHL had no function on the translation initiation of HIF-1α (Figure 5F; Figure S5E,F).

We further examined the role of OGDHL on the transactivating activity of the HIF-1α protein. We generated a construct containing

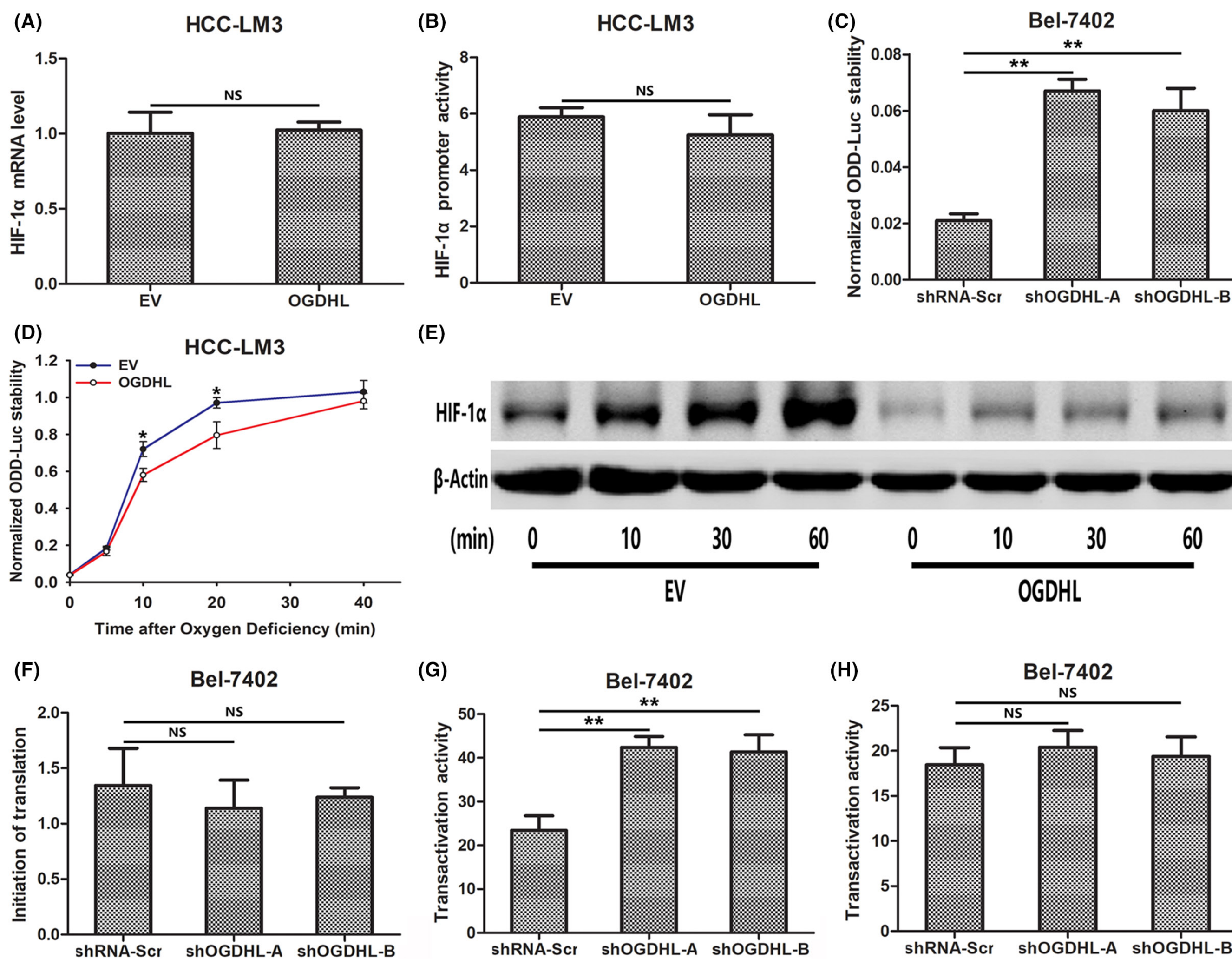

FIGURE 5 The depletion of oxoglutarate dehydrogenase-like (OGDHL) enhanced the stabilization and transactivating activity of hypoxia-inducible factor 1 (HIF)-1α in aerobic conditions. (A and B) HCC-LM3 (A) and HCC-LM3/pHIF-1α promoter-Luc cells (B) were transfected with Lenti-OGDHL or empty vector (EV) and subjected to qRT-PCR for HIF-1α mRNA expression (A) and luciferase analysis with pRL-SV40 as a normalized control to compare the relative HIF-1α promoter activity (B). (C) Bel-7402/ODD-Luc cells were infected with shRNA-Scr, shOGDHL-A or shOGDHL-B, and subjected to luciferase assays. (D–E) HCC-LM3/ODD-Luc (D) and HCC-LM3 (E) cells were infected with Lenti-OGDHL or EV and cultured under aerobic conditions. After oxygen deficiency, cells were collected for luciferase analysis (D) and immunoblotting assay (E). (F) Bel-7402 cells were transiently induced with pGL3/HIF-1α 5′UTR-Luc and subjected to luciferase analysis. (G and H) Bel-7402 cells were co-transfected with four different types of plasmids: either shRNA-Scr or OGDHL-shRNA (shOGDHL-A/-B), pG5H1bLuc, pRL-SV40, and either pcDNA3/HIF-1αTAD P564A-Gal4 DBD (G) or pcDNA3/HIF-1αTAD P564A with N803A-Gal4 DBD (H). Cells were incubated in aerobic conditions and subjected to the luciferase reporter assay. All data (except for E) are shown as means ± SD. $n = 3$. *$p < 0.05$. **$p < 0.01$. NS, not significant

the Gal4 DBD and HIF-1αTAD.[9] The transactivating activity of TAD was analyzed by substituting a proline residue for alanine (HIF-1αTAD P564A-Gal4DBD), which plays a critical function in the ODD domain of HIF-1αTAD- Gal4DBD (Figure S5G). The transactivating activity of TAD P564A was markedly enhanced through the knockdown of OGDHL (Figure 5G). Meanwhile, when a specific asparaginyl (N803), which plays a critical role in the FIH-1-regulated transactivating activity,[9] was changed to alanine (HIF-1αTAD P564A with N803A-Gal4 DBD), the activation effects of OGDHL knockdown were completely abrogated (Figure 5H; Figure S5H). Overall, these data demonstrated that the knockdown of OGDHL expression enhanced both the stability and transactivating activity of HIF-1α, leading to the accumulation of HIF-1.

3.6 | The depletion of oxoglutarate dehydrogenase-like suppressed the prolyl hydroxylation of hypoxia-inducible factor-1α by promoting the formation of L-2-HG

As the transactivating activity and stabilization of HIF-1α were modulated via PHDs and FIH-1, respectively,[20] we next considered

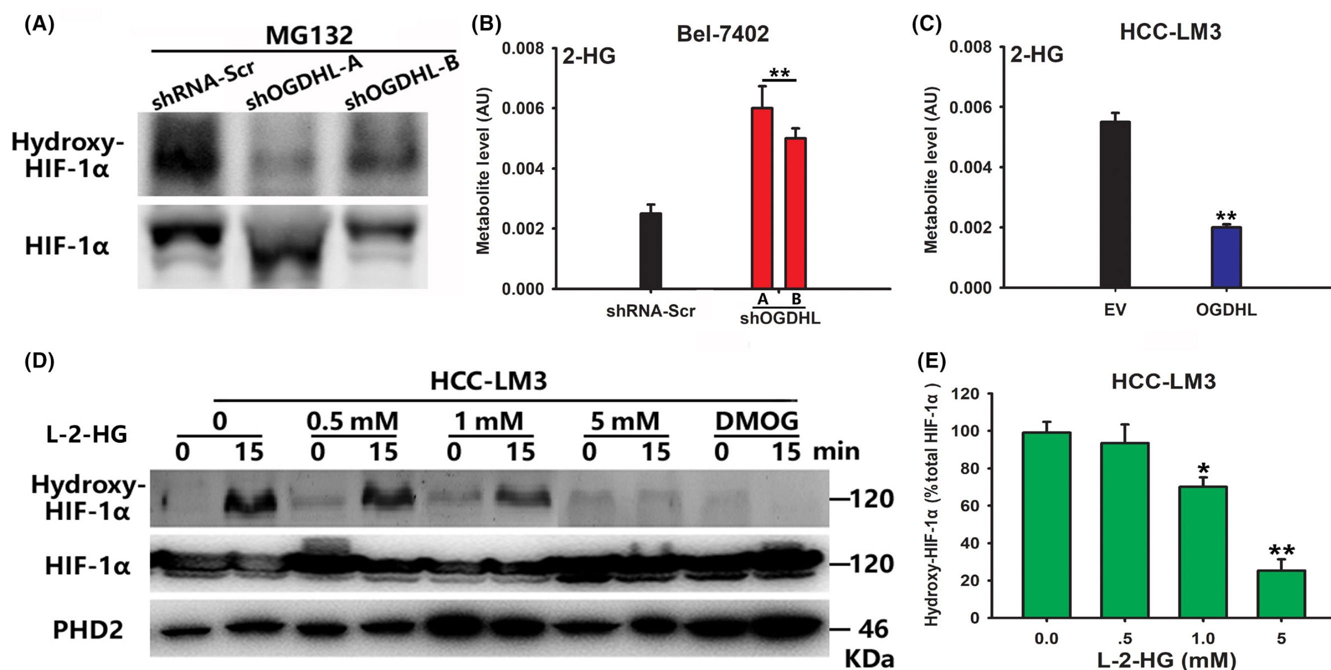

FIGURE 6 Oxoglutarate dehydrogenase-like (OGDHL)-mediated increase in 2-HG concentrations and hydroxylation of hypoxia-inducible factor 1 (HIF)-1α. (A) Bel-7402 cells were infected with shRNA-Scr, shOGDHL-A, or shOGDHL-B treated with 30 μM MG132 in aerobic conditions for 6 h and subjected to immunoblotting assays for hydroxyl-HIF-1α and HIF-1α to quantitatively analyze the hydroxylation of HIF-1α. (B and C) Bel-7402 (B) and HCC-LM3 (C) cells were infected with shRNA-Scr, shOGDHL-A, or shOGDHL-B (B) or treated with Lenti-OGDHL or EV (C) under aerobic conditions and subjected to LC/MS-based analysis to detect the intracellular abundance of 2-HG. (D and E) Recombinant hydroxyl-HIF-1α (P564) peptide was incubated with HCC-LM3 cell extracts with or without the incubation of L-2-HG (0.5–5 mM) for 0 or 15 min in aerobic conditions at 37°C and subjected to western blotting to detect the hydroxyl-HIF-1α (Pro564) (D) and compared to the total expression levels of HIF-1α protein (E). Values represent means ± SD. $n = 3$. $^*p < 0.05$. $^{**}p < 0.01$

whether the extent of HIF-1α hydroxylation could be affected by OGDHL in HCC cells. Notably, the hydroxylation of HIF-1α (P564), which was sensitive to the PHD antagonist $CoCl_2$ (Figure S6A), was significantly attenuated by the silencing of OGDHL (Figure 6A). Next, we investigated the underlying impact mechanisms of how the downregulation of OGDHL inhibited the hydroxylation of HIF-1α protein. Previous research showed that small TCA intermediates can reduce PHD activity under aerobic conditions.[14] Alternatively, Stephen et al demonstrated that L-2-HG, which has the potential to inhibit the levels of HIF-1α hydroxylation,[15] was significantly accumulated by a disruption in OGDHC activity.[18] Consistent with this opinion, we found that the concentrations of 2-HG were markedly reduced in both OGDHL-shA and OGDHL-shB clones when compared to the Bel-7402 control group (Figure 6B). In contrast, stable OGDHL-overexpressing cells exhibited reduced levels of 2-HG when compared to the empty vector (EV) control in HCC-LM3 cells (Figure 6C). Moreover, we found that the extent of HIF-1α hydroxylation was suppressed by L-2-HG, although there was no influence on the hydroxylation of HIF-1α protein following the addition of D-2-HG (Figure 6D,E; Figure S6B,C). The data suggested that the depletion of OGDHL in HCC cells increased the levels of intracellular L-2-HG to prevent the prolyl hydroxylation of HIF-1α and eventually enhanced its stabilization and transactivating activity.

3.7 | The downregulation of L-2-HG reduced hypoxia-inducible factor-1α stability following the accumulation of 2-OG in hepatocellular carcinoma cells

L-2-HG is a chiral compound derived from 2-OG (Figure 7A). Therefore, we investigated the role of 2-OG in the process of stabilizing HIF-1α in HCC cells. By using fluorometric assays, we demonstrated that the forced upregulation of OGDHL, and the depletion of endogenous OGDHL, led to a marked reduction and increase in 2-OG concentrations, respectively (Figure 7B,C). Consistent with these results, LC/MS-based data further showed that the depletion of OGDHL markedly increased the levels of 2-OG (Figure 7D). In the following experiments, we attempted to investigate whether exogenous 2-OG, a cell-permeable 2-OG analog (dimethyl-2OG; DM-2OG), was sufficient to stabilize HIF-1α by upregulating its stabilization and transactivating activity. We found that DM-2OG treatment markedly upregulated 2-HG levels when compared to the

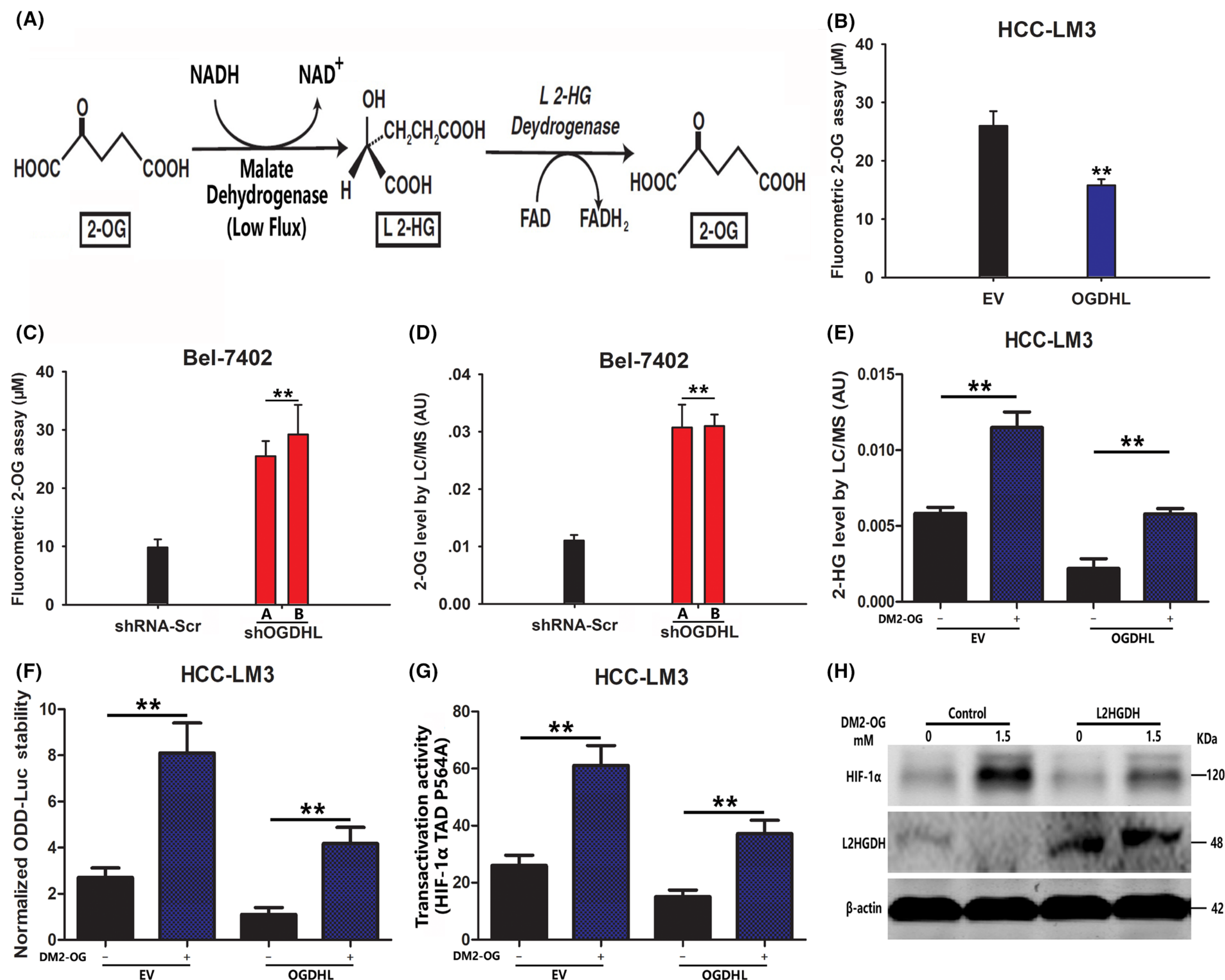

FIGURE 7 Decreasing L-2-HG reduced hypoxia-inducible factor 1 (HIF)-1α stability following the accumulation of 2-OG. (A) Schematic diagram of the formation of L-2-HG from 2-OG. (B and C) HCC-LM3 (B) and Bel-7402 (C) cells were infected with Lenti-OGDHL or empty vector (EV) (B), or treated with shRNA-Scr, shOGDHL-A, or shOGDHL-B (C) under aerobic conditions and subjected to the fluorometric 2-OG assay. (D) 2-OG levels in Bel-7402-Scr and Bel-7402-shOGDHL cells were tested using the LC/MS-based analysis. (E) EV and Lenti-OGDHL in HCC cells were incubated with or without DM-2OG (0.5 mM) in aerobic conditions for 24 h and then subjected to LC/MS-based analysis to detect the intracellular abundance of 2-HG. (F) HCC-LM3/ODD-Luc cells were treated with Lenti-oxoglutarate dehydrogenase-like (OGDHL) or EV, cultured with or without DM-2OG (0.5 mM) in aerobic conditions for 24 h, and then subjected to the luciferase assay. (G) HCC-LM3 cells were co-transfected with the following plasmids: either Lenti-OGDHL or its EV, pcDNA3/HIF-1αTAD P564A-Gal4DBD, pG5H1bLuc. Cells were incubated with or without DM-2OG (0.5 mM) in aerobic conditions for 24 h and then analyzed by luciferase assays. pRL-SV40 was implied as an internal control. (H) Bel-7402 cells overexpressing L-2HDGH were cultured with DM-2OG for 24 h. HIF-1α levels were examined by immunoblotting. All values represent means ± SD. $n = 3$. $^{**}p < 0.01$

control groups (Figure 7E) and consistently increased the transactivating activity and stabilization of HIF-1α when compared to the wide-type and cells overexpressing OGDHL in the ODD-Luc assay and TAD assay, respectively (Figure 7F,G). Consistent with this notion, we upregulated L-2-HGDH in cells treated with or without DM-2OG and confirmed that cells overexpressing L-2-HGDH abrogated the DM-2OG-mediated induction of HIF-1α protein expression (Figure 7H). Collectively, these data revealed that the upregulation of 2-OG under aerobic exposure is sufficient to induce the accumulation of L-2-HG and stabilize HIF-1α by enhancing its stability and transactivating activity.

3.8 | Aberrant oxoglutarate dehydrogenase-like expression inhibited hepatocellular carcinoma metastasis in nude mice

In our previous study, we demonstrated the positive effects of OGDHL on tumor growth.[13] In the present study, we investigated whether aberrant OGDHL expression could inhibit HCC metastasis in nude mice. To further study the impact of OGDHL in HCC metastasis, we constructed stable transfectants of HCC-LM3 cells with OGDHL-expressing lentivirus (HCC-LM3/OGDHL) or its empty vector (HCC-LM3/EV). Next, orthotopic xenograft

models were established by the injection of these cells into the liver parenchyma of nude mice. After 8 weeks, most mice in the EV group (5/6) developed lung metastatic nodules (6.2 ± 1.43 per mouse), while significantly fewer mice were found to have metastases in the OGDHL overexpression group (0.2 ± 0.05, $p = 0.004$; Figure 8A,B). The overexpression of OGDHL also markedly suppressed the growth of HCC-LM3/OGDHL xenograft tumors in immunodeficient mice ($p = 0.014$; Figure 8C,D); these findings were consistent with our previous report.[13] To account for the differences in growth of primary tumors arising from EV and OGDHL groups, we assessed the metastatic index, which normalizes the number of lung nodules to the weight of subcutaneous tumors. The metastatic index was much lower in the OGDHL overexpression group compared to the EV group (Figure 8E). To emphasize the importance of HIF-1α, PHD inhibitor DM2-OG promoted HCC metastasis process in OGDHL-overexpressing

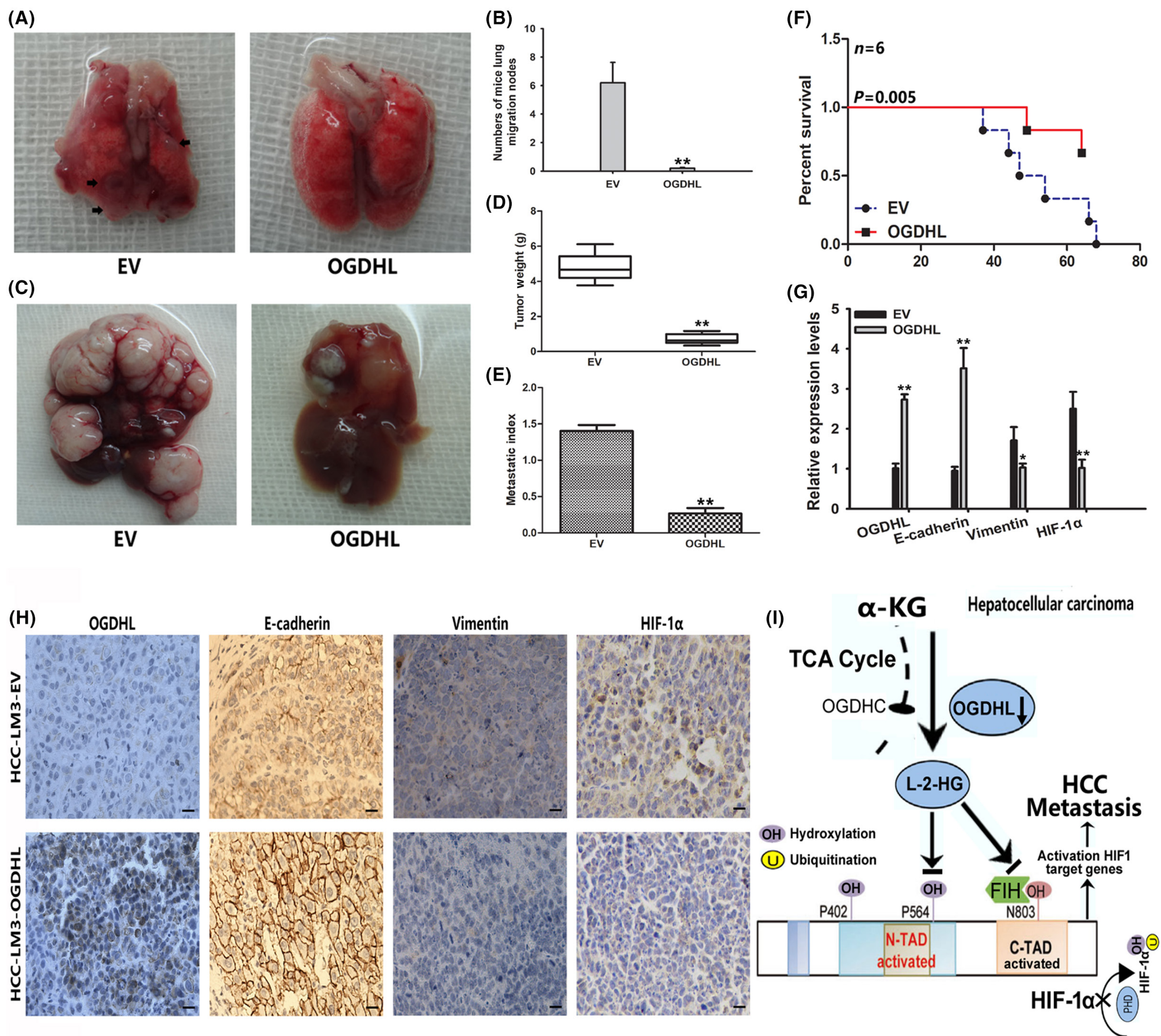

FIGURE 8 Aberrant oxoglutarate dehydrogenase-like (OGDHL) expression inhibited hepatocellular carcinoma (HCC) metastasis in mice. (A–C) After 64 days post-inoculation, representative images were taken of mice lung (A) and liver (C) from the EV and Lenti-OGDHL group; arrows point to metastases in lung nodes (A). The number of metastatic nodules per lung was tested manually (B). (D–F) Comparison of tumor weight (D), metastatic index (E), and survival (F) between mice inoculated with HCC-LM3-EV and HCC-LM3-OGDHL. Values represent means ± SD. $^{**}p < 0.01$. For survival analysis, the p-value was determined by the two-sided log-rank test. (G and H) Relative densitometry analysis and representative pictures of immunohistochemistry (IHC) assay for (IHChypoxia-inducible factor 1 (HIF)-1α, E-cadherin, OGDHL, and vimentin in HCC mice inoculated with HCC-LM3-EV or HCC-LM3-OGDHL. $^{*}p < 0.05$. $^{**}p < 0.01$. Scale bars, 20 μm. (I) A schematic diagram showing the proposed OGDHL-silencing HCC metastasis promotion mechanism

cells in mice (Figure S9C–F) by transient stabilization of HIF-1α (Figure S9A,B). In addition, mice in the OGDHL overexpression group (67%) survived to d54, much higher than the mice in the EV group ($p = 0.005$; Figure 8F). Compared to the EV group, we detected a reduction in HIF-1α and vimentin expression but an increase in E-cadherin expression in tumors from the OGDHL overexpression group (Figure 8G,H), thus showing that the inhibition of EMT by aberrant OGDHL expression played a critical role in modulating metastasis in HCC. Taken together, these data suggested that the OGDHL-regulated suppression of HIF-1α expression reduced the metastasis of HCC by inhibition of EMT in tumor cells and, therefore, can be implied as a potential target for cancer therapy.

4 | DISCUSSION

Until now, the molecular mechanisms underlying the invasiveness and metastasis of HCC were unclear. However, some genes have been found to be involved in HCC metastasis. Many reports have revealed that EMT plays an important role in the invasiveness and metastasis of cancer. However, clinical and basic studies suggest that EMT-related biomarkers are urgently needed to identify metastasis in HCC to improve the treatment and prognosis of HCC. In this study, we determined that aberrant OGDHL expression significantly suppressed the migration of HCC cells in vitro and lung metastasis in mice. Clinically, the low expression levels of OGDHL were significantly associated with tumor metastasis and invasiveness in two independent cohorts (cohort 2 and 3). Moreover, HIF-1α stabilization and activation of a hypoxia gene expression pattern, functioning as EMT programming,[21] was shown to be correlated with a reduction in OGDHL levels. Mechanistically, we revealed that OGDHL was an upstream modulator of HIF-1α. We identified that the silencing of OGDHL increased the transactivating activity and stabilization of HIF-1α by promoting the formation of L-2-HG as a component of OGDHC; this process subsequently induced an EMT response in HCC cells. Taken together, for the first time, we indicated OGDHL as an important effector that modulates the metastasis of HCC, which provides another opportunity for the diagnosis and treatment of HCC (Figure 8I).

Oxoglutarate dehydrogenase-like are enzymes in the TCA cycle that were originally shown to convert 2-OG to succinate.[22] OGDH, the E1 component of OGDHC, has recently received considerable attention as it stabilizes HIF-1α by upregulating L-2-HG, the so-called oncometabolite, from 2-OG.[18] Moreover, the role of the OGDH isoform, OGDHL, which is significantly downregulated in primary HCC tissues,[13] has yet to be investigated in EMT and the progression of metastasis in HCC. Following such a background, in our present study, we determined that wild-type OGDHL was a novel EMT-modulating enzyme.

The role of OGDHL in HIF-1α prolyl hydroxylation remains unsolved. First, a recent report suggested that OGDHC is critical to the modulation of HIF-1α prolyl hydroxylation in aerobic conditions.[18] These biological functions are multifaceted as depletion of all the OGDHC components stabilizes HIF-1α resulting from 2-OG accumulation and subsequently the formation of L-2-HG. However, this was unexpected, as the intracellular upregulation of 2-OG was unlikely to reduce PHD activity; furthermore, previous studies revealed that 2-OG can overcome the succinate-regulated suppression of HIF-1α prolyl hydroxylation.[23] Instead, in the progression of HCC metastasis, we confirmed that HIF-1α prolyl hydroxylation is reduced when 2-OG accumulates by disrupting OGDHL expression.

The relative role of 2-HG in modulating PHDs remains controversial, although it has been identified as an important metabolic regulator in tumors.[24] For example, Xu et al. suggested that without a concurrent reduction in 2-OG levels, 2-HG is unlikely to suppress PHD activity.[25] In contrast, we have determined that L-2-HG is not only able to reduce PHD activity but is also converted from increasing 2-OG under aerobic conditions in HCC cells. Our results are consistent with other reports in both hypoxia[26,27] and aerobic conditions.[18] Overall, our study suggests a more general function for 2-OG metabolism in modulating HCC HIF-1α hydroxylation.

In addition, further evidence for a feedback loop is provided by the role of HIF-1α in triggering the proteasome-regulated disruption of OGDH2, a short OGDH isoform, and the reduction of OGDHC activity.[28] Although we found silencing of OGDHL in the HIF-1α upregulated group (Figure 4C), we observed no obvious variations in 2-OG levels or OGDHC activity in the HIF-1α upregulated group (data not shown). In future studies, it will be of interest to investigate the relative function of the potential feedback mechanism in HCC cells. Our data revealed that the basic levels of OGDHL were low in various HCC cell lines. Clinically, OGDHL silencing in HCC is more apparent at sites of metastasis than at the primary site. Furthermore, the cellular transition of epithelial to mesenchymal phenotypes is regulated by OGDHL during cancer metastasis. Collectively, these data suggest that OGDHL might serve as a biomarker for HCC metastasis and recurrence. A clinical intervention targeting OGDHL-regulated EMT might provide more effective strategies for preventing and treating metastatic cancers.

AUTHOR CONTRIBUTIONS

Xuanfu Xu, Chuanyong Guo, and Wenhui Mo designed the experiments. Weiqi Dai, Yueyue Li, Weijie Sun, Renjun Bao, Shuqi Xu, Jianqing Chen, Ying Dai, Meng Ji, and Yiming Chen performed the experiments. Chao Ge, Wenjing Liu, and Wei Sun analyzed the data, Weiqi Dai wrote the manuscript. Xuanfu Xu and Chuanyong Guo supervised the study.

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DISCLOSURE

The authors declare no conflict of interests.

ETHICS STATEMENT

Approval of the research protocol by an Institutional Reviewer Board: For the HCC specimen study, this protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the Shidong Hospital Research Ethics Committee.

Informed Consent: Written informed consent was obtained from each patient.

Registry and the Registration No. of the study/trial: N/A.

Animal Studies: The animal research in our study was approved by the Animal Care and Use Committee of University of Shanghai for Science and Technology Affiliated Shidong Hospital.

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SUPPORTING INFORMATION

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