BPIFB1 inhibits vasculogenic mimicry via downregulation of GLUT1-mediated H3K27 acetylation in nasopharyngeal carcinoma

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Nasopharyngeal carcinoma (NPC) demonstrates significant regional differences and a high incidence in Southeast Asia and Southern China. Bactericidal/permeability-increasing-fold-containing family B member 1 (BPIFB1) is a relatively specific and highly expressed protein in the nasopharyngeal epithelium. BPIFB1 expression is substantially downregulated in NPC and is significantly associated with poor prognosis in patients with NPC. However, the specific molecular mechanism by which BPIFB1 regulates NPC is not well understood. In this study, we found that BPIFB1 inhibits vasculogenic mimicry by regulating the metabolic reprogramming of NPC. BPIFB1 decreases GLUT1 transcription by downregulating the JNK/AP1 signaling pathway. Altered glycolysis reduces the acetylation level of histone and decreases the expression of vasculogenic mimicry-related genes, VEGFA, VE-cadherin, and MMP2, ultimately leading to the inhibition of vasculogenic mimicry. To our knowledge, this is the first report on the role and specific mechanism of BPIFB1 as a tumor suppressor gene involved in regulating glycolysis and vasculogenic mimicry in NPC. Overall, these results provide a new therapeutic target for NPC diagnosis and treatment.

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BACKGROUND

Nasopharyngeal carcinoma (NPC) is a malignant tumor originating from the epithelium of the nasopharynx [1]. The incidence of NPC demonstrates substantial regional variations, with a high incidence in Southeast Asia and Southern China, and lower occurrence in North America, Northern Europe, and East Africa [2–4]. Etiological studies have shown that NPC results from a combination of genetic, environmental, and dietary factors as well as Epstein-Barr virus (EBV) infection, and dysregulated expression of various oncogenic and tumor suppressor genes [5–12].

BPIFB1 (bactericidal/permeability-increasing-fold-containing family B member 1), also known as LPLUNC1 (long palate, lung and nasal epithelium clone 1), is a member of the BPI/PLUNC superfamily as it contains two BPI domains [13–15]. In a previous study, we used cDNA microarrays and suppression subtractive hybridization to demonstrate that BPIFB1 is relatively specifically expressed in nasopharyngeal epithelium. However, it expressed at low levels in NPC tissues and is negatively associated with poor prognosis in patients with NPC [16]. Our previous data further demonstrated that BPIFB1 inhibits the growth of NPC cells in an IL-6-induced inflammatory environment, as well as their migration and invasion via vitronectin and vimentin interactions [17–19]. However, the other biological functions of BPIFB1 in NPC remain unclear.

Tumor neovascularization through angiogenesis or vasculogenic mimicry (VM), is essential for the rapid growth and metastasis of solid tumors [20]. Angiogenesis involves the migration of endothelial cells into tumors and formation of new blood vessels [21, 22]. However, tumor cells can also mimic endothelial cells to form tubular structures via a process called VM that was first identified in uveal melanoma and subsequently in other highly aggressive tumors including glioma, breast, ovarian, and liver cancers [23, 24]. However, VM and its regulatory mechanisms in NPC remain unexplored.

Hence, the current study sought to investigate the in vitro and in vivo effects of BPIFB1 on NPC-associated VM. Results show that BPIFB1 downregulates the expression of glucose transporter 1 (GLUT1), reduces histone acetylation and downregulates VEGFA, VE-cadherin and MMP2, ultimately inhibiting VM in NPC cells.

RESULTS

BPIFB1 inhibits VM in NPC

In this study, 52 NPC tissues and 26 non-tumor nasopharyngeal epithelial tissues (NPE, Table S1) were collected. Immunohistochemistry
results showed high BPIFB1 expression in NPE tissues, and low or undetectable expression in NPC tissues (Fig. 1A). VM structures were examined in these 52 NPC tissues by double staining for the endothelial cell marker CD31 and periodic acid-Schiff (PAS), a reagent that detects polysaccharides outlining the basement membrane [25]. The data showed that VM was more pronounced in the NPC tissues with low BPIFB1 expression but was less in cases with moderate BPIFB1 expression (Fig. 1B, C), suggesting that BPIFB1 may contribute to the inhibition of VM in NPC.

To investigate the effect of BPIFB1 on VM in NPC, a BPIFB1 overexpression vector tagged with Flag was transfected into the NPC cell lines HONE1 and HNE2 (Fig. S1A). Tube formation
assays were performed to investigate the effect of BPIFB1 on vascular formation via VM. The results showed that BPIFB1 significantly decreased the number of tubes in HONE1 and HNE2 cells (Fig. 1D) and downregulated the expression of VM-related molecules, namely, vascular endothelial growth factor A (VEGFA), vascular endothelial cadherin (VE-cadherin/CD144) and matrix metalloproteinase 2 (MMP2) at the mRNA (Fig. 1E) and protein (Fig. 1F) levels. These results suggest that BPIFB1 can inhibit VM in NPC cells.

**BPIFB1 reduces VM formation by inhibiting glycolysis in NPC cells**

Our previous study showed that BPIFB1 can inhibit NPC cell growth through the interleukin (IL)-6/signal transducer and activator of transcription 3 (STAT3) signaling pathway [17], while VEGFA and MMP2 were reported as downstream targets of STAT3 [26, 27]. Thus, to investigate whether BPIFB1 inhibits VM in NPC through the IL-6/STAT3 pathway, BPIFB1 was overexpressed in HONE1 and HNE2 cells, which were subsequently treated with IL-6 to activate STAT3. Results of the tube formation assay revealed that IL-6 did not impact the inhibitory activity of BPIFB1 against VM (Fig. S1B). Western blotting further demonstrated that BPIFB1 continued to significantly inhibit the expression of VEGFA, VE-cadherin and MMP2, although STAT3 was activated by IL-6 (Fig. S1C). In addition, tube formation was not significantly impacted following treatment of BPIFB1-overexpressing HONE1 and HNE2 cells with the STAT3 inhibitor, Statick (Fig. S1D).

Moreover, treatment with Statick did not affect the BPIFB1-inhibited induction of VEGFA, VE-cadherin and MMP2 expression (Fig. S1E). Collectively, these results suggested that BPIFB1 inhibited VM in NPC cells in an IL-6/STAT3-independent way.

To investigate the potential mechanism underlying BPIFB1-induced VM inhibition in NPC, signaling pathways dysregulated in NPC were assessed via gene set enrichment analysis (GSEA) using the gene expression profiling data from the normal nasopharyngeal tissues and 12 NPC tissues [28]. Results showed that the metabolic reprogramming pathway was dysregulated in NPC (Fig. 2A). Further analysis showed that the expression of BPIFB1 was negatively associated with several glycolysis-related genes in both GSE64634 and GSE53819 (another NPC gene expression profiling dataset, Fig. S2A and Table S2), indicating that BPIFB1 may regulate glycolysis and drive metabolic reprogramming in NPC carcinogenesis.

To identify whether BPIFB1 affects glycolysis in NPC cells, glycolysis (extracellular acidification rate, ECAR) were measured by Seahorse XF analyzer; the results showed that BPIFB1 could effectively inhibit glycolysis, including basal glycolysis and maximal glycolysis in HONE1 and HNE2 cells (Fig. 2C). BPIFB1 could also inhibit 2-NBDG uptake, glucose consumption, and lactate production in HONE1 and HNE2 cells (Fig. 2D, E). GLUT1 is an important glucose transporter protein with an important role in glycolysis. RT-PCR and western blotting analysis showed that BPIFB1 significantly downregulated the expression of GLUT1 at both the mRNA (Fig. S2B, C) and protein levels (Fig. 2F). Further, a significant negative correlation was observed between the expression of BPIFB1 and GLUT1 in the GSE64634 and GSE53819 datasets (Fig. S2D). We then knocked down GLUT1 in HONE1 and HNE2 cells using two siRNAs (Fig. S3A), and observed a significant decrease in glycolysis, including basal glycolysis and maximal glycolysis (Fig. S3B, C). GLUT1 knockdown also decreased 2-NBDG uptake, glucose consumption, and lactate production (Fig. S3D, E). Meanwhile, when BPIFB1 and GLUT1 were simultaneously overexpressed in HONE1 and HNE2 cells (Fig. 2G), GLUT1 attenuated glycolysis inhibition caused by BPIFB1 (Fig. 2H). Similar results were obtained in the 2-NBDG uptake assay, glucose consumption and lactate production assay (Fig. 2I, J). These results suggest that BPIFB1 inhibits glycolysis in NPC cells by downregulating GLUT1.

GLUT1 knockdown also significantly decreased tube formation in NPC cells as well as VEGFA, VE-cadherin, and MMP2 expression (Fig. 3A–C). In contrast, GLUT1 overexpression attenuated the BPIFB1-induced inhibition of tube formation in NPC cells as well as VEGFA, VE-cadherin, and MMP2 expression (Fig. 3D–F). These data indicate that BPIFB1 inhibited VM in NPC via downregulation of GLUT1-mediated glycolysis.

**BPIFB1 downregulates VEGFA, VE-cadherin, and MMP2 by inhibiting GLUT1-mediated H3K27 acetylation**

Glycolysis produces a large amount of pyruvate, a portion of which is converted into lactate via lactate dehydrogenase (LDH); in addition, pyruvate can enter the mitochondria where it is converted into acetyl coenzyme A (acyt-CoA) through a series of transformations. Acetyl-CoA is an important donor for histone acetylation [29–31]. We found that BPIFB1 reduced the amount of pyruvate in the mitochondria (Fig. S4A) and, subsequently, the acetyl-CoA abundance in HONE1 and HNE2 cells (Fig. 4A). Western blotting further revealed that BPIFB1 reduced the acetylation of histone H3 at the K9, K14, K18 and K27 sites (Fig. 4B and S4B). Moreover, sodium acetate (NaAC)-treated HONE1 and HNE2 cells had increased intracellular acetyl-CoA content (Fig. S4C). The inhibitory effect on acetylation histone H3 (Fig. S4D) and VEGFA, MMP2 and VE-cadherin expression (Fig. S4E) was attenuated by NaAC. These results suggest that BPIFB1 may regulate the expression of VEGFA, MMP2 and VE-cadherin by reducing intracellular acetyl-CoA and inhibiting histone H3 acetylation.

Acetylated histone H3, especially H3K27ac, promotes an open form of chromatin, and is generally associated with gene transcript activation [32, 33]. The ENCODE database [34] shows high H3K27ac signals in VEGFA, VE-cadherin and MMP2 genes (Fig. S5A, visualized by UCSC browser), suggesting that H3K27ac may be essential for inducing the expression of these genes. We further found that GLUT1 knockdown or treatment of NPC cells with glycolysis inhibitor 2-DG, reduced intracellular acetyl-CoA content and H3K27 acetylation (Fig. S5B–E), while GLUT1 overexpression attenuated these effects of BPIFB1 (Fig. 4C, D). These data suggested that BPIFB1 decreased H3K27ac via inhibiting GLUT1 and glycolysis.

To further confirm that BPIFB1 inhibits VEGFA, VE-cadherin, and MMP2 expression through GLUT1-mediated H3K27 acetylation, we overexpressed BPIFB1 or knocked down GLUT1 in HONE1 and HNE2 cells. Chromatin immune-precipitation (ChIP) results showed that BPIFB1 overexpression (Fig. 4E) or GLUT1 knockdown (Fig. S5F) significantly reduced H3K27ac in VEGFA, VE-cadherin, and MMP2 genes. While overexpression of GLUT1 attenuated the inhibitory effect of BPIFB1 on H3K27ac in VEGFA, VE-cadherin, and MMP2 genes (Fig. 4F).
BPIFB1 downregulates GLUT1 expression by inhibiting the JNK/AP1 signaling pathway

To investigate the mechanism of BPIFB1 in regulating GLUT1, vectors were constructed for luciferase expression under GLUT1 promoters (PGL3-GLUT1-P1, containing a −2516 bp−+112 bp fragment on GLUT1 promoter and PGL3-GLUT1-P2, containing a −312 bp−+112 bp fragment on the possible core promoter region of GLUT1). Dual luciferase reporter assay showed that BPIFB1 inhibited the activities of both reporters (Fig. 5A), suggesting that BPIFB1 regulates GLUT1 transcription primarily through the −312 bp−+112 bp region of the GLUT1 promoter. There are NF-κB and AP1 binding sites on the −312 bp−+112 bp region of the GLUT1 promoter.
promoter, predicted by the Evolutionary Conservation of Genomes (ECR) browser, an online transcription factor binding sites analysis tool. Dual luciferase reporter assays revealed that BPIFB1 significantly inhibited the activity of the classical AP1 reporter and PGL3-GLUT1-P2 reporter, which contains the NF-kB and AP1 binding sites. However, BPIFB1 did not inhibit the activity of the PGL3-GLUT1-P2-mut reporter when the AP1 binding site of the −312 bp−112 bp region was mutated (Fig. 5B). Meanwhile, mutation of the NF-kB binding site in the −312 bp−112 bp region did not affect the regulation of this promoter reporter by BPIFB1 (data not shown), suggesting that BPIFB1 regulates GLUT1 expression through the AP1 binding site on the GLUT1 promoter.

We then examined whether BPIFB1 regulates GLUT1 expression by downregulating the phosphorylation of JNK and c-Jun. Western blot results revealed that BPIFB1 could inhibit JNK and c-Jun phosphorylation (Fig. 5C). The JNK inhibitor SP600125 inhibited the phosphorylation of JNK and c-Jun and the expression of GLUT1 (Fig. 5D); it also significantly reduced the luciferase activities of the AP1 reporter and PGL3-GLUT1-P2 reporter (Fig. S6A). The JNK activator, Anisomycin, significantly increased the luciferase activities of the AP1 reporter and PGL3-GLUT1-P2 reporter and attenuated their suppression induced by BPIFB1 (Fig. S6B, C); further, it increased GLUT1 expression by attenuating the suppressed phosphorylation of JNK and c-Jun (Fig. 5E). These data indicate that BPIFB1 can regulate GLUT1 by inhibiting the JNK/AP1 signaling pathway.

**BPIFB1 inhibits VM formation by downregulating GLUT1 in vivo**

To investigate whether BPIFB1 could inhibit VM formation through GLUT1 in vivo, a subcutaneous NPC-bearing mouse model was generated. The results showed that BPIFB1 overexpression significantly inhibited the growth of transplanted tumor cells in nude mice, whereas overexpression of GLUT1 alone promoted their growth; moreover, GLUT1 overexpression weakened the growth inhibiting effect of BPIFB1 on the tumor cells in nude mice (Fig. 6A, B). The CD31/PAS double staining experiments on xenograft tissues revealed that VM occurred less often in the BPIFB1 overexpression group compared to the control group, while VM was most pronounced in the GLUT1 overexpression group. Moreover, VM occurred significantly more in the BPIFB1 and GLUT1 overexpression group than in the BPIFB1 overexpression group (Fig. 6C). Immunohistochemistry results revealed that GLUT1, VEGFA, VE-cadherin, and MMP2 expression in the BPIFB1 overexpression group was significantly lower than in the control group, whereas that in the group overexpressing both BPIFB1 and GLUT1 was higher than that in the BPIFB1 overexpressing group (Fig. 6D). These results suggest that BPIFB1 can inhibit VM by downregulating GLUT1 and can suppress VM-related molecules in vivo.

**BPIFB1 and VM-related proteins are negatively correlated in NPC clinical samples**

Immunohistochemistry, to examine the expression of VM-related molecules in clinical samples, showed that BPIFB1 expression was lower in NPC tissues compared to that in NPE tissues, whereas GLUT1, VEGFA, VE-cadherin, and MMP2 were highly expressed in NPC tissues, compared with those in NPE tissues (Fig. 7A, B). BPIFB1 expression was negatively correlated with that of GLUT1, VEGFA, VE-cadherin and MMP2 (Fig. 7C, D), suggesting that BPIFB1 plays an important role in the inhibition of VM formation in NPC.

**DISCUSSION**

In this study, we found that BPIFB1 could inhibit GLUT1 transcription through downregulation of the JNK/AP1 signaling pathway, which alters metabolic reprogramming and inhibits glycolysis in NPC cells. Inhibition of glycolysis reduces intracellular acetyl-CoA production, which decreases the acetylation of H3K27 and expression of VEGFA, VE-cadherin, and MMP2, ultimately leading to the inhibition of VM in NPC (Fig. 7E). This is an important discovery in the regulation of VM in NPC. Solid tumors cannot grow beyond 1–2 mm in diameter without neovascularization [24]. Several studies have demonstrated tumor angiogenesis in NPC, however, few have reported on VM in NPC [35–37]. Although there are many anti-tumor drugs targeting tumor angiogenesis, their clinical results remain unsatisfactory [38–40]. By inhibiting vascular endothelium-derived angiogenesis alone, tumor cells can reconstruct vascular networks in tumor tissues through VM; this may account, in part, for the poor effect of anti-angiogenic drugs.

Similar to angiogenesis, tumor VM aims to meet the metabolic demands of tumor cells. Multiple pro-tumor angiogenic signals are promote tumor VM [41, 42]. One such factor is VEGFA, which can activate the VEGFR signaling pathway by binding to its receptor, VEGFR, to promote tumor angiogenesis [43]. VEGFA can also promote VM in tumors. In salivary gland adenoid cystic carcinoma, VEGFA induced by hypoxia can cause epithelial to mesenchymal transition (EMT) in tumor cells and ultimately promote VM [41]. EBV infection is a clear causative factor for NPC. Indeed, EBV-encoded miR-BART1-5p can promote glycolysis in NPC by activating the AMPK/mTOR/HIF-1 signaling axis [44]. In addition, EBV-encoded LMP1 reportedly promotes VM via VEGFA/VEGFR1 signaling [45]. Lin Feng et al. also found that EBV-encoded LMP2A promotes VM formation through activation of PI3K/AKT/mTOR/HIF-1α signaling [25]. HIF-1α is an upstream transcription factor for key glycolysis genes such as GLUT1 and HK2 genes. These findings imply that EBV-encoded LMP2A may promote VM initiation in NPC by regulating intracellular metabolism. In this study, we found that the abnormal glycolysis caused by BPIFB1 overexpression could inhibit VM in NPC.
Fig. 3  BPIFB1 inhibits VM formation by downregulating GLUT1.  

A Tube formation assays were performed to detect tube formation by HONE1 and HNE2 cells after transfecting two GLUT1 siRNAs, respectively. 

B RT-PCR analysis of VEGFA, VE-cadherin, and MMP2 expression in HONE1 and HNE2 cells after GLUT1 knockdown. 

C Western blotting analysis of VEGFA, VE-cadherin, and MMP2 abundance in HONE1 and HNE2 cells after GLUT1 knockdown. 

D Tube formation assays were performed in HONE1 and HNE2 cells after co-transfecting the BPIFB1 and GLUT1 overexpression plasmids. 

E RT-PCR analysis of VEGFA, VE-cadherin, and MMP2 expression in HONE1 and HNE2 cells after co-transfection with BPIFB1 and GLUT1 overexpression plasmids. 

F Western blotting analysis of VEGFA, VE-cadherin, and MMP2 abundance in HONE1 and HNE2 cells after co-transfection with BPIFB1 and GLUT1 overexpression plasmids. 

Data shown are representative images or expressed as the mean ± s.d. of each group from three separate experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
In this study, the abnormal glycolysis caused by BPIFB1 was found to inhibit histone acetylation. Glycolysis provides energy to tumor cells as well as regulates gene expression through various epigenetic modifications, such as histone methylation and acetylation [46–48]. Acetyl-CoA is an important acetyl donor of histone acetylation; thus, production of acetyl-CoA can alter the histone acetylation status. Histone acetylation weakens the interaction between DNA and histones, thus promoting chromatin opening. In addition, acetylated histones form recognition sites for bromodomain-containing proteins, which are generally found in transcriptional co-regulators, ultimately promoting the expression of genes and initiation of cell biological processes [49, 50]. H3K27 acetylation, specifically, plays an important role in the development and treatment of tumors as it can induce the expression of a variety of pro-oncogenic molecules in tumor cells [51]. For instance, in colorectal cancer, H3K27 acetylation induces the expression of the lncRNA EIF3J-AS1, which promotes colorectal cancer cell...
proliferation and reduces apoptosis [52]. In oral squamous carcinoma, H3K27 acetylation promotes cell proliferation and migration by activating the IncRNA CCAT1 [53]. In breast cancer, H3K27 acetylation activates EMT and promotes trastuzumab resistance through IncRNA TINCR [54]. Glycolysis can also promote T helper cell differentiation by increasing H3K27 acetylation levels in T helper cells, leading to increased expression of interferon (IFN)-γ [29].

In this study, we found that BPIFB1 reduced acetyl-CoA by downregulating glycolysis, which reduced acetylation of histone 3 on H3K9, H3K14, H3K18 and H3K27. Through the ENCODE database, high H3K27ac signal was found in VEGFA, VE-cadherin,
radiotherapy in our previous study [14]. These results indicate that restoring BPIFB1 expression in NPC alone or in combination with targeting drugs, can inhibit the conversion of tumor cells to endothelial types. In addition, inhibition of VM by BPIFB1 can contribute to the inhibition of NPC cell migration and invasion.

An increase in glycolysis is reported to promote radioresistance in NPC cells [56, 57]. In this study, BPIFB1 was found to downregulate glycolysis in NPC by inhibiting GLUT1. Meanwhile, BPIFB1 enhanced the sensitivity of NPC cells to radiotherapy in our previous study [14]. These results indicate that restoring BPIFB1 expression in NPC alone or in combination with glycolytic inhibitors, may provide new targets and strategies for the treatment of NPC. Hence, this study is the first to reveal that BPIFB1 inhibits VM in NPC by altering the glycolytic process, thereby expanding the current knowledge regarding the regulatory role of BPIFB1 in NPC, and providing a new reference direction and theoretical basis for the treatment of NPC and for development of anti-tumor angiogenesis targeting drugs.

**MATERIAL AND METHODS**

**Cell culture and antibodies**

NPC cell lines HONE1 and HNE2 were maintained in our laboratory. The cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (Invitrogen, Shanghai, China), penicillin (100 U/mL), and streptomycin (100 µg/mL) in a humidified incubator under 5% CO₂ at 37 °C. The antibodies used are listed in Table S3.

**Constructs and reagents**

The p-IRES-Flag-BPIFB1 plasmid was constructed previously; p-CMV3-GLUT1 was purchased from Sino Biological (Beijing, China). GLUT1 siRNAs were purchased from GenePharma (Shanghai, China). The Neoect Reagent (Neoect biotech Co., Ltd. China) was used for plasmid transfection and Hiperfect (Qiagen, Hilden, Germany) was used for siRNA transfection according to the manufacturer’s protocol. Reagents 2-deoxy-D-glucose (2-DG) and 2-deoxy-2-(1-nitro-2-1,3-benzoxadiazol-4-yl)aminoo-D-glucopyranoside (2-NBDG) and Stattic were purchased from MedChemExpress (MCE USA); SP600125 and Anisomycin were purchased from Selleck Chemicals (Houston, TX, USA). IL-6 was obtained from Sino Biological (Beijing, China). Sodium acetate (NaAc) and the Acetyl-CoA Test Kit were purchased from Sigma–Aldrich (USA). The Mitochondria Isolation Kit was purchased from Beyotime Biotechnology (Jiangsu, China), while the Pyruvate Test Kit was purchased from Solarbio (Beijing, China).

**NPC clinical samples**

In total, 26 non-tumor NPE tissues and 52 NPC samples (Table S1) were collected at the Second Xiangya Hospital of Central South University (Changsha, China). The study was approved by the Joint Ethics Committee of the Central South University Health Authority and informed consent was obtained from all participants. The diagnoses of all specimens were confirmed via histopathological examination.

**RNA isolation and quantitative real-time PCR**

Total RNA was isolated using Trizol reagent (Invitrogen, USA) according to the manufacturer’s protocol. cDNA synthesis was performed using a reverse transcription kit (Vazyme, Nanjing, China) according to the manufacturer’s instructions. SYBR Green (Vazyme) was used for RT-PCR analysis on the MiniOpticon system (Bio-Rad, Hercules, CA, USA). After the reactions were completed, relative gene expression levels were calculated using the 2⁻ΔΔCt method, and β-actin was used as the endogenous control. The primer sequences used are listed in Table S4.

**Western bloting**

Total proteins were lysed using RIPA buffer (Beyotime Biotechnology, Jiangsu, China) containing a protease/phosphatase inhibitor cocktail (Roche Applied Sciences, Mannheim, Germany), separated by 10% SDS-PAGE, and transferred onto PVDF membranes (Millipore, Billerica, MA, USA). After washing, the membrane was incubated with HRP-labeled secondary antibodies (CUSBIO, Wuhan, China) for 1 h at room temperature. The proteins were then detected using ECL reagent (Millipore).

**Measurement of glucose uptake, lactate production, and 2-NBDG uptake**

Cells were seeded in 12-well plates (10⁵ cells/well), after incubation at 37 °C for 8 h, the medium was replaced with fresh complete medium, and cells were incubated at 37 °C for another 24 h. Glucose and lactate levels were measured using the Automatic Biochemical Analyzer (AU680, Beckman Coulter International, Brea, CA, USA). The relative glucose consumption rate and lactate production rate were normalized to the cell number in each sample.

For the 2-NBDG uptake assay, cells were treated with fresh glucose-free medium containing 100 µM 2-NBDG for 45 min and the mean fluorescence intensity was immediately measured using flow cytometry.

**Glycolysis assay**

For the glycolysis assay, a Glycolysis Stress Test Kit (Agilent, USA) was used to measure the extracellular acidification rate according to the manufacturer’s instructions (Seahorse Bioscience, North Billerica, MA, USA).

**Tube formation assay**

For the tube formation assay, 50 µl of Matrigel (BD) was plated in 96-well plates and incubated at 37 °C for 1 h to allow Matrigel polymerization. Then, 3 × 10⁴ cells per well were seeded onto the Matrigel layer and incubated at 37 °C for 4 h. Randomized fields were captured using microscope. The numbers of capillary-like structures were quantified using ImageJ software. The data are presented as the average numbers of tubes ± standard deviation (s.d.).

**Luciferase reporter assay**

All luciferase reporter constructs were generated from the PGL3-basic vector (Promega, Madison, WI, USA). The wild type and mutant sequences were amplified using PCR. Hiperfect (Qiagen, Hilden, Germany) was used for dual luciferase reporter assay. AP1 reporter activity was used as a positive control. Western blotting analysis of p-JNK1/2, JNK1/2, p-c-Jun, c-Jun, and GLUT1 abundance in HONE1 and HNE2 cells after BPIFB1 overexpression. Western blotting analysis of p-JNK1/2, JNK1/2, p-c-Jun, c-Jun, and GLUT1 expression in HONE1 and HNE2 cells after treatment with 10 µM SP600125, examined by western blotting. Data shown are representative images or expressed as the mean ± s.d. of each group from three separate experiments. *p < 0.05, **p < 0.01.
Fig. 6  **BPIFB1 reduces VM formation via GLUT1 downregulation in vivo.**

A. Pictures of tumors formed in nude mice after transplantation of HONE1 cells transfected or co-transfected with the BPIFB1 and GLUT1 overexpression plasmids. B. The statistical results of tumors formed in nude mice after transplanting HONE1 cells transfected or co-transfected with the BPIFB1 and GLUT1 overexpression plasmids. \( n = 5 \), two-tailed Mann–Whitney test. C. H&E staining was performed on these tissues, BPIFB1 expression was examined via immunohistochemistry and VM was detected via CD31/PAS double staining in nude mice xenograft tissues. Magnification: 100×, Scale bar = 100 μM; Magnification: 400×, Scale bar = 20 μM. D. H&E staining was performed on these tissues, immunohistochemistry showed the expression of BPIFB1, GLUT1, VEGFA, VE-cadherin, and MMP2 in xenograft tissues. Magnification: 200×, Scale bar = 50 μM. Data shown are representative images or expressed as the mean ± s.d. of each group from one separate experiment (in vivo). *\( p < 0.05 \), **\( p < 0.001 \), ***\( p < 0.0001 \). Student’s t test or two-way ANOVA.
Fig. 7  Relationship between BPIFB1 and GLUT1, VEGFA, VE-cadherin, and MMP2 in NPC tissues. A Immunohistochemistry was performed to detect the expression of BPIFB1, GLUT1, VEGFA, VE-cadherin, and MMP2 in 26 NPE tissues and 52 NPC tissues. Magnification: 200×, Scale bar = 50 μM. B The statistical results of BPIFB1, GLUT1, VEGFA, VE-cadherin, and MMP2 expression in 26 NPE and 52 NPC tissues. C Representative images showing the correlation between BPIFB1 and GLUT1, VEGFA, VE-cadherin, and MMP2 expression in NPC tissues. Magnification: 200×, Scale bar = 50 μM. D Correlation between BPIFB1 and GLUT1, VEGFA, VE-cadherin, or MMP2 expression in 26 NPE and 52 NPC tissues. E Model for inhibition of VM by BPIFB1 through downregulation of GLUT1-mediated H3K27 acetylation. Data shown are representative images or expressed as the mean ± s.d. of each group from one separate experiment (in vivo). ****p < 0.0001.
of GLUT1 promoter, named PGL3-GLUT1-WT and PGL3-GLUT1-mut, were amplified and cloned into the PGL3-basic vector. The primers used are shown in Table S4. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega). The relative luciferase signal was presented as firefly luciferase activity normalized to the Renilla luciferase activity. The AP1-luc reporter was purchased from Beyotime Biotechnology (Jiangsu, China).

CHIPS

Cells were fixed with 1% formaldehyde for 10 min at room temperature, and the fixation was stopped with 0.125 M glycine; the cell lysis buffer was then added and the samples were sonicated to generate 200–1000 bp fragments. The resulting cell lysates were immunoprecipitated using the H3K27ac antibody (active motif, USA) and analyzed by RT-PCR. The primers used are shown in Table S4.

Animal experiments

Female 4-week-old, nu/nu-BALB/c athymic nude mice were randomly divided into four groups (five mice each). First, $5 \times 10^4$ HONE1 cells transfected or co-transfected with the empty vector, BPIFB1 overexpression vector, or GLUT1 overexpression vector were injected subcutaneously into respective mice. Tumor growth was monitored every 3 days. Tumor size was assessed by measuring the largest perpendicular diameters, and the tumor volume was calculated as follows: $V = \frac{1}{2} \times (\text{length}) \times (\text{width}) \times \text{height}$. After 33 days of subcutaneous inoculation, the mice were euthanized by cervical dislocation and the tumor tissue was excised. The formed tumor masses were removed and weighed. All animal protocols were approved by the Institutional Laboratory of Animal Care and Use Committee at Central South University (2020syaw0896).

Immunohistochemistry and CD31/PAS staining

Immunohistochemistry was performed on formalin-fixed paraffin-embedded sections of clinical NPC tissues and mouse xenograft tissues. Briefly, the tissues were deparaffinized and dehydrated, and the samples were then subjected to EDTA-mediated high-temperature antigen retrieval; the cell lysis buffer was added and the samples were sonicated to generate 200–1000 bp fragments. The staining was scored according to the staining intensity and the distribution of stained cells. Distribution was evaluated as none (0), faint (1), moderate (2), strong (3). The sections were reviewed by two pathologists. The VM structure criteria were negative for CD31 but positive for PAS (CD31 positive and the distribution of stained cells. Distribution was evaluated as none (0), faint (1), moderate (2), strong (3). The sections were reviewed by two pathologists. The VM structure criteria were negative for CD31 but positive for PAS (CD31+ and the distribution of stained cells. Distribution was evaluated as none (0), faint (1), moderate (2), strong (3). The sections were reviewed by two pathologists.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 7.0 software. Differences between groups were analyzed using the Student’s t test when there were only two groups or using one-way ANOVA when there were more than two groups. Spearman’s correlation coefficient was used to determine the correlations between BPIFB1 and GLUT1, VEGFA, VE-cadherin, or MMP2. A two-tailed value of $p < 0.05$ was considered statistically significant. **p < 0.01; ***p < 0.001; ****p < 0.0001.

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Author Contributions

XJ performed all Experiments; ZZ designed this study; XD, JW, YM and LS collected tissue samples and the clinical data; FW, SZ, ZG and YH gave guidance on experimental methods; FX, YW, CG, BX, MZ, QL, XL, GL, WX analyzed and interpreted the data; XJ and ZZ drafted the paper. All authors read and approved the final paper.

Competing Interests

The authors declare no competing interests.

Additional Information

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