


Upregulation of Nogo-B by hypoxia inducible factor-1 and activator protein-1 in hepatocellular carcinoma

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Abstract

Nogo-B is an important regulator of tumor angiogenesis. Expression of Nogo-B is remarkably upregulated in multiple tumor types, especially hepatocellular carcinoma (HCC). Here, we show the transcriptional regulation mechanisms of Nogo-B in liver cancer. In response to hypoxia, expression of Nogo-B significantly increased in HCC tissues and cells. The distal hypoxia-responsive element in the promoter was essential for transcriptional activation of Nogo-B under hypoxic conditions, which is the specific site for hypoxia inducible factor-1 α (HIF-1 α) binding. In addition, Nogo-B expression was associated with c-Fos expression in HCC tissues. Nogo-B expression was induced by c-Fos, yet inhibited by a dominant negative mutant A-Fos. Deletion and mutation analysis of the predicted activator protein-1 binding sites revealed that functional element mediated the induction of Nogo-B promoter activity, which was confirmed by ChIP. These results indicate that HIF-1 α and c-Fos induce the expression of Nogo-B depending on tumor microenvironments, such as hypoxia and low levels of nutrients, and play a role in upregulation of Nogo-B in tumor angiogenesis.

KEYWORDS

c-Fos, hepatocellular carcinoma, HIF-1 α , hypoxia, Nogo-B

1 | INTRODUCTION

Hepatocellular carcinoma is the fifth most common malignancies and is the third leading cause of cancer-related death worldwide.¹ The prognosis for HCC is poor, due to low positive rates of diagnosis and lack of effective therapy.² Angiogenesis plays an essential role

in growth and metastasis of many different cancers, including HCC.³ Nogo-B is a member of the reticulon (RTN) family.⁴ Nogo-B KO mice showed reduced angiogenesis and arteriogenesis, indicating that it is involved in physiological angiogenesis.⁵ Our previous studies have shown that the expression level of Nogo-B was significantly upregulated in HCC.⁶ Increased Nogo-B promoted tumor growth

Abbreviations: AP-1, activator protein-1; C/EBP, CAAT/enhancer binding protein; DFO, desferrioxamine; DIG, digoxigenin; HCC, hepatocellular carcinoma; HIF-1, hypoxia inducible factor-1; HRE, hypoxia responsive element; NgBR, Nogo-B receptor; PHD, prolyl hydroxylase domain; TME, tumor microenvironment; TPA, 12-O-tetradecanoylphorbol-13-acetate; TRE, TPA response element; TSS, transcription start site; VEGF, vascular endothelial growth factor; VHL, von Hippel-Lindau.

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and angiogenesis.^{6,7} However, the regulatory mechanism of Nogo-B expression in HCC still remains unclear.

It is well established that tumor angiogenesis is regulated by stress-prone TME. Among these stress conditions, hypoxia is the predominant regulator that upregulates many of the angiogenic growth factors, such as VEGF.⁸ Hypoxia inducible factor plays a critical role in response to the hypoxic environment. The HIF family comprises three α subunit members (HIF-1 α , HIF-2 α , and HIF-3 α) and a β subunit member (HIF-1 β). Hypoxia inducible factor-1 has been widely examined, which contains an oxygen-dependent HIF-1 α subunit and a constitutively expressed HIF-1 β subunit. Under normoxia, HIF-1 α is targeted by the VHL E3 ubiquitin ligase complex for proteasomal degradation. A family of oxygen- and iron-dependent PHD enzymes hydroxylates HIF-1 α at prolines residues 402 and 564, which is required for the binding of VHL protein to HIF-1 α .⁹ Under hypoxia, HIF-1 α is stabilized from hydroxylation and ubiquitination due to limited oxygen supply. Furthermore, in normal oxygen condition, hypoxic signaling can also be mimicked by certain chemical mimics, such as DFO¹⁰ and CoCl₂.¹¹ In this process, the DFO and cobalt stabilize HIF-1 α from degradation by inhibiting the PHD activity through Fe²⁺ chelation or substitution, respectively. Hypoxia inducible factor-1 α stabilized by either oxygen depletion or hypoxia-mimic chemicals forms a heterodimer with HIF-1 β to bind the HRE in the promoter of target genes. The activated angiogenic factors in turn promote the sprouting of new blood vessels to supply oxygen and nutrients for tumor growth.¹² Numerous studies have found that expression of HIF-1 α in HCC tissues are higher compared with that in corresponding adjacent tissues.¹³⁻¹⁵ Overexpression of HIF-1 α indicates poor prognosis in patients with HCC.¹⁶ In addition to HIF-1, AP-1 is important in the induction of many angiogenic factors in cancer.¹⁷⁻²² Activated protein-1 is a heterodimer or homodimer that comprises components from JUN, FOS, ATF, and MAF families.²³ Changes in TME, including low levels of glucose, stimulate AP-1 to recognize the TRE to regulate cell proliferation, differentiation, apoptosis, angiogenesis, and tumor invasion.²⁴ The major components of the AP-1 dimer, c-Jun and c-Fos, show a different pattern in HCC. The expression of c-Fos is significantly higher in tumor compared to adjacent tissue, whereas c-Jun shows high levels of expression in both tumor and adjacent nontumor tissue.²⁵ Increased c-Fos expression leads to the induction of VEGF.²⁶⁻²⁸ To date, the Nogo-B regulation by HIF-1 or AP-1 has not been reported.

In this study, we investigated the mechanism of upregulated Nogo-B expression in HCC. We showed that both HIF-1 and AP-1 participate in Nogo-B transcription. Their active binding elements in the Nogo-B promoter were also identified.

2 | MATERIALS AND METHODS

2.1 | Tumor specimens

Fresh surgical specimens of HCC, including tumor and adjacent nontumorous liver tissues, were collected from Zhongshan Hospital

(Fudan University, China). Specimens were immediately frozen in liquid nitrogen after operation and stored at -80°C . This study was approved by the Ethics Committee of the School of Life Sciences of Fudan University according to the Declaration of Helsinki. Informed consent was obtained from all patients to approve the use of their tissues for research purposes.

2.2 | Plasmid constructs

The full-length human Nogo-B promoter from -4041 to $+295$ relative to the TSS was cloned into pGL3-basic vector (Promega) with forward primer 5'-TTCTCTGAGTCTGGTGTTCACGTCTGC-3' and reverse primer 5'-CCCAAGCTTTGGAGGGTGGAGATG-3' (p4041-Luc). To generate progressive deletion constructs, a series of forward primers 5'-GCCCTCGAGCTCCAGAGTAGGC-3' (p991-Luc), 5'-GCCCTCGAGCTAAGGTGACTGAAATG-3' (p770-Luc), 5'-GCCCTCGAGTTACCATGTAAGAGTCCAG-3' (p664-Luc), 5'-GCCCTCGAGTCTCTCCCTCCCG-3' (p17-Luc), and 5'-GCCCTCGAGCAAACCTGGGCGG-3' (p + 36-Luc) were used in combination with the same reverse primer of the full-length promoter construct. Site-directed mutagenesis was carried out using the QuikChange Mutagenesis Site-Directed Kit (Stratagene) according to the manufacturer's instructions. The p4041m1-Luc and p4041m2-Luc were generated by changing the CGTG core sequence of HRE into AAAG. The pTREM1-Luc, pTREM2-Luc, and pTREM3-Luc were generated by changing the consensus AP-1 binding site TGACTCA to TcctaCA.²⁹ The A-Fos and A-C/EBP plasmids were kindly provided by Dr Charles Vinson (Laboratory of Metabolism, NCI).

2.3 | Lentiviral transduction

The lentiviral vectors carrying EGFP as well as shRNAs against human HIF-1 α (si1HIF1A sense, 5'-CUGAUGACCAGCAACUUGA-3'³⁰; si2HIF1A sense, 5'-GAUGGAAGCACUAGACAAA-3')³¹, HIF-2 α (siHIF2A sense, 5'-GCAUCAUGUGUGUCAACUACG-3'), and non-silencing control with scrambled sequence were constructed and packaged by Genechem (China). SMMC-7721 cells were transduced at a multiplicity of infection of 10. Silencing effects were confirmed by exposure of cells to hypoxia for 24 hours and detected by western blot.

2.4 | Cell culture and transfection

Immortal HCC cells, SMMC-7721 and SK-HEP-1, were grown in DMEM plus 10% (v/v) FBS (Gibco) at 37°C in a 5% humidified CO₂ atmosphere or in a low-oxygen chamber with 1% O₂ for hypoxia treatment. Cells were transfected at 80% confluence using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Isolation and culture of primary rat hepatocytes were carried out

as described previously.³² Briefly, primary hepatocytes were obtained from male adult Sprague-Dawley rats (Shanghai SLAC Laboratory Animal Co., Ltd), and were maintained in DMEM supplemented with 10% FBS (Gibco), penicillin (50 U/mL), and streptomycin (50 mg/mL). Insulin (4 µg/mL; Gibco) and dexamethasone (1 µM; Sigma) were added to the above medium before use. Twenty-four hours after cell plating, the medium was replaced. Complete medium was used as the control (Normoxia). Dulbecco's modified Eagle's medium supplemented with 0–150 µM CoCl₂ (Sigma) or DFO (Sigma), DMEM-low glucose medium (Gibco), and DMEM supplemented with 0.1% FBS (low-serum) were used in different experimental groups. Cell lysates were collected at the indicated times for analysis.

2.5 | Reverse transcription–quantitative PCR

Total RNA was extracted by TRIzol (Invitrogen) and reverse transcribed with M-MLV Reverse Transcriptase (Promega). cDNAs were quantified with the Power SYBR Green PCR Master Mix (Applied Biosystems) and calculated by the $\Delta\Delta CT$ method. The primers targeting HIF1A (forward, 5'-TATGAGCCAGAAGAAGCTTTAGGC-3'; reverse, 5'-CACCTCTTTGGCAAGCATCCTG-3'), HIF2A (forward, 5'-CGCTAGACTCCGAGAACATG-3'; reverse, 5'-GTTGCGAGG GTTGATAGATGA-3'), and HIF3A (forward, 5'-CTGGAGCAAACGG AGCAACAC-3'; reverse, 5'-ATCTGGGAGATCAGCCGAAAG-3') were used. Beta-actin served as a reference control (forward, 5'-GC GTGACATTAAGGAGAAGC-3'; reverse, 5'-CCACGTCACACTTCAT GATGG-3').

2.6 | Luciferase assay

Luciferase assay was used to detect the promoter activity of Nogo-B. The full-length, mutant, and a series of truncated human Nogo-B promoters were inserted into constructs with firefly luciferase reporter as described above. Cells grown in triplicate in 24-well plates were transfected with 150 ng Nogo-B promoter firefly reporter plasmids and 10 ng *Renilla* luciferase plasmids pRL-CMV as an internal control. In cotransfection experiments, indicated amounts of expression vectors were added. The luciferase activities were measured by using the Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase activity was normalized with *Renilla* luciferase activity in each cell lysate.

2.7 | Liver artery ligation assay

Liver artery ligation was carried out in male adult Sprague-Dawley rats (Shanghai SLAC Laboratory Animal Co., Ltd) according to the reported protocol.³³ Rats were randomly grouped, and three independent experiments were undertaken.

2.8 | Western blot analysis

Western blot analysis was carried out as described previously with Ab against HIF-1 α (1:1000 dilution; BD Biosciences), Myc (1:1000 dilution; Sigma), c-Fos (1:500 dilution; Santa Cruz Biotechnology), Nogo-B (1:700 dilution; Santa Cruz Biotechnology), or β -actin (1:1000 dilution; Sigma).³⁴ The membrane was developed with enhanced chemiluminescence detection reagents (Santa Cruz Biotechnology).

2.9 | Chromatin immunoprecipitation

Chromatin immunoprecipitation assay was carried out as described previously.³⁵ In brief, SMMC-7721 cells were cross-linked with formaldehyde, neutralized with glycine (125 mM), lysed, and sonicated. Chromatin was immunoprecipitated overnight at 4°C with 2 µg Abs or IgG as a negative control, and was collected with 40 µL protein-A/G agarose. After they were reversed and purified, the DNA samples were amplified by PCR. Primers that cover the -3829/-3822 putative HRE in the human Nogo-B promoter were used: forward, 5'-CCAGTTTAGGTGTCATTTAGCATTCTGC-3'; and reverse, 5'-ATTGAGAGGTGCTCTTGAACACAGGC-3'. A fragment of VEGF promoter covering the HRE was also amplified as a positive control:³⁶ forward, 5'-GTAGTGTGAATCATCACGCAGG-3'; and reverse, 5'-GCACCAAGTTTGTGGAGCTGA-3'. The primers for the -782/-776 putative TRE in the Nogo-B promoter are 5'-CTGCAACT ACTTTCTTTAACTCTCCAGCCTCAT-3' and 5'-CGGGGAGACAGG AAAGTGGAAATACAAAGAT-3'.

2.10 | Electrophoretic mobility shift assay

Nuclear extracts from both normoxic and hypoxic SMMC-7721 cells were prepared as described previously.³⁷ Electrophoretic mobility shift assay was carried out by using a DIG Gel Shift Kit (Roche) according to the manufacturer's instructions. Briefly, 5-µg aliquots of nuclear extracts of SMMC-7721 cells were incubated with 1 µg poly[d(A-T)], 0.1 µg poly-L-lysine, and 1 ng DIG-labeled Nogo-B-HRE probe for 20 minutes at 4°C. For competition analysis, 100-fold molar excess of unlabeled oligonucleotide was also added just prior to the addition of the labeled probe. The WT-Nogo-B-HRE probe (5'-CACAAACGTGCTTGCATGTTT-3') contains a putative HRE at -3829/-3822 of the Nogo-B promoter. The MUT-Nogo-B-HRE probe (5'-CACAAAAAGCTTGCATGTTT-3') was used as a negative control. For supershift analysis, 1 µL HIF-1 α Ab (Abcam) was added after the initial incubation, and then the mixture was further incubated for 30 minutes at 4°C. DNA-binding complexes were run on a 5% nondenaturing PAGE with 0.5 \times TBE, electroblotted onto a nylon membrane, and then subjected to DIG-chemiluminescence detection.

2.11 | Statistical analysis

All experiments were repeated at least three times. The results were expressed as the mean \pm SD. Statistical significance was determined with Student's *t* test.

3 | RESULTS

3.1 | Induction of Nogo-B expression by hypoxia in HCC

Our previous study indicated that Nogo-B expression is significantly elevated in HCC and drastically promotes tumor angiogenesis.⁷ Given hypoxia plays a critical role in the induction of angiogenic factors, Nogo-B might also be induced by the hypoxic environment in tumor tissue. Our result showed that, compared to the tumor adjacent tissue, expression levels of Nogo-B were increased in HCC tumors, which correlated with the expression of HIF-1 α (Figure 1A). In physiological conditions, hepatocytes express Nogo-B at trace level.³⁸ To simulate the stress of low oxygen and nutrients during tumorigenesis, we ligated the rat hepatic artery. Nogo-B expression was markedly increased under hypoxic conditions in the ligated rat livers compared to the control livers (Figure 1B). In isolated primary rat hepatocytes, expression of Nogo-B was also induced under chemically mimicked hypoxic conditions (DFO and CoCl₂) (Figure 1C). Moreover, consistent increases in HIF-1 α and Nogo-B expression levels were observed in an HCC cell line (SMMC-7721) cultured in a hypoxia chamber with low oxygen

(Figure 1D). These results strongly suggested that the expression of Nogo-B could be induced by hypoxia.

3.2 | Hypoxia induced Nogo-B expression through HIF-1 α

To determine whether the induction of human Nogo-B transcription by hypoxia was mediated by HIF-1 α , the luciferase reporter containing the intact Nogo-B promoter region was constructed. Overexpression of HIF-1 α increased the transcriptional activity of Nogo-B promoter in SMMC-7721 cells (Figure 2A). Lentivirus carrying shRNA, which targets HIF-1 α , was then delivered into SMMC-7721 cells (Figures 2B and S1A). In association with knockdown of HIF-1 α , activation of the Nogo-B promoter in response to hypoxia was dramatically reduced (Figures 2C and S1B). Thus, HIF-1 α could play a key role in modulating Nogo-B expression under hypoxic stress in tumors.

Similar experiments were also undertaken to examine the involvement of HIF-2 α and HIF-3 α . Compared to HIF-1 α , only a slight, nonsignificant increase or decrease of Nogo-B transactions was induced by HIF-2 α and HIF-3 α , respectively (Figure S2A,B). The basal mRNA level of these two HIFs in SMMC-7721 cells was then determined using quantitative PCR. Surprisingly, HIF-3 α was expressed at a very low level, compared to HIF-1 α and HIF-2 α in HCC cells (Figure S2C). A previous study also indicated that the expression of HIF-3 α is not consistent with HCC progression.³⁹ Thus, we only delivered shRNA targeting HIF-2 α to SMMC-7721 cells to study its

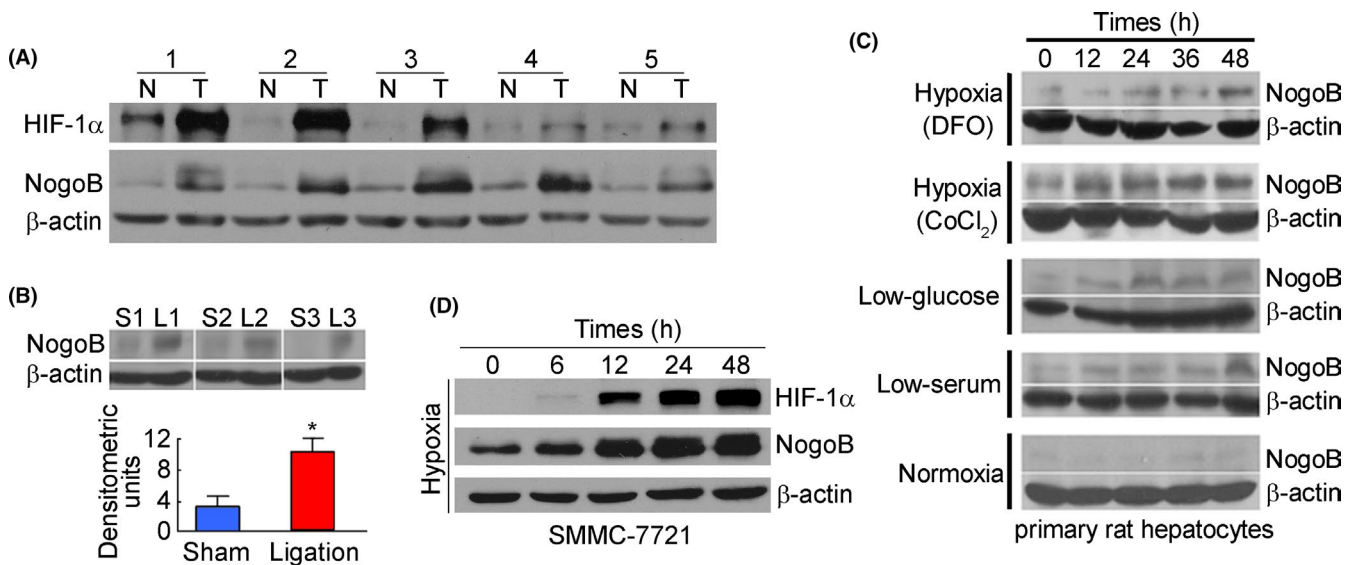


FIGURE 1 Nogo-B expression is induced by hypoxia in hepatocellular carcinoma (HCC). A, Western blot analysis of Nogo-B and hypoxia inducible factor-1 α (HIF-1 α) in HCC samples (T) and adjacent nontumorous liver tissues (N). β -Actin was used as a loading control. B, Western blot analysis of Nogo-B protein in cellular extract of livers obtained from rats that underwent hepatic artery ligation (L) or sham operation as control (S). The densitometric unit of each band was measured with Quantity One (Bio-Rad) using the same parameters. β -Actin served as an internal control for normalization. Data are shown as mean \pm SD ($n = 3$, * $P < .05$). C, Western blot analysis of Nogo-B expression in primary rat hepatocytes under hypoxia mimics, low glucose, low serum, and normoxia conditions. D, Western blot analysis of both HIF-1 α and Nogo-B expression in SMMC-7721 cells cultured in a low-oxygen chamber with 1% O₂ atmosphere. DFO, desferrioxamine

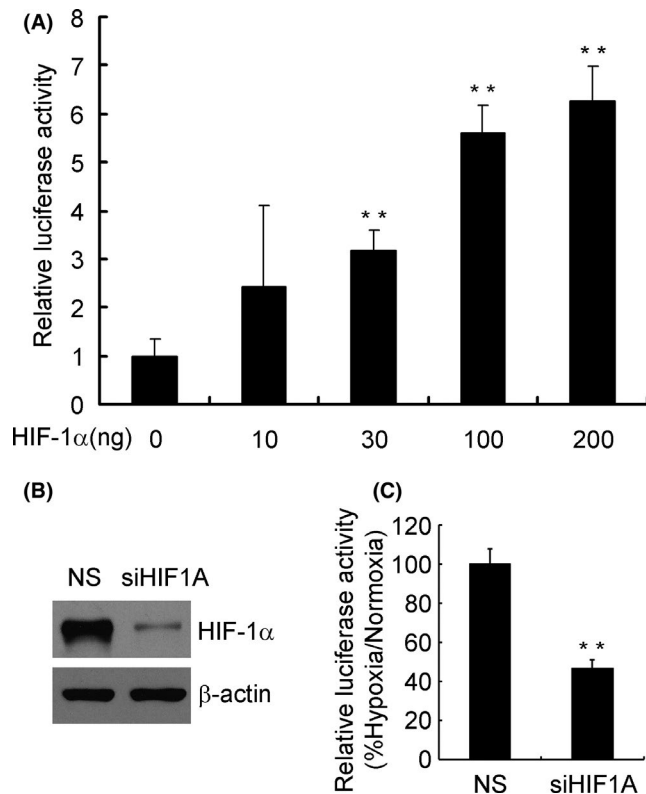


FIGURE 2 Hypoxia induction of Nogo-B expression is mediated by hypoxia inducible factor-1 α (HIF-1 α). A, Relative luciferase activity of the Nogo-B promoter in SMMC-7721 cells after being transfected with indicated amounts of pCMV-Myc-HIF-1 α . Luciferase activity was measured 24 h after transfection and was normalized to cells transfected with the control (empty vector). B, Western blot analysis of HIF-1 α expression in SMMC-7721 cells transfected with lentivirus carrying either control shRNA (NS) or HIF-1 α shRNA (siHIF1A). C, Relative luciferase activity of the Nogo-B promoter in hypoxia condition after HIF-1 α knockdown. Data are shown as mean \pm SD ($n = 3$, ** $P < .01$)

transaction role on the Nogo-B promoter. Knockdown of HIF-2 α in HCC cells, however, did not significantly affect Nogo-B promoter activity under hypoxia (Figure S2D,E). These results were consistent with previous findings that, although both HIF-1 α and HIF-2 α are important regulators of angiogenesis, only HIF-1 α is unambiguously reported to show an increasing expression pattern like Nogo-B in the context of HCC progression, whereas expression of HIF-2 α is not related to tumor prognosis.^{40,41}

3.3 | Hypoxia inducible factor-1 regulated Nogo-B expression by binding distal HRE

Bioinformatics analysis of the Nogo-B promoter region led to the identification of two putative HREs (HRE1 and HRE2) to which HIF-1 may bind (Figure 3A). Several luciferase reporter constructs were generated, including the control of the WT Nogo-B promoter (p4041-Luc) and its variants lacking one (p991-Luc) or both (pLuc) HREs. Their transactivation activities were investigated in two HCC

cell lines, SMMC-7721 and SK-HEP-1 (Figure 3B). Under hypoxic conditions, the WT reporter carrying both HREs (p4041-Luc) was strongly induced in comparison to the vector control. In contrast, truncated Nogo-B promoter without the distal HRE (-3829/-3822) led to the abrogation of its response to hypoxia, suggesting that the distal HRE is necessary for the activation of Nogo-B promoter by hypoxia. To further verify the importance of the distal HRE, we mutated the CGTG core sequence into AAAG and found that this mutation completely abolished hypoxia responsiveness, further confirming that the distal HRE is crucial for hypoxia-induced activation of the Nogo-B promoter.

We then assessed whether HIF-1 is bound to the putative HRE in HCC cells in vitro by EMSA and in vivo by ChIP. In the EMSA, a 21-bp double-stranded probe containing the putative HRE of the Nogo-B promoter formed a complex in nuclear extracts prepared from SMMC-7721 cells grown under hypoxic conditions (Figure 3C). A 100-fold excess of unlabeled oligonucleotide (WT) completely abolished the complex, whereas an oligonucleotide containing a 3-bp substitution in the HRE of Nogo-B promoter (MT) failed to compete for binding to the protein. The protein-DNA complex disappeared in the presence of an anti-HIF-1 α Ab, indicating that a protein component of the complex was HIF-1. Interestingly, we did not observe a supershifted protein-DNA complex. It is possibly due to the mutually exclusive binding of the Ab and the HRE oligonucleotide to HIF-1.⁴²⁻⁴⁴ We then used ChIP to investigate the association between HIF-1 protein and the HRE region of Nogo-B within cells. As shown in Figure 3D, when SMMC-7721 cells were cultured in the low-oxygen chamber, the distal HRE in the Nogo-B promoter was detected to be pulled down by HIF-1 α protein. As a positive control, the known HRE of VEGF was also recognized by HIF-1 under the same conditions. Similar results were obtained from another hepatoma cell line, SK-HEP-1.

3.4 | c-Fos upregulated Nogo-B expression in HCC

Angiogenic factor VEGF is well-characterized to be regulated by HIF-1 α in response to hypoxia. Furthermore, the expression of VEGF is also enhanced by c-Fos to induce tumor angiogenesis.¹⁷ Our result has shown that, in addition to hypoxia, elevation of Nogo-B expression might also be triggered by low glucose (Figure 1C). It has been reported that low glucose induces the expression of c-Fos in tumor cells.⁴⁵ Therefore, we examined whether the expression level of Nogo-B was modulated by c-Fos in HCC. We first detected the c-Fos expression in HCC tissues and observed an elevated expression of c-Fos along with Nogo-B (Figure 4A). Furthermore, the upregulation of Nogo-B and c-Fos expression is significantly positive correlated in primary HCC tumor tissues (Figure S3). Then we treated SMMC-7721 or SK-HEP-1 cells with an endogenous AP-1 activator, TPA. The results indicated that both promoter activity and protein expression of Nogo-B were significantly increased by TPA exposure in a dose-dependent manner (Figure 4B,C). Similar results were also observed by overexpressing c-Fos in SMMC-7721 and SK-HEP-1 cells (Figure 4D,E). However, overexpression of

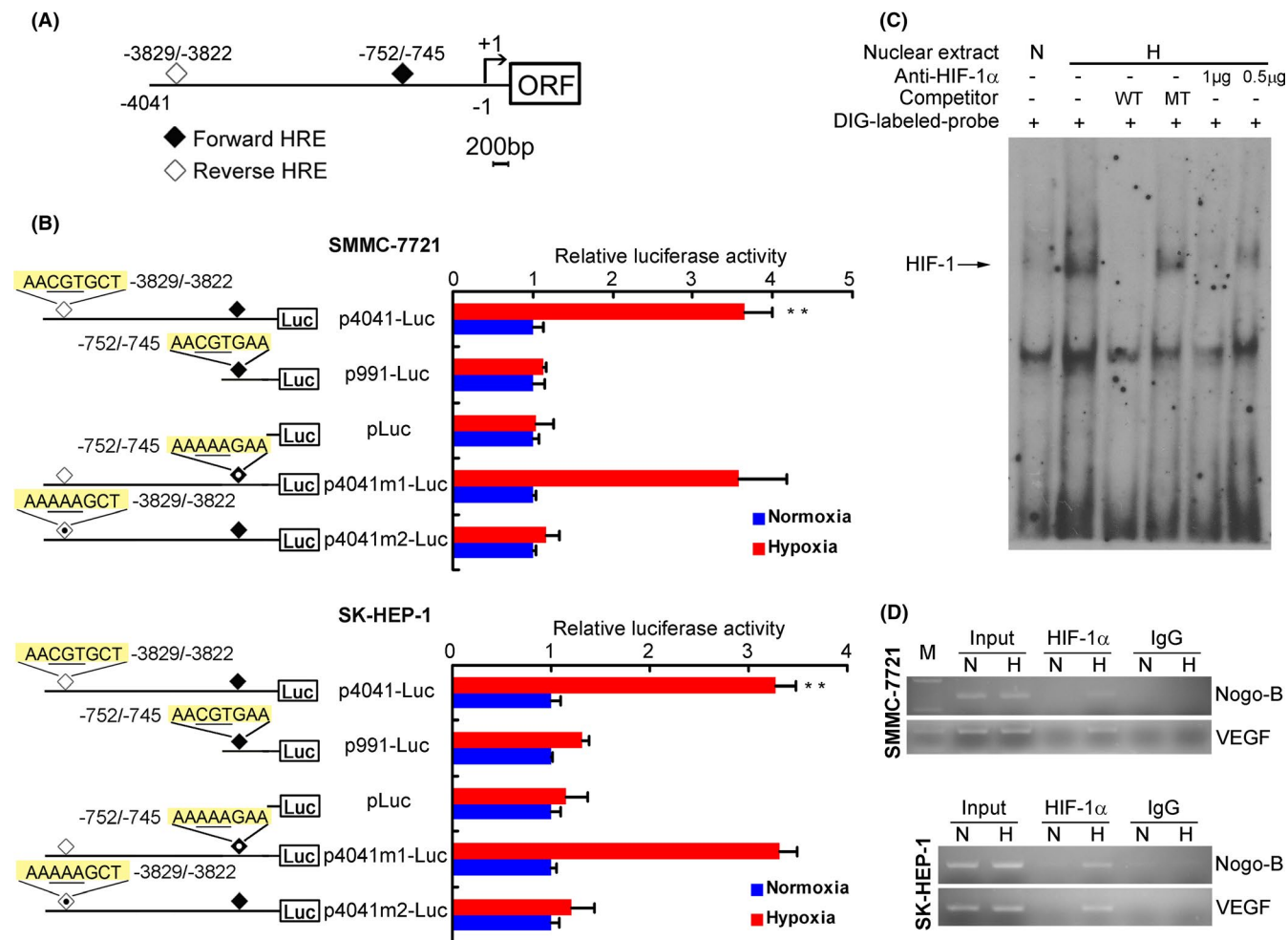


FIGURE 3 Hypoxia induced Nogo-B expression by the binding of hypoxia inducible factor-1 (HIF-1) to the distal hypoxia responsive element (HRE) element in Nogo-B promoter. A, Schematic representation of the HREs in the 5'-UTR of the human Nogo-B gene. Two putative HREs at $-3829/-3822$ and $-752/-745$ are marked. Filled diamond, HRE in forward orientation; open diamond, HRE in reverse orientation. B, Schematic representation of luciferase reporter constructs (left panel) and their activities in SMMC-7721 and SK-HEP-1 cells cultured under hypoxic conditions (right panel). Mutant HREs are marked in the center of diamond symbols. Luciferase activity is normalized against normoxic controls. Data are shown as mean \pm SD ($n = 3$, $**P < .01$). C, Gel mobility shift assay to detect the interaction between HIF-1 and the functional HRE in Nogo-B promoter. The digoxigenin (DIG)-labeled 21-bp double-stranded probe containing the functional HRE in Nogo-B promoter was incubated with nuclear extract from hypoxic SMMC-7721 cells in the absence or presence of excess unlabeled WT or mutant (MT) probes. The band of shifted HIF-1-DNA complex is indicated. Shifted bands were further attenuated by the preincubation of nuclear extracts with anti-HIF-1 α Ab. D, CHIP analysis of the functional HRE in Nogo-B promoter. Soluble chromatin was prepared from SMMC-7721 and SK-HEP-1 cells cultured in normoxia (N) or hypoxia (H) and immunoprecipitated by an anti-HIF-1 α Ab or IgG control. The HRE-containing region in the vascular endothelial growth factor (VEGF) promoter was used as a positive control. M, DNA ladder

AP-1 component c-Jun led to a slight induction of Nogo-B (2.04-fold in SMMC-7721 cells and 1.73-fold in SK-HEP-1 cells), compared to c-Fos (8.83-fold in SMMC-7721 cells and 3.77-fold in SK-HEP-1 cells). This indicated that the heterodimer containing c-Fos was the dominant functional form of AP-1 in the Nogo-B modulation in HCC (Figure 4F).

To further illustrate the role of c-Fos in the induction of Nogo-B, A-Fos, a dominant negative to c-Fos, was expressed in SMMC-7721 cells with the TPA treatment or c-Fos overexpression. As shown in Figure 4G, A-Fos completely abolished the TPA induction of Nogo-B transcription. In addition, A-Fos also drastically reduced c-Fos-mediated Nogo-B activation. A dominant negative to C/EBP, A-C/EBP, was used as a negative control and failed to inhibit c-Fos-dependent activation of the Nogo-B promoter. These results

indicated that c-Fos plays a crucial role in the induction of human Nogo-B in HCC.

3.5 | Induction of Nogo-B expression by c-Fos through TRE

The AP-1 complex recognizes and binds to the consensus TPA response element of 5'-TGAG/CTCA-3' to regulate the transcription of target genes. By analyzing the Nogo-B promoter sequence, we identified three putative TREs (Figure 5A), including two distal TREs at $-782/-776$ (TRE1) and $-676/-670$ (TRE2) relative to the TSS and one proximal TRE at $+26/+32$ (TRE3). To determine the AP-1 responsive

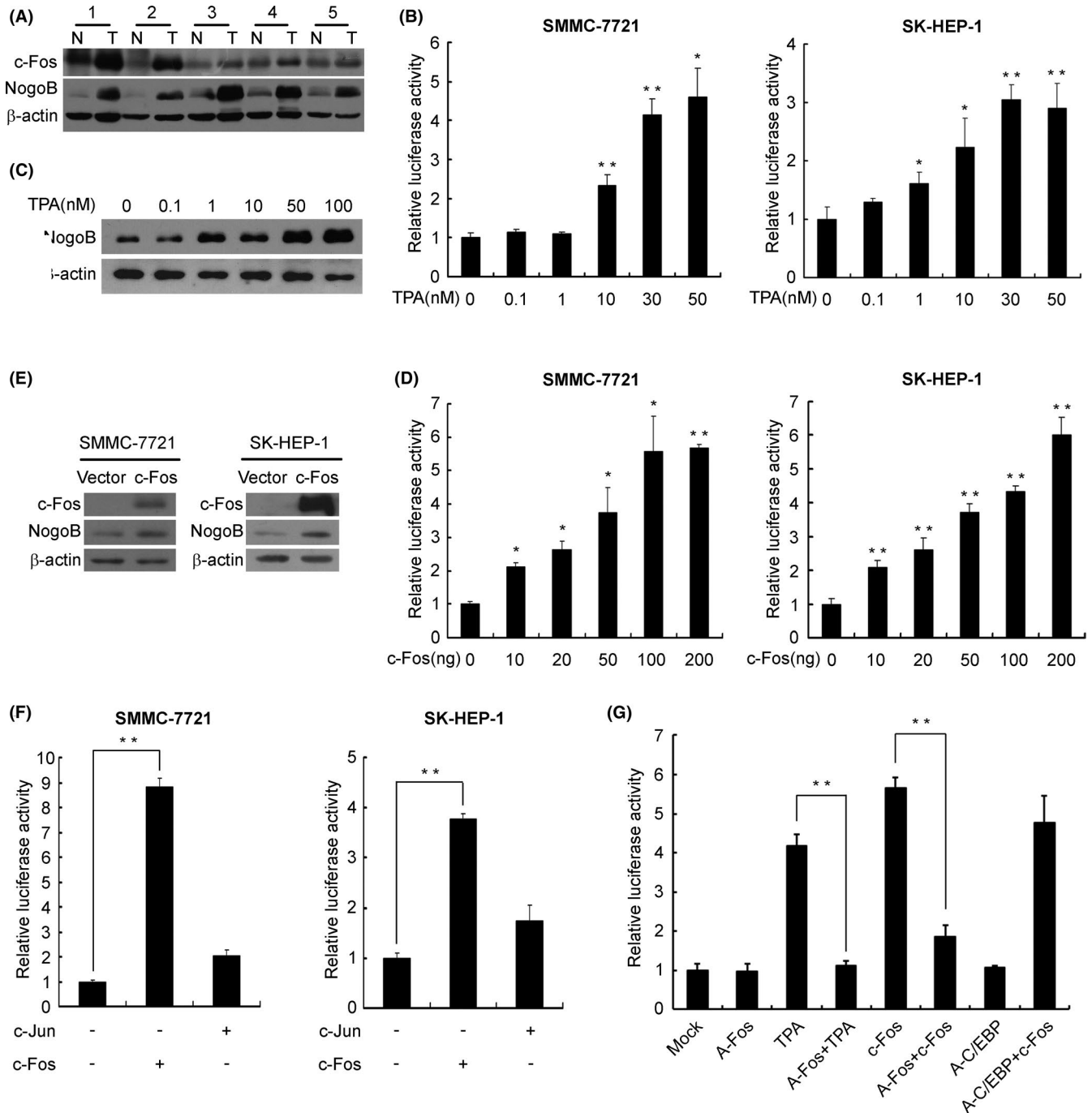


FIGURE 4 Nogo-B expression upregulated by c-Fos in hepatocellular carcinoma (HCC). A, Western blot analysis of Nogo-B and c-Fos in HCC samples (T) and adjacent nontumorous liver tissues (N). β -actin was used as a loading control. B, Relative luciferase activity of the Nogo-B promoter in SMMC-7721 and SK-HEP-1 cells with serum-starved for 24 h and treated with indicated amount of 12-O-tetradecanoylphorbol-13-acetate (TPA) for another 6 h. The fold induction of luciferase activity was calculated by defining the value for TPA untreated as 1. C, SMMC-7721 cells were treated with various concentrations of TPA for 6 h. The expression of Nogo-B was detected by western blot against Nogo-B. β -Actin was used as a loading control. D, Relative luciferase activity of the Nogo-B promoter in SMMC-7721 and SK-HEP-1 cells after being transfected with indicated amount of pCMV-Myc-c-Fos. E, SMMC-7721 and SK-HEP-1 cells were transfected with c-Fos or empty vector. After 24 h, cell lysates were subjected to western blot analysis using anti-Myc Ab (top panel), anti-Nogo-B Ab (middle panel), or anti- β -actin Ab (bottom panel). F, Relative luciferase activity of the Nogo-B promoter in SMMC-7721 and SK-HEP-1 cells after transfected with indicated expression vector. G, SMMC-7721 cells were transfected with indicated expression vector or treated with 50 nM TPA. Luciferase activity was measured and normalized to cells transfected with the control (empty vector). Data are shown as mean \pm SD ($n = 3$, * $P < .05$, ** $P < .01$). EBP, enhancer binding protein

region in the Nogo-B promoter, the progressive deletion constructs of the promoter was investigated in SMMC-7721 cells (Figure 5B). The deletion from -991 bp to -770 bp relative to the TSS resulted in a marked reduction of the Nogo-B promoter activity. However, no more obvious loss of induction by c-Fos was observed when further sequences were deleted. These results suggested that the

region located between -991 bp and -770 bp upstream of the TSS is essential for c-Fos induction of Nogo-B transcription. Moreover, site-directed mutation of this TRE from TGACTCA to TCCTACA at -782 bp to -776 bp (TREM1) completely abolished the promoter activation induced by c-Fos, whereas mutations at the other two putative TREs (TREM2 and TREM3) were unable to reduce the activity

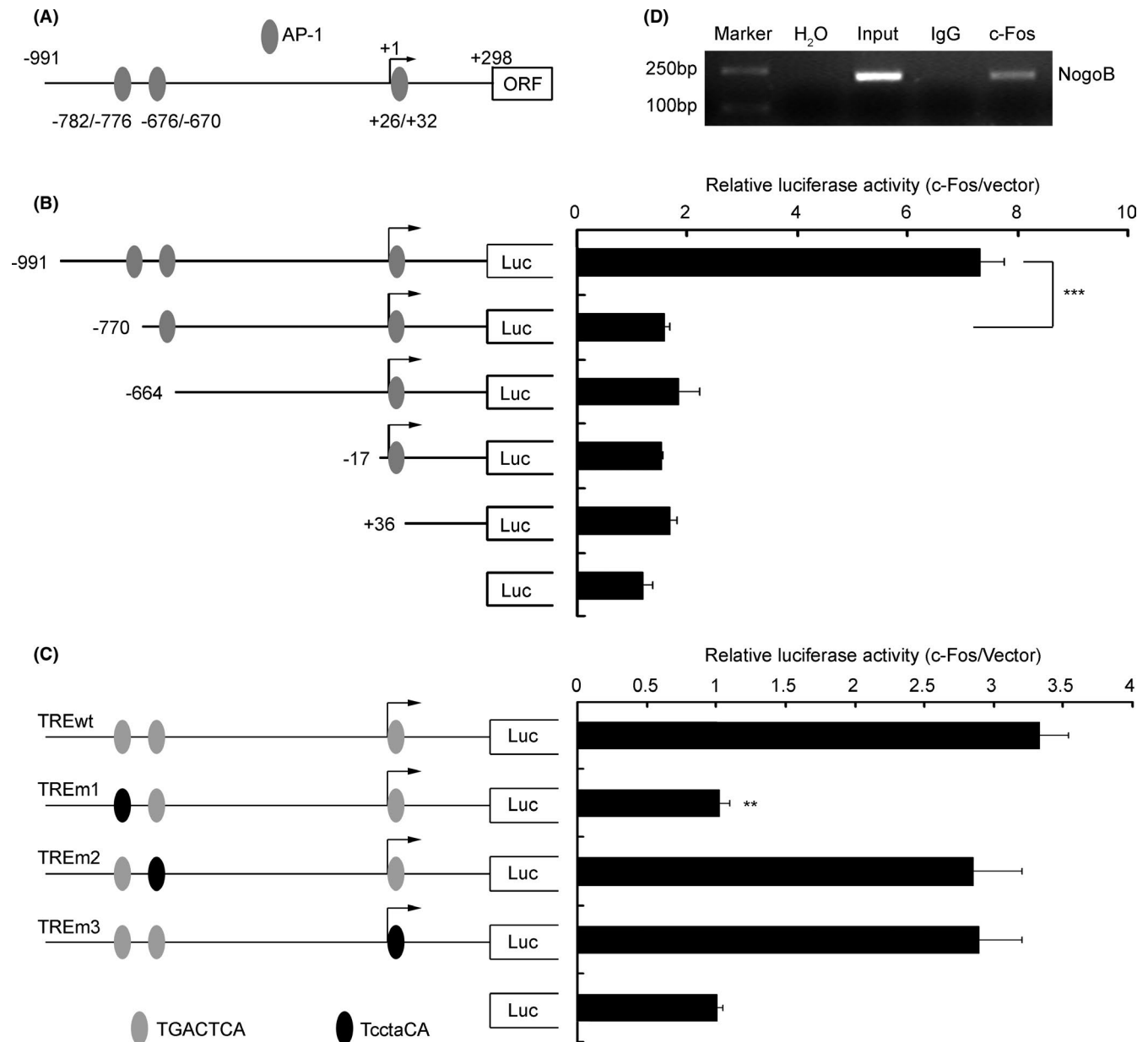


FIGURE 5 c-Fos upregulated Nogo-B expression through the 12-O-tetradecanoylphorbol-13-acetate response element (TRE) in Nogo-B promoter. A, Schematic diagram of the TREs in Nogo-B promoter. Arrow indicates the transcription start site (TSS). Potential binding sites for activator protein-1 (AP-1) at -782/-776 (TRE1), -676/-670 (TRE2), and +26/+32 (TRE3) relative to the TSS are indicated as gray ellipses. -, upstream to the TSS; +, downstream to the TSS. B, Deletion analysis of the functional TRE in Nogo-B promoter. Relative luciferase activity of the Nogo-B promoter deletion reporters that were cotransfected with c-Fos or empty vector in SMMC-7721 cells. C, Mutation analysis of the TREs in Nogo-B promoter. Wild-type AP-1 elements are indicated as gray ellipses. Mutant AP-1 elements are indicated as black ellipses. Relative luciferase activity of the Nogo-B promoter mutant constructs were cotransfected with c-Fos or empty vector in SMMC-7721 cells. Luciferase activity was measured 24 h after transfection and normalized to cells transfected with the empty vector control. Data are shown as mean \pm SD ($n = 3$, $**P < .01$, $***P < .001$). D, AP-1 binding to the TRE of human Nogo-B promoter in vivo. SMMC-7721 cells were transfected with pCMV-Myc-c-Fos. After 24 h, ChIP assay was carried out using anti-Myc Ab, mouse IgG, or corresponding input, and analyzed by PCR. H₂O was used as a negative control in PCR

by c-Fos (Figure 5C). This is consistent with the result of the deletion analysis and suggested the important role of distal TRE. The ChIP assay was undertaken to confirm the interplay of AP-1 and this TRE region of the Nogo-B promoter in SMMC-7721 cells (Figure 5D). The result showed that the distal TRE1 in the Nogo-B promoter was pulled down by c-Fos Ab, but no fragment was detected by IgG, which suggested the role of distal TRE1 in the binding of AP-1 to Nogo-B promoter.

To examine the relationship between HIF-1 and AP-1 in Nogo-B regulation, we compared WT and mutant Nogo-B promoter activity in the condition of HIF-1 or AP-1 overexpression. We found that the HRE mutant significantly abolished the transcription activity of full length Nogo-B promoter, whereas no significant effect of TRE mutant could be detected under hypoxia (Figure S4A). Similarly, following c-Fos overexpression, the Nogo-B promoter activity was dramatically reduced by mutation of TRE but not HRE (Figure S4B). Furthermore, overexpression of both HIF-1 α and c-Fos did not further enhance the promoter activity of Nogo-B compared with expressing one of them alone (Figure S4C). These results suggested that HIF-1 and AP-1 regulated Nogo-B transcription in an independent manner.

4 | DISCUSSION

Vascular endothelial growth factor is the most well-characterized angiogenic factor. Drugs targeting the VEGF pathway have been used for first-line therapy of cancers.⁴⁶ However, the resistance to anti-VEGF treatment suggests the existence of alternative pathways in parallel to the VEGFA/VEGFR axis.⁴⁷ Both Nogo-B and VEGF can induce angiogenesis through NgBR.^{48,49} However, the role of NgBR in tumor development is still in debate.⁵⁰⁻⁵² Our previous study reported a novel Nogo-B/integrin pathway that may promote tumor angiogenesis in multiple cancers, especially HCC.⁷ Expression levels of Nogo-B are significantly upregulated in HCC. Elevated Nogo-B is positively correlated with HCC tumor vessel density and promotes tumor growth and angiogenesis, which was inhibited by Nogo-B depletion. Moreover, blocking of Nogo-B binding to its receptor integrin $\alpha_v\beta_3$ in vivo selectively inhibits tumor angiogenesis. However, it has not been clarified whether these two pathways are activated by related regulatory mechanisms in HCC. Interestingly, consistent with our previous findings on Nogo-B, knockdown of HIF-1 or AP-1 was also reported to attenuate proliferation, migration, and invasion of HCC cells.^{53,54} As most of the angiogenic factors, including VEGF, are induced by a hypoxic TME, we first examined the association between Nogo-B expression and hypoxic conditions. Not unexpectedly, our data indicated that Nogo-B expression was induced in vivo and in vitro by hypoxia through direct binding of HIF-1 to its promoter, which is very similar to the regulating manner of VEGF in the progress of tumor angiogenesis. In addition, VEGF has been reported to be induced by c-Fos in tumor angiogenesis.¹⁷ Consistent with this, our results showed that c-Fos, a component of the AP-1 dimer,

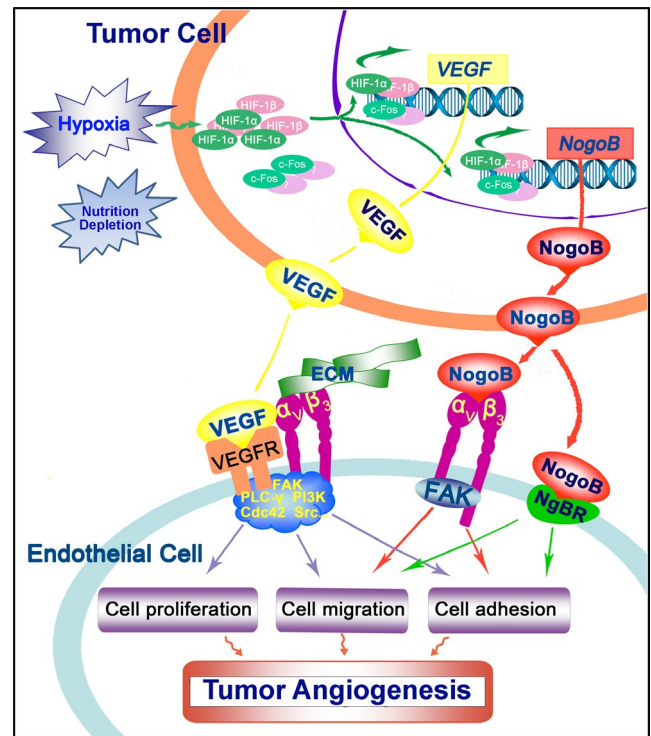


FIGURE 6 Schematic model for Nogo-B-induced tumor angiogenesis. In response to hypoxia, expression of Nogo-B is transcriptionally activated by hypoxia inducible factor-1 (HIF-1) similarly to vascular endothelial growth factor (VEGF). Activator protein-1 (AP-1), comprising c-Fos, also activates the transcription of VEGF and Nogo-B. Both overexpressed VEGF and Nogo-B are secreted into the extracellular microenvironment (ECM). Through binding with VEGF receptor (VEGFR), VEGF could activate multiple intracellular signaling pathways (including FAK, PLC- γ /Ras, and PI3K/Akt) to regulate endothelial cell proliferation in addition to migration and adhesion. Furthermore, through binding integrin $\alpha_v\beta_3$ and activating the FAK pathway or through binding Nogo-B receptor (NgBR), Nogo-B regulates endothelial cell adhesion and migration without affecting cell proliferation, thereby promoting tumor angiogenesis

was also involved in the upregulation of Nogo-B in HCC. The AP-1 transcription factor is a dimeric complex that can be modulated by the dimer composition, which contributes to the determination of cell fate. For example, the c-Jun protein can form a homodimer and binds to the cis-elements in the promoter of target genes, while c-Fos needs to form a heterodimer with the other AP-1 family members, such as c-Jun.²⁴ In this study, we found that c-Fos, but not c-Jun, upregulated Nogo-B expression. Considering the previous studies, VEGF seems to be more likely to be regulated by the c-Fos subunit of AP-1 in angiogenesis.^{17,21} This could also be a regulatory feature of Nogo-B. Nevertheless, the counterpart of c-Fos in AP-1 heterodimers in these two pathways still needs further investigation. As c-Fos could be promoted by hypoxia⁵⁵ or low glucose,⁴⁵ the stimuli initiating c-Fos expression are worthy of examination in following studies. Moreover, the coordination between HIF-1 and AP-1 in the modulation of both Nogo-B and VEGF transcription remain to be clarified.

Taking all results from our present and previous studies into consideration, it is now possible to formulate a rudimentary working model on how Nogo-B participates in the regulation of tumor angiogenesis (Figure 6). Stressing the TME, including hypoxia and nutrient deprivation, activates Nogo-B as well as VEGF expression through HIF-1, accompanied by the regulation of AP-1. Secreted Nogo-B could bind to both NgBR and integrin $\alpha_v\beta_3$ to activate downstream pathways, resulting in cell cytoskeletal changes and enhanced cell adhesion and migration. Thus, Nogo-B played a complementary and parallel role in tumor angiogenesis to that of VEGF, while sharing a common induction mechanism, which will shed light on future studies of novel effective therapeutics.

Hypoxia is one of the hallmarks of solid tumors. Overexpression of HIFs, mainly HIF-1 α , has been linked with various primary tumors.⁵⁶ However, the role of AP-1 in cancers is more complicated, as AP-1 is a homo- or heterodimer formed by members of four subfamilies, namely, Jun, Atf, Fos, and Maf. For example, c-Jun is known to be overexpressed in colorectal, pancreatic, breast, and non-small-cell lung cancers, whereas increased expression of c-Fos is more specific in endometrial, cervical, ovarian, esophageal, and hepatocellular cancers.⁵⁷ It has been suggested that lower expression of Nogo-B is correlated with the survival from lung, breast, cervical, kidney, and ovarian cancers.⁵⁸ Thus, Nogo-B is possibly regulated by HIF-1 together with or without different AP-1 members, depending on the type of cancers. Although our present study focused on the effects of HIF-1 α and c-Fos on the induction of Nogo-B in HCC, especially following our previous studies of the fascinating functions of Nogo-B in HCC progression,^{6,7} a comprehensive description of the regulatory mechanisms of Nogo-B in the other kinds of cancers will be intriguing for the further investigations.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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