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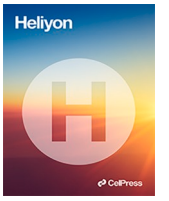

Research article

Hypoxia/inflammation-induced upregulation of HIF-1α and C/EBPβ promotes nephroblastoma cell EMT by improving HOXA11-AS transcription

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ABSTRACT

Background: Homeobox (HOX) A11 antisense RNA (HOXA11-AS) has been identified as a cancer promoting lncRNA and is overexpressed in nephroblastoma. However, how HOXA11-AS is regulated in a hypoxic inflammatory environment has not been studied.
Methods: In this study, gene expression and epithelial-mesenchymal transition (EMT) ability were detected in the nephroblastoma cell line WiT49 under conditions of hypoxia and inflammation. Next, HOXA11-AS transcription factors were predicted by datasets and subsequently confirmed by CHIP-QPCR, EMSA, and dual-luciferase reporter assays. Moreover, the regulatory relationships of HOXA11-AS and its transcription factors were further confirmed by rescue experiments.
Results: Our results showed that a hypoxic microenvironment promoted HOXA11-AS expression and nephroblastoma progression, induced EMT, and activated the Wnt signaling pathway. Combined hypoxia and inflammation had a more substantial effect on nephroblastoma than either hypoxia or inflammation alone. HIF-1α and C/EBPβ were confirmed to be the transcription factors for HOXA11-AS. Silencing of HIF-1α or C/EBPβ downregulated HOXA11-AS expression and suppressed EMT and the Wnt signaling pathway in nephroblastoma cells exposed to a hypoxic or inflammatory microenvironment. HOXA11-AS overexpression partly reversed the effect of HIF-1α or C/EBPβ knockdown.
Conclusion: We demonstrated that hypoxia/inflammation-induced upregulation of HIF-1α and C/EBPβ promoted nephroblastoma EMT by improving HOXA11-AS transcription. HOXA11-AS might be a therapy target for nephroblastoma.

1. Introduction

Nephroblastoma, also known as Wilms tumor, is one of the most common cancers in children. It currently affects approximately 1 in 10,000 children, and approximately 10 million new cases of nephroblastoma are diagnosed each year [1,2]. The current treatments for nephroblastoma are surgery, radiation, and chemotherapy. Although the survival rate of nephroblastoma patients exceeds 90%, some survivors may experience severe chronic diseases and are at high risk for disease recurrence [2–5]. Therefore, there is an urgent need to explore the molecular pathogenesis of nephroblastoma and develop new therapeutic strategies.

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Cancer is often characterized by the presence of hypoxia and inflammation. A hypoxic microenvironment is one of the important characteristics of a solid tumor, and plays an important role in the occurrence and development of tumors [6]. Hypoxia can promote tumor cell proliferation, migration, metastasis, and apoptosis [7]. An in-depth study of the hypoxic microenvironment can further our understanding of the growth characteristics of a tumor, and the diagnosis, treatment, and prognosis of cancer. Research on how a hypoxia microenvironment influences tumor occurrence, development, metastasis, response to treatment, and prognosis has received a great deal of attention. The effect of chronic inflammation is another hot spot of cancer research. As reported, chronic inflammation can promote the occurrence and development of cancer, and participate in various pathological processes related to cancer occurrence, development, and metastasis [8]. It has also been suggested that cancer is a type of chronic inflammatory disease. It is estimated that at least 15% of cancers are caused by inflammation; however the exact mechanism by which inflammation promotes cancer development remains unclear. Therefore, there is an urgent need to investigate the molecular mechanisms by which inflammation and hypoxia affect nephroblastoma and identify biomarkers for those two factors.

Long noncoding RNAs (lncRNAs) contain >200 nucleotides, and are involved in almost every biological process in the human body. Many lncRNAs have been found to play essential roles in different human cancers, and could possibly serve as therapeutic targets for certain cancers, including nephroblastoma [9,10]. Homeobox (HOX) A11 antisense RNA (HOXA11-AS), is characterized by highly conserved homeodomains, which are involved in embryo implantation, endometrial development, and cervix carcinogenesis by regulating HOXA11 [11–13]. In addition, HOXA11-AS might be a novel regulator of cancer cell proliferation and metastasis [14,15]. Some studies have revealed that HOXA11-AS is overexpressed and promotes cell migration and invasion by modulating the Wnt1/β-catenin pathway in gastric cancer [14,16]. Knockdown of HOXA11-AS was found to reverse the expression of epithelial-mesenchymal transition (EMT)-related genes in cervical cancer cells [17]. In our previous study, we revealed the effects of HOXA11-AS on nephroblastoma, in terms of apoptosis and cell cycle progression [18]. However, the mechanism by which transcription factors for HOXA11-AS regulate both EMT and the Wnt/β-catenin signaling pathway in nephroblastoma remains elusive. Considering the effects of hypoxia and an inflammatory microenvironment on cancers, we investigated whether transcription factors for HOXA11-AS affect nephroblastoma cell EMT and the Wnt/β-catenin signaling pathway under conditions of hypoxia and inflammation.

First, we detected the expression levels of HOXA11-AS, and investigated its effects on nephroblastoma progression in a hypoxic/inflammatory microenvironment. Next, we predicted the potential transcription factors and their binding sequences for the HOXA11-AS promoter, and analyzed their regulatory relationships under conditions of hypoxia and inflammation. This study is the first attempt to elucidate the regulatory mechanism of lncRNA HOXA11-AS in the hypoxic and inflammatory microenvironment of nephroblastoma. Our results suggest a potential pathogenic mechanism and therapeutic biomarker for nephroblastoma.

2. Materials and methods

2.1. Patient tissue collection

15 pairs of cancer tissues and corresponding paracancerous tissues were collected from the patients with nephroblastoma, who received surgery in our hospital. And the patients did not receive chemotherapy or radiotherapy before. The samples were used to detect the level of HOXA11-AS by qRT-PCR.

2.2. Cell culture

Monocyte THP-1, normal human embryonic kidney cells HEK-293A, nephroblastoma cell line WiT49 and SK-NEP-1 were bought from the National Collection of Authenticated Cell Cultures (Shanghai, China) and nephroblastoma cell line HFWT was bought from RIKEN BioResource Research Center. THP-1 cells were cultured in RPMI-1640 medium with 0.05 mM β-mercaptoethanol. HEK-293 and WiT49 cells were cultured in DMEM medium,. SK-NEP-1 cells were cultured in McCoy's 5A medium. HFWT cells were cultured in Ham F12 medium. All medium were added 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin, except 15% FBS for HFWT and SK-NEP-1. THP-1 cells were induced to differentiate into macrophages by treatment with phorbol ester-12-myristate-13-acetate (PMA). WiT49 cells were exposed to normoxic or hypoxic (95% N2 and 5% CO_2) conditions, and then co-cultured with macrophages in Transwell plates (Millipore, Burlington, MA, USA) to induce inflammation. THP-1-induced macrophages were seeded into the upper chamber and WiT49 cells were added to the lower chamber.

2.3. Vector construction and transfection

SiRNAs for hypoxia-inducible factor 1α (HIF-1α), CCAAT/enhancer-binding protein β (C/EBPβ), as well as for pcDNA HOXA11-AS and their negative controls, were synthesized by GenePharma (Shanghai, China). Transfections were performed using a Lipofectamine 2000 kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's instructions.

2.4. Cell viability, migration, and invasion assays

Cell viability was evaluated using the Cell counting kit-8 (CCK-8) assay. The cells were seeded into 96-well plates, incubated at 37 °C with 5% CO_2, and the OD450 values were calculated every 6 h for a period of 48 h.

Transwell assays were conducted to evaluate cell migration and invasion capabilities. Transwell plates (24 wells) and 8.0-μm pore

membranes (Corning, Corning, NY, USA) coating with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) were used for cell invasion experiments. Approximately 1.0×10^5 cells in 100 mL of serum-free DMEM were seeded into the top chamber and incubated for 6 h at 37 °C, and 500 mL of medium containing 10% FBS was added to the lower chamber. Next, the Transwell chambers were washed, fixed with glutaraldehyde, and stained with 0.1% crystal violet. Finally, five microscopic visual fields were randomly selected, and the numbers of cells in each field were counted under a x400 microscope. Cell migration assays were performed in a manner to similar to the invasion assays but without the use of FBS and Matrigel.

Cell migration was further confirmed by the wound-healing assay. Stable transfected cells were seeded into 6-well plates and cultured overnight; after which, the cell monolayer was scratched with a sterile plastic pipette tip, and then washed with culture medium. At the end of the experiment, the cells were further cultured for 48 h in medium containing 1% FBS.

2.5. Quantitative real-time PCR (qRT-PCR) analysis

The levels of *HOXA11-AS*, *HIF-1α*, and *C/EBPβ* gene expression were evaluated in WiT49 cells. Total cellular RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The PCR primers were designed and synthesized by Sangon (Shanghai, China), and are listed in Table 1 qRT-PCR analysis was conducted by using a HiScript II One Step qRT-PCR SYBR Green Kit (#Q221-01), on an ABI 7900 PCR system (Foster City, CA, USA). Relative levels of gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method, with *β-actin* serving as an internal control gene.

2.6. Enzyme-linked immunosorbent assay (ELISA)

The levels of pro-inflammatory cytokines, including interleukin-1β (IL-1β), interferon γ (IFN-γ), and tumor necrosis factor-α (TNF-α) were measured using ELISA kits (Abcam, Cambridge, UK), according to the manufacturer's instructions.

2.7. Fluorescence in situ hybridization (FISH) and immunofluorescence (IF) assays

Transfected cells were fixed with 4% formaldehyde, washed by PBS, treated with 0.5% Triton X-100, and then cultured at 4 °C for 5 min. Next, the cells were treated with a HOXA11-AS FISH probe (RiboBio; Guangzhou, China) mixed in 100 μL of hybridization buffer at 37 °C overnight. After hybridization, the slides were washed and treated with 4′6-diamidine 2-phenylindole (DAPI; Shanghai Beyotime Biotechnology Co., Ltd., China).

IF assays were conducted in a manner similar to FISH assays. Briefly, the cells were fixed with 4% formaldehyde, blocked with 5% BSA, and then incubated with anti-HOXA11 antibody (#ab72591, Abcam; 1:800), followed by incubation with a goat anti-mouse fluorescent secondary antibody (#ab150115, Abcam; 1:500) for 48 h. The cells were then stained with DAPI and observed under a fluorescence microscope (Leica, Germany). Five randomly selected visual fields in each group were observed, photographed, and analyzed for staining.

2.8. Western blot analysis

The levels of HOXA11, β-catenin, receptor–related protein 6 (LRP6), phosphorylation-LRP6 (p-LRP6), E-cadherin (E-cad), N-cadherin (N-cad), Vimentin, HIF-1α, C/EBPβ, and β-actin protein expression were assessed in WiT49 cells. The total cellular proteins were isolated with RIPA lysis buffer (#R0278, Sigma), and a standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method was used to separate the proteins [19]. Next, the separated protein bands were transferred onto membranes that were subsequently blocked, and then cultured with primary antibodies against HOXA11 (#ab54365; 1:1000), β-catenin (#ab32572; 1:800), LRP6 (#ab134146; 1:1000), p-LRP6 (#ab76417; 1:800), E-cad (#ab6528; 1:800), N-cad (#ab6258; 1:800), Vimentin (#ab92547; 1:800), HIF-1α (#ab51608; 1:1000), C/EBPβ (#ab32358; 1:1000), and β-actin (#ab32572; 1:5000) (Abcam, Cambridge, MA, USA). Next, the membranes were then washed and incubated with HRP conjugated Goat Anti-Rabbit IgG H&L (1: 5000, #ab6721, Abcam). Finally, the immunostained bands were observed using a Tanon 6600 Luminescence imaging workstation (Tanon, China).

2.9. Bioinformatics analysis

The PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) and JASPAR (http://jaspar.genereg.net/) databases were used to predict HOXA11-AS transcription factors.

Table 1
The primer list used in the qRT-PCR analysis.

Genes	Forward primer (5′-3′)	Reverse primer (5′-3′)	References
HOXA11-AS	GAGTGTTGGCCTGTCCTCAA	TTGTGCCCAGTTGCCTGTAT	Su et al. (Su et al., 2021)
HIF-1α	CCGCAACTGCCACCACTGATG	TGAGGCTGTCCGACTGTGAGTAC	Li et al. (X. Li et al., 2020)
C/EBPβ	ACAAGGCCAAGATGCGCAAC	TTCCGCAGGGTGCTGAGCT	Xi et al. (Xi et al., 2016)
β-actin	CCAACCGTGAAAAGATGACC	CCAGAGGCATACAGGGACAG	

2.10. Chromatin immunoprecipitation (ChIP)-qPCR

The ChIP-qPCR assay was performed to verify the binding of HIF-1α and C/EBPβ onto the HOXA11-AS promoter region using EZ-Magna ChIP kit (Merck Millipore, Germany). In brief, DNA-protein complexes were cross-linked with 1% formaldehyde at room temperature. 15 min later, 0.125 M glycine was added to end the cross-linking. Then, isolated the DNA-protein complexes using the buffer in the kit. Control (IgG, #ab172730) or antibodies against HIF-1α or C/EBPβ (#ab308433, #ab264305) were used to immunoprecipitated the DNA-protein complexes. DNA was eluted and purified from the complexes with a spin column, followed by PCR amplification and qPCR detection. Fold-enrichment has calculated as the ratio of amplification efficiency of HIF-1α or C/EBPβ to IgG.

2.11. Dual-luciferase reporter assay

Dual-luciferase reporter assays were performed to detect the transcription factors that bind to regions in the HOXA11-AS promoter. Truncated fragments of the HOXA11-AS promoter were amplified and inserted into a pGL3-Basic luciferase reporter (Promega, Madison, WI, USA) by using the restriction enzymes *Mlu1 I* and *Xho*I (TaKaRa, Japan); they were then ligated by use of T4 DNA ligase (TaKaRa, Japan) for subsequent transfection into WiT49 cells. Cultured cells that were 80%–90% confluent were transfected using a Lipofectamine 2000 kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). After 48 h of transfection, firefly and Renilla luciferase activities were measured using a Dual Luciferase Reporter Assay System (Promega). Relative luciferase activity was determined by using Renilla luciferase activity as an internal control.

Next, dual-luciferase reporter assays were performed to confirm the binding of HIF-1α, HOXA11-AS, C/EBPβ, and HOXA11-AS. First, the binding sequences for HIF-1α or C/EBPβ on HOXA11-AS were synthesized (WT) and mutated (Mut); after which, pGL3-promoter vectors (Thermo Fisher Scientific) containing the binding sites were constructed by GenePharma (Shanghai, China). Next, the vectors were transfected into WiT49 cells treated with control conditions or hypoxia. Finally, a Dual-Luciferase Reporter Assay System (Promega) was used to detect luciferase activity.

2.12. Electrophoretic mobility shift assay (EMSA) and shift western blotting

Biotin-labeled oligonucleotide probes specific for the HOXA11-AS-binding site of the *HIF-1α* (5′-ACGTGG-3′) and the *C/EBPβ* gene (5′-GCTCAAT-3′) were synthesized (Generay Biotech; Shanghai, China) and detected by using a DIG Gel Shift Kit (Roche, Mannheim, Germany). The competitor probes were the same as normal probes but had not biotin-conjugation. HOXA11-AS binding specificity was confirmed by mutant oligonucleotide probes (Mut competitor). Recombinant Human HIF-1α protein and HIF-1α antibody were bought from Abcam (#ab154478 and #ab308433). Nuclear extracts were incubated for 15 min at room temperature with probes, HIF-1α protein and HIF-1α antibody. Next, the complexes were separated on 6% non-denaturing polyacrylamide gels. After electrophoresis, the complexes were transferred onto nylon membranes, examined for chemiluminescence, and analyzed using an ImageQuant LAS 4000 Scanner (GE Healthcare. Chicago, IL, USA).

2.13. Statistical analysis

GraphPad Prism 8 software (San Diego, CA, USA) was used to analyze all statistical date and draw graphs. The student's *t*-test and one-way ANOVA were used to compare differences between groups, depending on the number of groups. Data are expressed as a mean value ± standard deviation (SD). A *P*-value <0.05 was considered to be statistically significant.

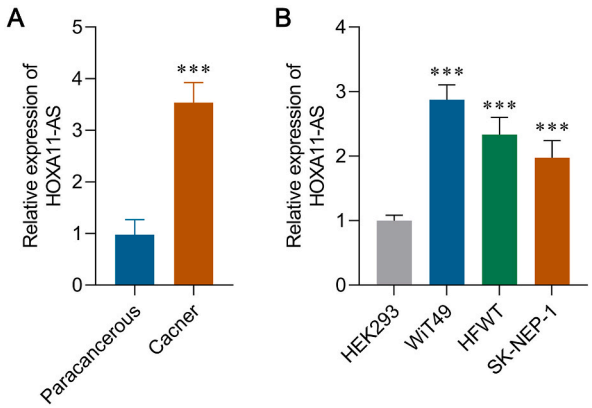

Fig. 1. HOXA11-AS was overexpressed in nephroblastoma. (A) The expression of HOXA11-AS in 15 pairs of nephroblastoma tissues and corresponding paracancerous tissues were detected by qRT-PCR. (B) The HOXA11-AS level in normal human embryonic kidney cells HEK-293 and nephroblastoma cell lines (WiT49, HFWT, SK-NEP-1) were detected by qRT-PCR.

3. Results

3.1. HOXA11-AS was overexpressed in nephroblastoma

We firstly detected the expression of HOXA11-AS in nephroblastoma tissues. The results showed that HOXA11-AS was overexpressed in nephroblastoma tissues, compared with paracancerous tissues (Fig. 1A). Next, HOXA11-AS level was also detected in normal human embryonic kidney cells HEK-293A and nephroblastoma cell lines (WiT49, HFWT, SK-NEP-1). As showed in Fig. 1B, nephroblastoma cell lines had higher HOXA11-AS expression compared with HEK-293, and WiT49 had the highest expression. Therefore, WiT49 cells were used for the following experiments.

A hypoxic microenvironment promoted HOXA11-AS expression, nephroblastoma progression, induced EMT, and activated the Wnt signaling pathway.

To investigate the role of HOXA11-AS in nephroblastoma in a microenvironment, the effects of HOXA11-AS dysregulation must be assessed in some initial experiments. Our data showed that cell viability was significantly promoted by a hypoxic microenvironment when compared with a normal microenvironment ($P < 0.001$; Fig. 2A). The hypoxic microenvironment also significantly increased the levels of HOXA11-AS expression ($P < 0.001$; Fig. 2B). Because HOXA11-AS expression during hypoxia reached a maximum level at 48 h, we chose 48 h as the hypoxia treatment time for subsequent experiments. To analysis the expression of HOXA11-AS and HOXA11, those expression levels were assessed by FISH or IF. Our results showed that HOXA11-AS expression was significantly upregulated, and HOXA11 was significantly downregulated in a hypoxia microenvironment when compared with a control environment (Fig. 2C and D).

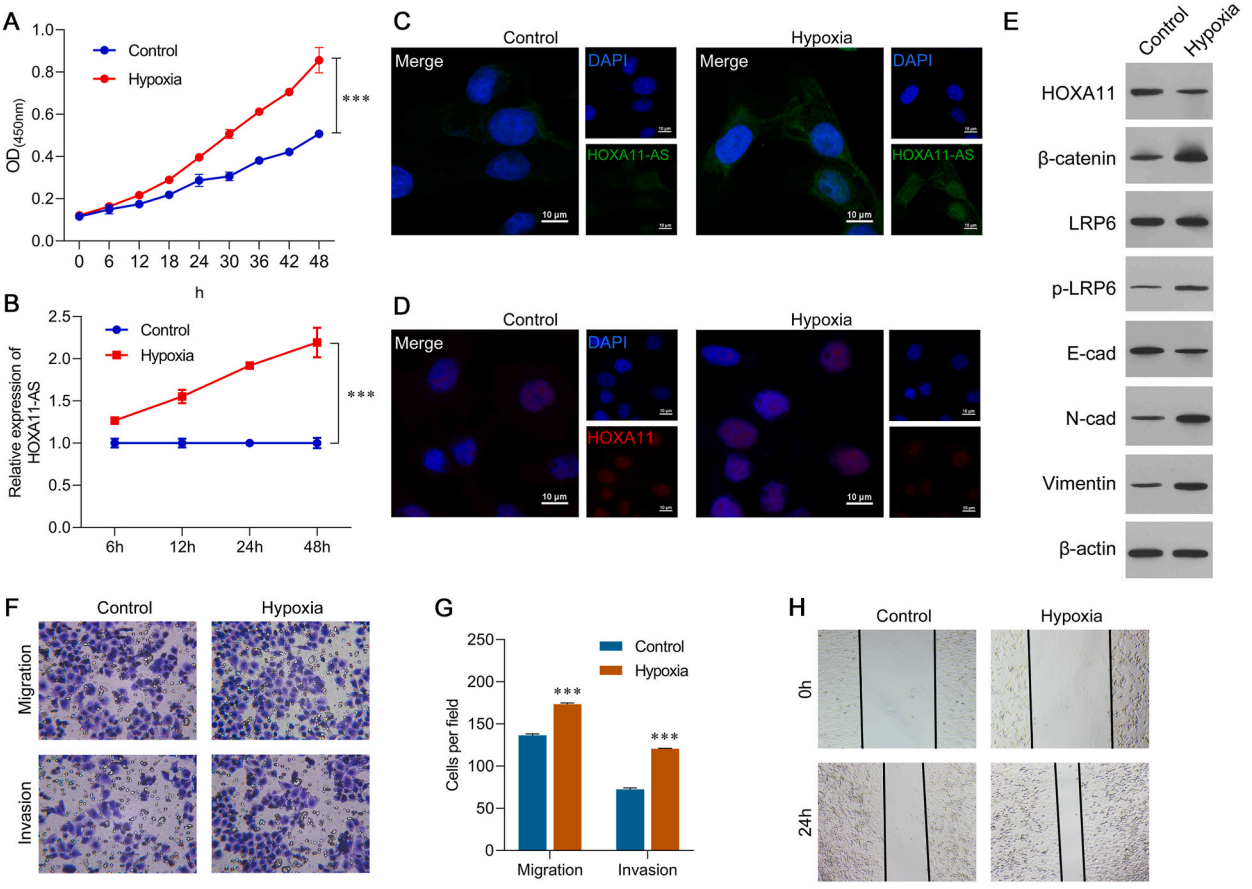

Fig. 2. A hypoxic microenvironment promoted HOXA11-AS expression, cell migration, invasion, and EMT, and activated the Wnt signaling pathway. (A) Cell viability was evaluated every 6 h until 48 h using the CCK-8 assay. (B) HOXA11-AS expression in hypoxic and control WiT49 cells was evaluated at 6 h, 12 h, 24 h, and 48 h by qRT-PCR. (C, D) FISH and IF staining for HOXA11-AS and HOXA11 expression, respectively, in WiT49 cells treated with control conditions or hypoxia (HOXA11-AS, green; HOXA11, red; DAPI, blue). (E) The levels of HOXA11, Wnt signaling pathway-related factors (β-catenin, LRP6, and p-LRP6), and EMT biomarkers (E-cad, N-cad, and Vimentin) were evaluated by western blotting, with *β-actin* serving as a housekeeping gene. (F, G) Transwell assays were performed to determine the migration and invasion capabilities of WiT49 cells treated with control or hypoxic conditions. (H) The wound-healing assay was used to assess the invasion ability of cells under conditions of a control or hypoxic microenvironment. ***$P < 0.001$ compared with Control. Full images of gels and blots were provided in Supplementary material 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Next, we confirmed the levels of HOXA11 protein expression by western blotting, and found them to be consistent with those shown by IF (Fig. 2E). The EMT-associated biomarkers E-cad, N-cad, and vimentin, as well as the Wnt signaling pathway-related factors β-catenin and LRP6 in the control and hypoxia groups were detected by western blotting. We found that the levels of E-cad protein were significantly decreased, and those of N-cad and vimentin were significantly increased by hypoxia (Fig. 2E), which demonstrated that a hypoxic microenvironment induce EMT in nephroblastoma cells. As for the Wnt signaling pathway, the levels of β-catenin and p-LRP6 protein were significantly increased (Fig. 2E), revealing an activated Wnt signaling pathway in the hypoxic microenvironment. We also evaluated the migration and invasion abilities of nephroblastoma cells in the hypoxic microenvironment, and found that hypoxia significantly promoted migration and invasion ($P < 0.001$; Fig. 2F–H). When taken together, our data showed that a hypoxic microenvironment promoted HOXA11-AS expression, cell viability, and EMT, and also activated the Wnt signaling pathway.

3.2. Combined hypoxia and inflammation had a stronger effect on nephroblastoma EMT than either hypoxia or inflammation alone

To further investigate the combined effect of hypoxia and inflammatory on nephroblastoma, THP-1 cells were induced to transform into macrophages, which were subsequently co-cultured with WiT49 cells to induce inflammation. The inflammation microenvironment was confirmed by detection of inflammatory cytokines. As shown in Fig. 3A, the levels of inflammatory cytokines (IL-1β, IFN-γ, and TNF-α) were higher in the Hypoxia and Co-culture groups, and highest in the Co-culture + Hypoxia group superior to the control group ($P < 0.001$). We found that HOXA11-AS expression was significantly upregulated in the co-culture microenvironment ($P < 0.05$; Fig. 3B). It is worth noting that a combined hypoxic and inflammatory microenvironment significantly promoted HOXA11-AS expression as compared with either a hypoxic or inflammatory microenvironment alone ($P < 0.001$; Fig. 3B). Furthermore, the levels of HOXA11-AS expression detected by FISH analysis were consistent with those shown by qRT-PCR (Fig. 3C)., Co-culture + hypoxia produced a stronger inhibitory effect on HOXA11 expression than did hypoxia and co-culture (Fig. 3D). Next, we verified the levels of HOXA11 expression by western blotting (Fig. 4A), and found they agreed with those shown by IF analysis. As shown in Fig. 4A, E-cad expression was the lowest in Co-culture + Hypoxia group, while the levels of N-cad and vimentin expression were the

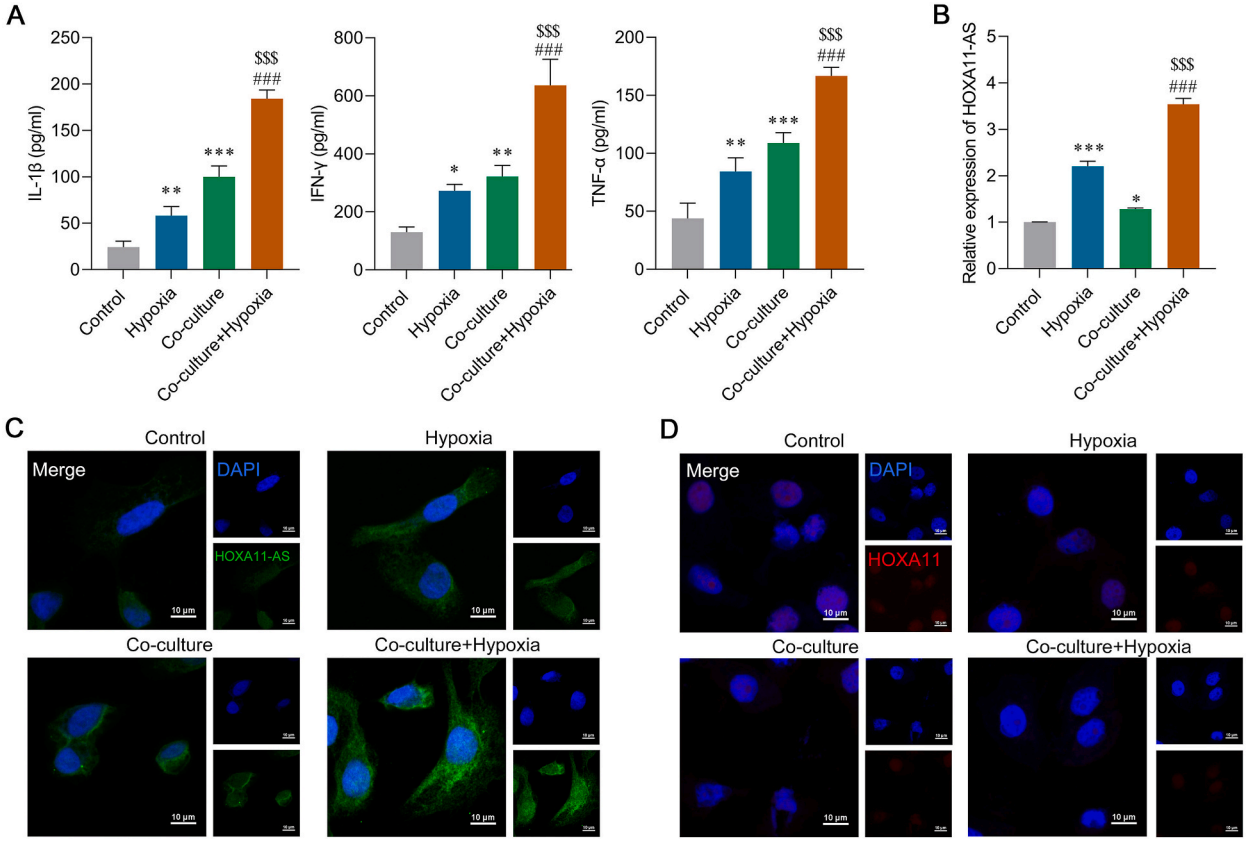

Fig. 3. Combined hypoxia and inflammation had a stronger effect on HOXA11-AS and HOXA11 expression than either hypoxia or inflammation alone. (A) The levels of inflammatory cytokines (IL-1β, IFN-γ, and TNF-α) were measured by ELISA. (B) HOXA11-AS expression was evaluated by qRT-PCR in the Control, Hypoxia, Co-culture, and Hypoxia + Co-culture groups. (C, D) FISH and IF staining for HOXA11-AS and HOXA11, respectively, in WiT49 cells (HOXA11-AS, green; HOXA11, red; DAPI, blue). *$P < 0.05$ *vs.* Control. **$P < 0.01$ *vs.* Control. ***$P < 0.001$ *vs.* Control. $$$ $P < 0.001$ *vs.* Hypoxia. ###$P < 0.001$ *vs.* Co-culture. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

highest in that group. Activation of the Wnt signaling pathway was more highly promoted by co-culture + hypoxia when compared with either hypoxia or co-culture alone (Fig. 4A). The EMT process was also confirmed by Transwell and wound-healing assays. Those results showed that cell migration and invasion were significantly increased by either hypoxia or co-culture when compared with the control environment ($P < 0.001$; Fig. 4B–E). Moreover, treatment with co-culture + hypoxia induced higher rates of cell migration and invasion than treatment with hypoxia or co-culture alone ($P < 0.001$; Fig. 4B–E). When taken together, our data showed that a microenvironment of combined hypoxia and inflammation had a stronger effect on nephroblastoma cell EMT than a microenvironment of either hypoxia or inflammation alone.

3.3. HIF-1α and C/EBPβ were confirmed to be the transcription factors for HOXA11-AS

We next predicted and verified the transcription factors for HOXA11-AS. A database search indicated that HIF-1α and C/EBPβ were the transcription factors for HOXA11-AS, and their expression levels in hypoxic and inflammatory microenvironments were assessed to further verify their response effects. As expected, the levels of HIF-1α in cells treated with co-culture + hypoxia were greater than those

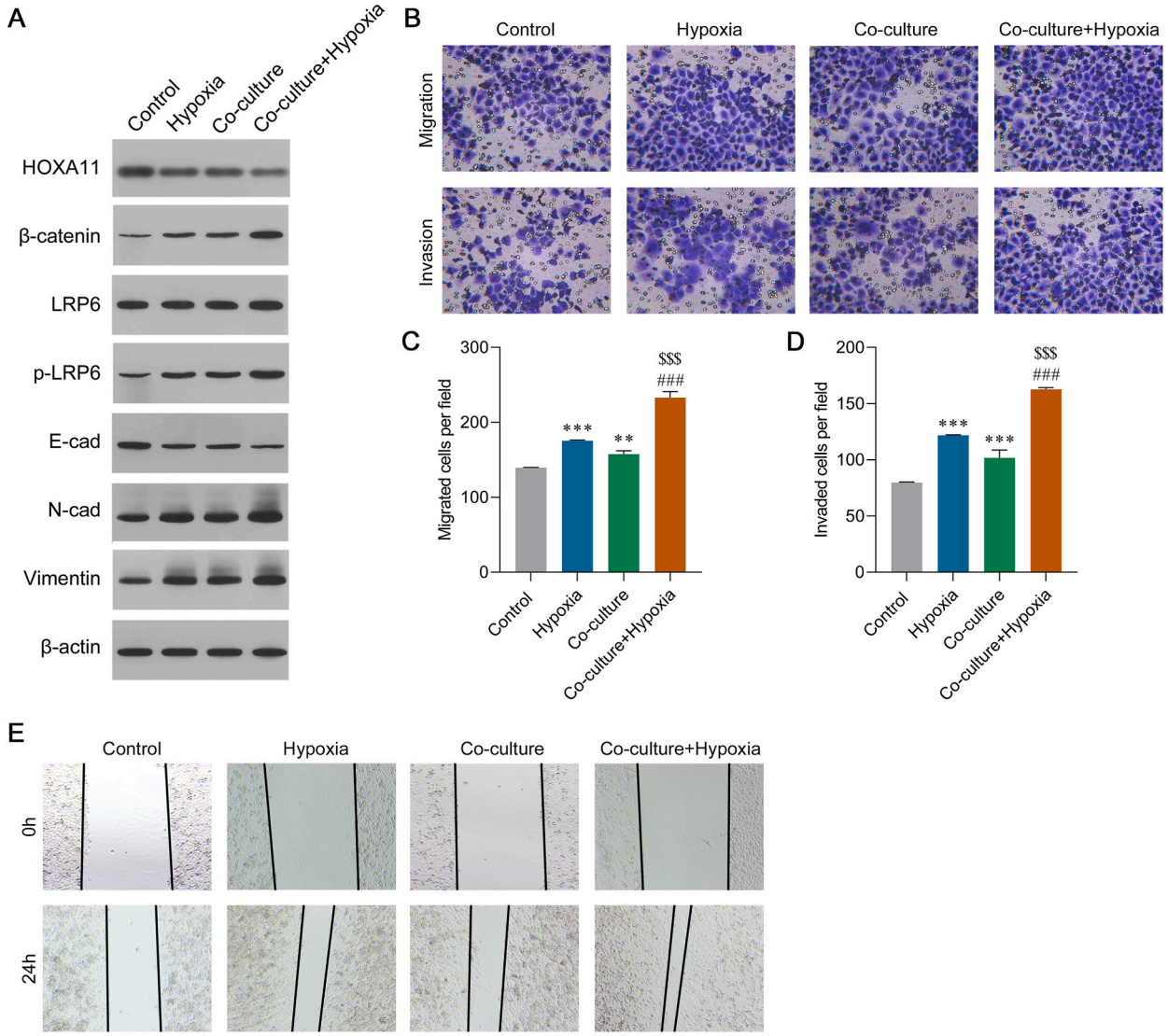

Fig. 4. Combined hypoxia and inflammation had a stronger effect on nephroblastoma EMT than either hypoxia or inflammation alone. (A) The levels of HOXA11 protein, Wnt signaling pathway-related factors (β-catenin, LRP6, and p-LRP6), and EMT biomarkers (E-cad, N-cad, and Vimentin) were evaluated by western blotting, with *β-actin* serving as a housekeeping gene (B–D) Transwell assays were performed to assess the migration and invasion of capabilities of WiT49 cells treated with control conditions, hypoxia, co-culture, and co-culture + hypoxia. (E) The wound-healing assay was used to assess the invasion ability of cells in the Control, Hypoxia, Co-culture, and Co-culture + Hypoxia groups ***$P < 0.001$ *vs.* Control. $$$ $P < 0.001$ *vs.* Hypoxia. ###$P < 0.001$ *vs.* Co-culture. Full images of gels and blots were provided in Supplementary material 3.

in cells treated with co-culture but not hypoxia, suggesting that HIF-1α is more sensitive to hypoxia than to co-culture (Fig. 5A). While, the levels of C/EBPβ expression were different from those of HIF-1α, C/EBPβ expression was significantly upregulated in the Hypoxia or Co-culture group when compared with the Control group, and was continually significantly upregulated in the Co-culture + Hypoxia group when compared with the Hypoxia or Co-culture group (Fig. 5A). Hypoxia and co-culture treatment had a similar effect on regulating C/EBPβ, expression, and their combination strengthened the effect.

The binding sequences of HIF-1α and C/EBPβ on the HOXA11-AS promoter were also predicted by databases. The sequence from

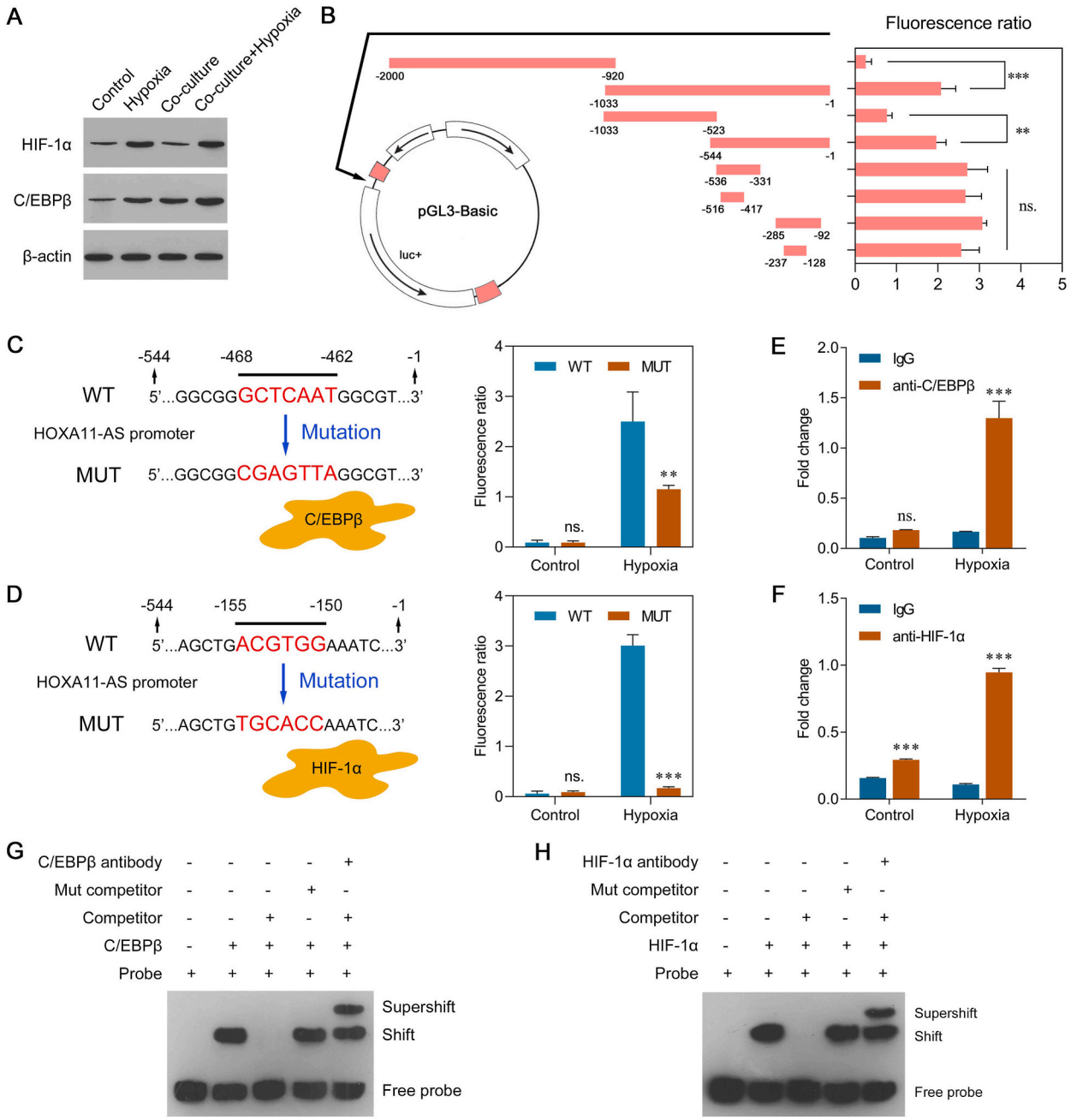

Fig. 5. Prediction and verification of HOXA11-AS transcription factors (HIF-1α and C/EBPβ). (A) The levels of HIF-1α and C/EBPβ expression were assessed by qRT-PCR. (B) HOXA11-AS promoters were amplified and inserted into the pGL3-Basic vector. (C, D) Dual-luciferase reporter activity was measured to evaluate the binding relationship between HOXA11-AS and its transcription factors (HIF-1α and C/EBPβ). (E, F) CHIP-qPCR analysis of HIF-1α and C/EBPβ binding to the HOXA11-AS locus. (G, H) Nuclear extracts were subjected to HOXA11-AS DNA binding and HIF-1α and C/EBPβ antibody assays by using the EMSA supershift assay. *$P < 0.05$ *vs.* Control. **$P < 0.01$ *vs.* Control. ***$P < 0.001$ *vs.* Control. $$$ $P < 0.001$ *vs.* Hypoxia. ###$P < 0.001$ *vs.* Co-culture. Full images of gels and blots were provided in Supplementary material 3.

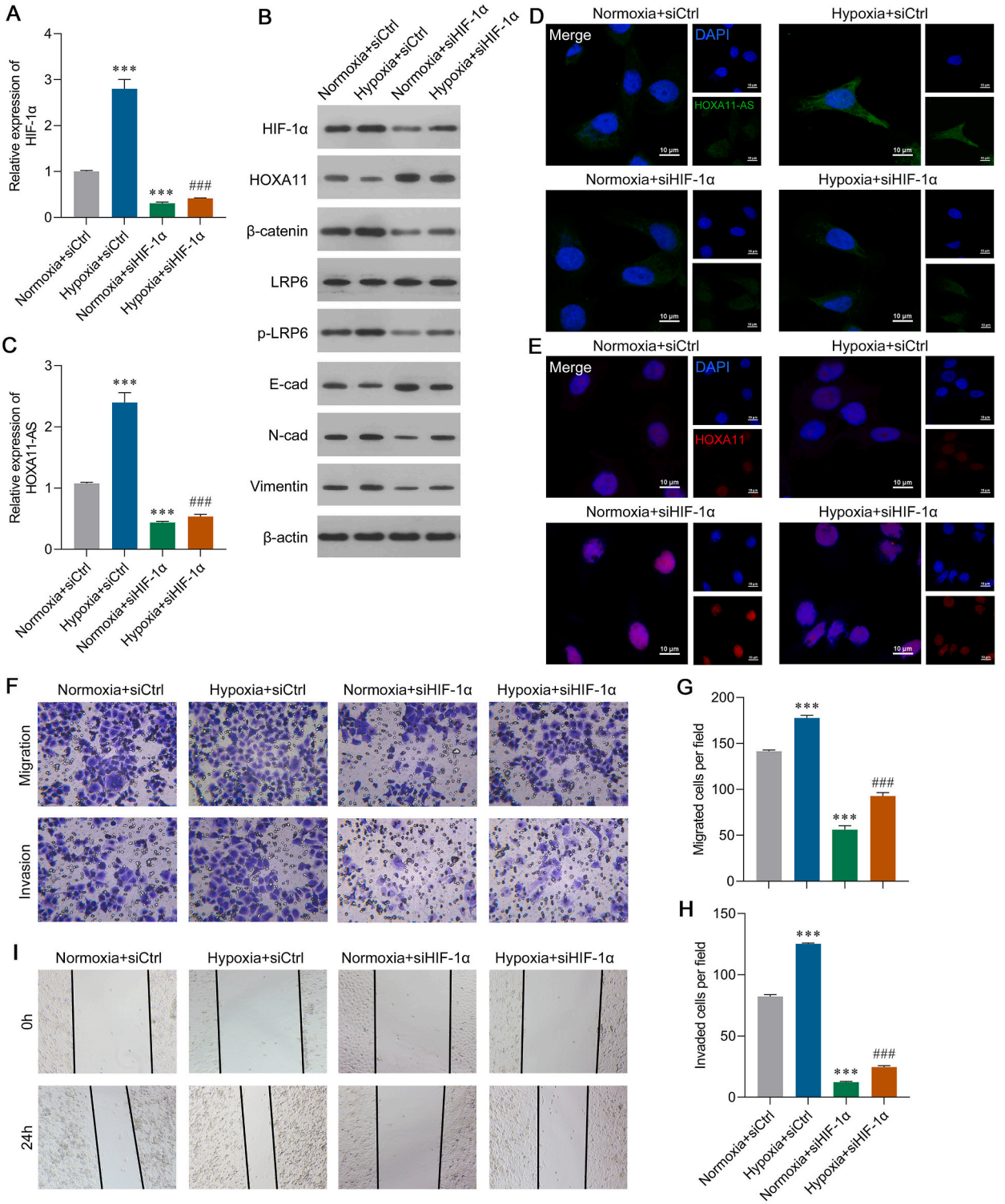

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Fig. 6. Silencing of HIF-1α downregulated HOXA11-AS expression, suppressed EMT and the Wnt signaling pathway, and inhibited the migration and invasion of nephroblastoma cells in hypoxia microenvironments. (A) HIF-1α levels in transfected cells were evaluated by qRT-PCR. (B) Western blotting was used to assess the expression levels of HIF-1α, HOXA11, EMT biomarkers (E-cad, N-cad, and Vimentin), and Wnt signaling pathway -associated proteins (β-catenin, LRP6, and p-LRP6) in transfected cells, with *β-actin* serving as a housekeeping gene. (C) HOXA11-AS expression in transfected cells was evaluated by qRT-PCR. (D, E) FISH and IF staining for HIF-1α and HOXA11-AS, respectively, in WiT49 cells (HOXA11-AS, green; HOXA11, red; DAPI, blue). (F–H) Transwell assays were performed to assess the migration and invasion capabilities of transfected cells. (I) The wound-healing assay was used to assess the invasion ability of transfected cells. ***$P < 0.001$ *vs*. Normoxia + siControl. ###$P < 0.001$ *vs*. Hypoxia + siCtrl. Full images of gels and blots were provided in Supplementary material 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

−468 to −462 was predicted to be the C/EBPβ binding region and the sequence from −155 to −150 was predicted to be the HIF-1α binding region. To confirm those hypotheses, truncated fragments of the HOXA11-AS promoters were amplified and inserted into a pGL3-Basic luciferase reporter, which was transfected into WiT49 cells for subsequent detection of luciferase activity. The results showed that the luciferase activity of pGL3 containing the HOXA11-AS promoter ranged from −516 to −417, and the region from 237 to −128 had the highest activity (Fig. 5B). To further confirm the binding of HIF-1α and HOXA11-AS, and C/EBPβ and HOXA11-AS, a luciferase reporter containing the WT and Mut promoter was designed and constructed. As shown in Fig. 5C and **D**, the fluorescence activities of the WT promoters (both C/EBPβ and HIF-1α) were obviously higher than those of the MUT promoters in the hypoxic environment. These results confirmed the binding of HIF-1α and HOXA11-AS, and C/EBPβ and HOXA11-AS, and further revealed the change in HIF-1α and C/EBPβ transcriptional regulatory ability due to hypoxia. We also performed CHIP-qPCR assays and EMSA experiments to further determine whether HIF-1α and C/EBPβ could bind to HOXA11-AS. As shown in Fig. 5E and F, CHIP-qPCR assays revealed that the fold-enrichments in anti–HIF–1α and anti-C/EBPβ binding to the promoter site were higher than that of IgG under conditions of hypoxia ($P < 0.001$). Moreover, EMSA experiments conducted with the mutant unlabeled competition probe (Mut Competitor) showed that the binding of HIF-1α and C/EBPβ to the biotinylated probe was affected (band 4 compared with band 3; Fig. 5G and H), indicating the binding of HIF-1α or C/EBPβ with HOXA11-AS. These results confirmed that HIF-1α and C/EBPβ are two transcription factors for HOXA11-AS.

3.4. A hypoxic microenvironment upregulated HOXA11-AS expression, and promoted EMT and the wnt signaling pathway in nephroblastoma cells by activating HIF-1α

We next investigated whether a hypoxic microenvironment could affect nephroblastoma cells by regulating HIF-1α and HOXA11-AS. WiT49 cells were cultured in a normoxia/hypoxia microenvironment, and then transfected with siCtrl/siHIF-1a. We found that HIF-1α expression was upregulated by hypoxia and downregulated by siHIF-1α. ($P < 0.001$; Fig. 6A and B). A hypoxic environment significantly upregulated HOXA11-AS expression, but that upregulation was reversed by siHIF-1α ($P < 0.001$; Fig. 6C). The levels of HOXA11-AS expression as determined by FISH were consistent with those shown by qRT-PCR analysis (Fig. 6D). HOXA11 expression as detected by western blotting exhibited a trend that was opposite to that of HOXA11-AS, which was upregulated by silencing of HIF-1α (Fig. 6B). That trend was consistent with that shown by IF studies (Fig. 6E). These results showed that silencing of HIF-1α downregulated HOXA11-AS expression, and upregulated HOXA11 expression. To further investigate whether hypoxia regulates EMT and the Wnt signaling pathway via HIF-1α, we examined the levels of related protein expression. Our data revealed that E-cad expression was significantly downregulated, and the expression levels of N-cad, Vimentin and Wnt signaling pathway biomarkers were significantly upregulated in the hypoxic environment. Furthermore, all those changes could be reversed by siHIF-1α (Fig. 6B). The migration and invasion abilities of cells were also significantly reduced by silencing of HIF-1α ($P < 0.001$; Fig. 6F–I). These results indicated that the hypoxic microenvironment activated HIF-1α expression, which further upregulated HOXA11-AS, and thus activated EMT and the Wnt signaling pathway in nephroblastoma cells.

3.5. An inflammatory microenvironment upregulated HOXA11-AS expression and promoted EMT and the wnt signaling pathway in nephroblastoma cells by activating C/EBPβ

After exploring the role of HIF-1α, we further investigated whether an inflammatory microenvironment affects nephroblastoma cells by regulating C/EBPβ and HOXA11-AS. WiT49 cells were cultured alone or co-cultured with THP-1-source macrophages to induce inflammation; before which, the cells were transfected with siCtrl (Control + siCtrl; Co-culture + siCtrl) or siC/EBPβ (Control + siC/EBPβ; Co-culture + siC/EBPβ). After co-culture with macrophages, the expression levels of C/EBPβ and HOXA11-AS were significantly increased, HOXA11 expression was significantly downregulated, and the EMT process and Wnt signaling pathways were significantly promoted in WiT49 cells, and all those effects were reversed by siC/EBPβ ($P < 0.001$; Fig. 7A–E). Fig. 7F–H shows that the effects of an inflammatory microenvironment on cell migration and invasion were also reversed by siC/EBPβ ($P < 0.001$). When taken together, our findings show that an inflammatory microenvironment activates C/EBPβ expression, which further regulates HOXA11-AS, and thus promotes EMT and the Wnt signaling pathway in nephroblastoma cells.

3.6. HIF-1α and C/EBPβ promoted the EMT process, the wnt signaling pathway, and nephroblastoma progression by regulating HOXA11-AS in a unidirectional manner

Finally, we further confirmed the regulatory relationship between transcription factors and HOXA11-AS. HOXA11-AS

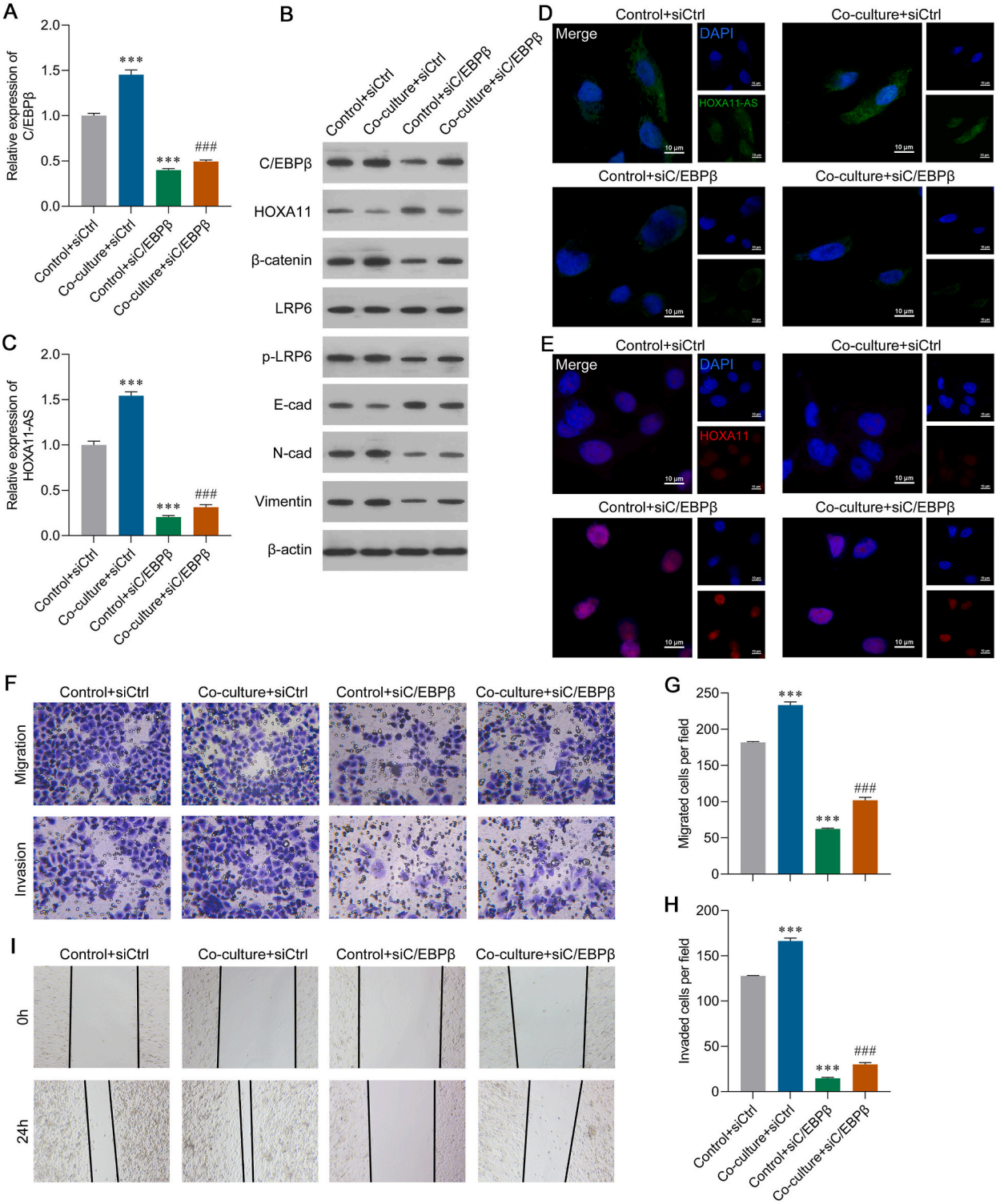

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Fig. 7. Silencing of C/EBPβ downregulated HOXA11-AS expression, suppressed EMT and the Wnt signaling pathway, and inhibited the migration and invasion of nephroblastoma cells in an inflammatory microenvironment. (A) C/EBPβ expression in transfected cells was evaluated by qRT-PCR. (B) Western blotting was used to assess the levels of C/EBPβ, HOXA11, EMT biomarkers (E-cad, N-cad, and Vimentin), and Wnt signaling pathway-associated proteins (β-catenin, LRP6, and p-LRP6) in transfected cells, with *β-actin* serving as a housekeeping gene. (C) HOXA11-AS expression in transfected cells was evaluated by qRT-PCR. (D, E) FISH and IF staining for C/EBPβ and HOXA11-AS, respectively, in WiT49 cells (HOXA11-AS, green; HOXA11, red; DAPI, blue). (F–H) Transwell were performed to assess the migration and invasion capabilities of transfected cells. (I) The wound-healing assay was used to assess the invasion ability of transfected cells. ***$P < 0.001$ *vs*. Normoxia + siControl. ###$P < 0.001$ *vs*. Hypoxia + siCtrl. Full images of gels and blots were provided in Supplementary material 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

overexpression vectors were constructed and transfected into WiT49 cells. We found that overexpression of HOXA11-AS did not affect the regulatory function of HIF-1α ($P > 0.05$; Fig. 8A and B). However, silencing of HIF-1α significantly downregulated HOXA11-AS expression ($P < 0.001$; Fig. 8C). These results indicated that HIF-1α regulates HOXA11-AS in a unidirectional manner. Western blot (Fig. 8B) and IF (Fig. 8D) studies showed that HOXA11 was upregulated after silencing of HIF-1α, and the effect could be reversed by overexpression of HOXA11-AS. siHIF-1α induced E-cad expression and inhibited N-cad, Vimentin, β-catenin, and p-LRP6 expression, and those changes were reversed by overexpression of HOXA11-AS (Fig. 8B). The migration and invasion abilities of cells were also significantly inhibited by silencing of HIF-1α, and those effects were reversed by overexpression of HOXA11-AS ($P < 0.001$; Fig. 8E–H).

As for C/EBPβ, Fig. 9A–H shows that the effects of siC/EBPβ on HOXA11 and Wnt-related protein expression, as well as on cell migration and invasion, were all reversed by overexpression of HOXA11-AS, which was similar to HIF-1α. These results further confirmed that HIF-1α and C/EBPβ were transcription factors for HOXA11-AS, and regulated nephroblastoma cell EMT, the Wnt pathway, and nephroblastoma progression via HOXA11-AS.

4. Discussion

Hypoxia and inflammation are two essential reasons for cancer. However, the molecular mechanism by which hypoxia and inflammation in a microenvironment regulate HOXA11-AS in nephroblastoma has been rarely been studied. In this study, we found that a hypoxic and inflammatory microenvironment upregulated HOXA11-AS expression, downregulated HOXA11 expression, promoted nephroblastoma progression, induced EMT, and activated the Wnt signaling pathway.

Hypoxia and inflammation are typical features of microenvironments in nearly all solid tumors. As a biomarker of hypoxia, HIF-1α was found to be overexpressed in 93%–100% of human nephroblastoma samples. Silencing of HIF-1α was shown to inhibit nephroblastoma growth and angiogenesis *in vivo* [20]. We also compared the function of WiT49 cells transfected with siHOXA11-AS or siCtrl in normoxic conditions, and the results showed that wnt signaling proteins' expressions, migration and invasion ability were inhibited by siHOXA11-AS, although the alteration of migration and invasion ability was relatively little (Supplementary material 1). This may imply that HOXA11-AS will play more functions in an abnormal environment, like hypoxia or inflammation. Liu et *al.* [21] demonstrated that echinomycin could target HIF-1α, and block the growth and metastasis of recurrent anaplastic nephroblastoma by reducing IGF1-AKT signaling. In addition to hypoxia, inflammation is also an essential factor that induces cancer. An inflammatory signaling profile was found to promote the proliferation of tumor cells, which is consistent with our present study. It also promotes a microenvironment rich in growth factors, activated inflammatory cells, and factors that support angiogenesis, migration, and invasion [21]. Although inflammation has been widely studied in many cancers, little is known about inflammation in nephroblastoma [22]. Our study found that combined treatment with hypoxia and inflammation strongly upregulated HOXA11-AS expression and promoted nephroblastoma cell EMT. We also studied the mechanism by which of hypoxia and inflammation regulate nephroblastoma.

To further reveal the mechanism by which hypoxic and inflammatory microenvironments regulate HOXA11-AS, we conducted a database search to identify transcription factors for HOXA11-AS, and the search identified HIF-1α and C/EBPβ as HOXA11-AS transcription factors. We found that C/EBPβ and HIF-1α regulate HOXA11-AS expression, induce EMT, activate the Wnt signaling pathway, and thereby promote nephroblastoma progression. The role of transcription factor HIF-1α in cancers has been intensively studied. There were differences in the numbers of normal cells and tumor cells that able to adapt and survive in an oxygen-deprived environment. During that process, HIF-1α activation generates the Warburg effect through multiple mechanisms, including enhanced glucose uptake, transcription of glycolytic enzymes, and a downregulation of mitochondrial activity [6,23]. It has been shown that HIF-1α regulates the switch from pyruvate catabolism and oxidative phosphorylation to glycolysis in both hypoxic and normoxic cells [24]. Studies conducted by Wang et al. [25] and Chen et al. [25] demonstrated that a higher level of HIF-1α expression is associated with renal cell carcinoma progression.

C/EBPβ is a transcription regulator involved in the G2/M cell cycle stage [26], that regulates cellular proliferation, differentiation, apoptosis, and tumorigenesis. C/EBPβ has been studied in several cancers, including ovarian cancer [27], breast cancer [28], and gastric cancer [29]. Yang et al. [30] revealed that TNIP1 inhibits renal cell carcinoma progression by targeting C/EBPβ. Zahid et al. [31] demonstrated that knockdown of C/EBPβ reduces inflammation, which is consistent with our present study. Inflammation-induced miR-155 inhibits the self-renewal of neural stem cells by suppressing C/EBPβ expression [32]. Those studies revealed the relationship between inflammation and C/EBPβ, and also demonstrated the importance of non-coding RNA in regulating the process via C/EBPβ. In the present study, we found that inflammation-induced C/EBPβ upregulated HOXA11-AS expression, and suppressed EMT and the Wnt signaling pathway, thus inhibiting the migration and invasion of nephroblastoma cells. Our study provides novel sights into the effects of hypoxia/inflammation-induced lncRNA on nephroblastoma progression, and may lead to new strategies for treatment of nephroblastoma.

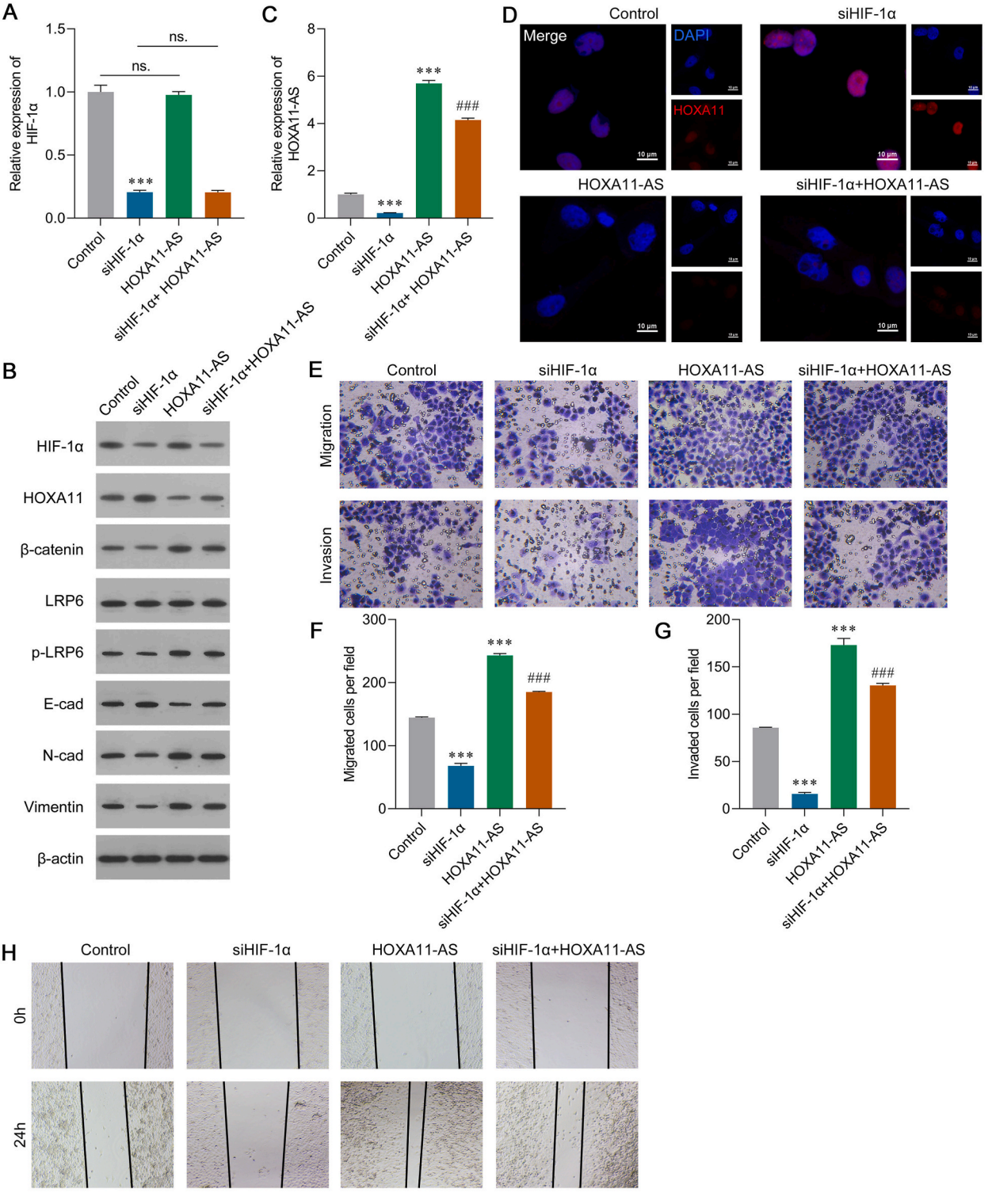

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Fig. 8. HIF-1α influenced the EMT process, Wnt signaling pathway, and nephroblastoma progression by regulating HOXA11-AS in a unidirectional manner. (A) The effects of silencing and overexpression transfections on HIF-1α expression were assessed by qRT-PCR. (B) Western blotting was used to assess the levels of HIF-1α, HOXA11, EMT, and Wnt signaling pathway proteins. (C) HOXA11-AS expression was assessed by qRT-PCR. (D) IF staining for HOXA11 in transfected cells (HOXA11, red; DAPI, blue). (E–G) Transwell assays were performed to assess the migration and invasion abilities of transfected cells. (H) The wound-healing assay was used to assess the invasion ability of transfected cells. ***$P < 0.001$ Control *vs.* ###$P < 0.001$ *vs.* siHIF-1α. Full images of gels and blots were provided in Supplementary material 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

In this study, HOXA11-AS was found to activate wnt pathway and promote cell migration and invasion of nephroblastoma cell. HOXA11-AS was also reported to induce metastasis in melanoma [33] and breast cancer [34]. And other cancers also find wnt pathway induced by HOXA11-AS, such as hepatocellular carcinoma [35] and gastric cancer [36]. Besides wnt pathway, HOXA11-AS also regulates JAK/STAT [37], PI3K/AKT [38], MEK/ERK [39]and TGF-β pathway [40], which reminds us that HOXA11-AS1 can modulate several cell processes by multiple mechanisms. LncRNA can play their role by several ways, like sponging to miRNA or binding with proteins. However, the downstream mechanism of HOXA11-AS is not studied in this study, which needs to be further explored. Moreover, clinical sample and *in vivo* experiments are needed to further confirm the role of HOXA11-AS. Many RNA-based therapies have been developed, such as siRNA, shRNA, CRISPR-Cas9 gene editing [41]. Our research provides a potential RNA-therapy target. However, it needs more clinical sample and *in vivo* experiments to further study the function of HOXA11-AS before applying it in clinic.

In summary, our present study revealed for the first time the molecular mechanism by which combined hypoxia and inflammation affect nephroblastoma. Hypoxia and inflammation were found to activate both HIF-1α and C/EBPβ expression, which in turn, promoted *HOXA11-AS* transcription, which downregulated HOXA11 expression, and promoted EMT and Wnt signaling in nephroblastoma (Supplemental material 2). The present study might provide novel sights into therapeutic biomarkers for nephroblastoma. However, these results need to be further confirmed in clinical samples and *in vivo* experiments.

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Ethics approval

This research is approved by the Ethical Committee of Guangzhou Women and Children's Medical Center (2022-041B00).

Data availability statement

The data generated in this study are available within the article.

Consent to participate

Informed consents were obtained from the patients' parents.

Consent to publish

Not applicable.

CRediT authorship contribution statement

Shibo Zhu: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Rui Zhou:** Writing – review & editing, Validation, Resources, Methodology, Formal analysis, Data curation. **Xiangliang Tang:** Writing – original draft, Validation, Methodology, Formal analysis, Data curation. **Wen Fu:** Writing – review & editing, Validation, Supervision, Software, Data curation. **Wei Jia:** Writing – review & editing, Visualization, Validation, Software, Methodology.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

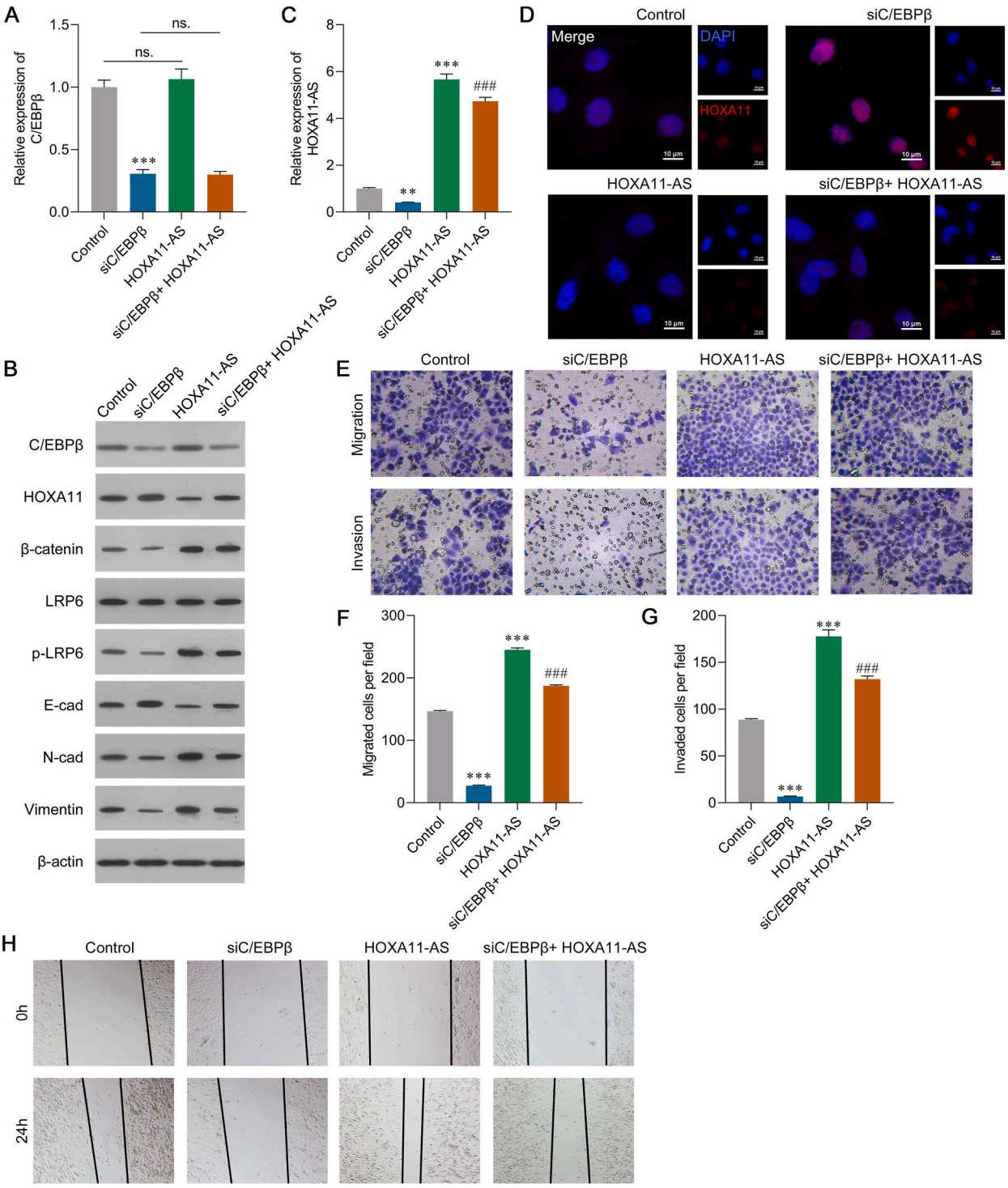

Fig. 9. C/EBPβ influenced the EMT process, Wnt signaling pathway, and nephroblastoma progression by regulating HOXA11-AS in a unidirectional manner. (A) The effects of silencing and overexpression transfections on C/EBPβ levels were assessed by qRT-PCR. (B) Western blotting was used to assess the levels of C/EBPβ, HOXA11, EMT, and Wnt signaling pathway proteins. (C) HOXA11-AS expression levels were assessed by qRT-PCR. (D) IF staining for HOXA11 in transfected cells (HOXA11, red; DAPI, blue). (E–G) Transwell assays were performed to assess the migration and invasion abilities of transfected cells. (H) The wound-healing assay was used to assess the invasion ability of transfected cells. $^{***}P < 0.001$ *vs.* Control. $^{\#\#\#}P < 0.001$ *vs.* siC/EBPβ. Full images of gels and blots were provided in Supplementary material 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Acknowledgement

A preprint has been previously published [42].

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e27654.

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