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

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Abstract

Homeobox (HOX) A11 antisense RNA (HOXA11-AS) has been identified as a cancer promoter lncRNA and overexpressed in nephroblastoma. However, how HOXA11-AS is regulated in hypoxia and inflammation environment has not been studied. In this study, genes expression and EMT ability were detected in nephroblastoma cell line WiT49 under hypoxia and inflammation environment. Then HOXA11-AS transcription factors were predicted by datasets and they were confirmed by CHIP-QPCR, EMSA and Dual-luciferase reporter assay. Next, the regulation relationships of HOXA11-AS and its transcription factors were further confirmed by rescue experiments. Our results showed that the hypoxia microenvironment promoted the expression of HOXA11-AS and nephroblastoma progression, induced epithelial-mesenchymal transition (EMT), and activated the Wnt signaling pathway. Combined hypoxia and inflammation have a more substantial effect than single hypoxia or inflammation on nephroblastoma. HIF-1α and C/EBPβ were confirmed to be the transcription factors of HOXA11-AS. Silencing HIF-1α or C/EBPβ downregulated the expression of HOXA11-AS, suppressed EMT and Wnt signaling pathway in nephroblastoma cells under hypoxia or inflammation microenvironments. HOXA11-AS overexpression partly reversed the effect of HIF-1α or C/EBPβ knockdown. In 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 of nephroblastoma.

Highlights

1. Hypoxia and inflammation microenvironment promoted the expression of HOXA11-AS and nephroblastoma progression, induced EMT, and activated the Wnt signaling pathway
2. HIF-1α and C/EBPβ were confirmed to be the transcription factors of HOXA11-AS
3. Hypoxia/inflammation-induced upregulation of HIF-1α and C/EBPβ, promoted nephroblastoma EMT by improving HOXA11-AS transcription

Introduction

Nephroblastoma, also known as Wilms tumor, is one of the most common cancer in children, affecting approximately 1 in 10,000 children, and there are about 10 million new cases every year [1, 2]. At present, the treatments for nephroblastoma are still surgery, radiation and chemotherapy. Although the survival rate of above treatment is over 90%, some of the survivors may experience severe chronic diseases and face high risk of recurrence [2–5]. Therefore, exploring the molecular mechanism pathogenesis and new therapeutic strategies of nephroblastoma remains an urgent task.

Cancer is often characterized by the presence of hypoxia and inflammation. Hypoxic microenvironment is one of the important characteristics of the solid tumor, which plays an important role in the occurrence and development of tumors [6]. Hypoxia can promote tumor cell proliferation, migration, metastasis, apoptosis [7]. The in-depth study of the hypoxic microenvironment can further understand the growth characteristics of the tumor, the diagnosis, treatment, and prognosis of cancer. The research of tumor

hypoxia microenvironment on tumor occurrence, development, metastasis, treatment and prognosis has become a hot spot for exploring new cancer treatment methods. Chronic inflammation is another hot spot. As reported, chronic inflammation can promote the occurrence and development of cancer, and participate in various pathological processes of the occurrence, development and metastasis of cancer [8]. It was also suggested that cancer is also a chronic inflammatory disease. It is estimated that at least 15% of cancers are caused by inflammation, but the exact mechanism is not yet clear. Therefore, investigating the molecular mechanism and therapeutic biomarkers of hypoxia and inflammatory on nephroblastoma is urgently needed.

Long noncoding RNAs (lncRNAs), over 200 nucleotides, are involved in almost every aspect of biological processes in the human body. Many lncRNAs have been found to play essential parts in different sorts of human cancers and could be a potential therapeutic target, 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 can be a novel regulator in cancer cell proliferation and metastasis [14–16]. Some studies have revealed that HOXA11-AS was overexpressed and promoted migration and invasion through modulating the Wnt1/β-catenin pathway in gastric cancer [15, 17]. Knockdown HOXA11-AS reversed epithelial-mesenchymal transition (EMT)-related genes in cervical cancer cells [18]. In our previous study, we revealed the role of HOXA11-AS on nephroblastoma in terms of apoptosis and cell cycle progression [19]. However, the role of transcription factors of HOXA11-AS regulates both EMT and Wnt/β-catenin signaling pathway on nephroblastoma had not been recovered. Considering the effects of hypoxia and inflammatory microenvironments on cancers, we wonder whether the transcription factors of HOXA11-AS affects nephroblastoma EMT and Wnt/β-catenin signaling pathways under hypoxia and inflammatory microenvironments.

In the present study, we investigated the role of transcription factors of lncRNA HOXA11-AS on EMT and Wnt signaling pathway of nephroblastoma under hypoxia and inflammatory microenvironments. Firstly, we detected the expression levels of HOXA11-AS, and its functions on nephroblastoma progression under hypoxic/inflammatory microenvironmental. Next, we predicted the potential transcription factors and their binding sequences of HOXA11-AS promoter, and analyzed their regulatory relationship under hypoxia and inflammatory microenvironments. This study is the first attempt to elucidate the regulatory mechanism of lncRNA HOXA11-AS under hypoxia and inflammatory microenvironments of nephroblastoma, which might provide a potential pathogenic mechanism or therapeutic biomarker for nephroblastoma.

Materials And Methods

Cell culture

THP-1 and nephroblastoma cell line WiT49 was bought from National Collection of Authenticated Cell Cultures (Shanghai, China), and cultured in minimal essential medium (MEM) supplemented with 10%

fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37 ℃, 5% CO_2. THP-1 cells were induced to differentiate to macrophages by Phorbol ester-12-myristate-13-acetate (PMA). WiT49 cells were treated in Normoxia or Hypoxia (95% N2 and 5% CO_2) conditions, and then they were co-cultured with macrophages using Transwell inserts (Millipore) to induce inflammation, in which THP-1-induced macrophages in the upper and WiT49 in the lower chamber.

Vector construction and transfection

The siRNAs of hypoxia-inducible factor 1α (HIF-1α) and CCAAT/enhancer-binding protein β (C/EBPβ), the pcDNA of HOXA11-AS, and their negative control were synthesized by GenePharma (Shanghai, China). According to the manufacturer's manual, the transfection treatment was performed using Lipofectamine 2000 kit (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Cell viability, migration, and invasion analyses

The cell viability was evaluated by Cell counting kit-8 (CCK-8) assay. The cells were seeded into 96-well plates, incubated at 37℃ with 5% CO_2, and it's OD450 values were calculated every 6h of 48h.

For cell migration and invasion experiments, the Transwell assays were conducted. The 24-well Transwell chamber and 8.0-μm pore membranes (Corning USA) coating with Matrigel (BD Biosciences) were employed for the cell invasion experiment. Approximately 1.0×10^5 cells/well in 100 mL serum-free DMEM were seeded in the top chamber for 6 h at 37℃, and 500mL medium containing 10% FBS was placed into the lower chamber. Subsequently, the Transwell was washed, fixed with glutaraldehyde, and stained with 0.1% crystal violet. Finally, five visuals were randomly selected, and the cell numbers were counted under a 400× microscope. The cell migration analysis was similar with that of invasion, except the supplementation of DMEM and culture.

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

Quantitative real-time PCR (qRT-PCR) analysis

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

Enzyme-linked immunosorbent assay (ELISA)

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

Fluorescence in situ hybridization (FISH) and immunofluorescence (IF) analyses

The transfected cells were fixed with 4% formaldehyde, washed by PBS, and supplemented with 0.5% Triton X-100, and culture at 4°C for 5min. Then, the cells were treated with HOXA11-AS FISH probe (RiboBio; Guangzhou, China) mix in 100 μL hybridization buffer at 37°C overnight. After hybridization, the slide was washed and mounted with 4'6-diamidine 2-phenylindole (DAPI; Shanghai Beyotime Biotechnology Co., Ltd., China).

The IF analysis was conducted similarly with FISH analysis. Briefly, the cells were fixed with 4% formaldehyde, blocked with 5% BSA, and incubated with anti-HOXA11 antibody (#ab72591, Abcam; 1:800) and goat anti-mouse fluorescent secondary antibody (#ab150115, Abcam; 1:500) for 48 h. The cells were stained with DAPI and observed under a fluorescence microscope (Leica, Germany). Five randomly selected visuals in each group were observed, photographed, and calculated.

Western blot and analyses

The protein expression 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 were assessed in WiT49 cells. The proteins were isolated by RIPA lysis buffer (#R0278, Sigma). Standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method was used to separate the proteins [20]. Then, the gels were transferred, blocked, and 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) bought from Abcam (Cambridge, MA, USA). The membranes were washed and incubated with the HRP conjugated Goat Anti-Rabbit IgG H&L (1: 5000, #ab6721, Abcam). Finally, the bands were observed under a Tanon 6600 Luminescence imaging workstation (Tanon, China).

Bioinformatics analysis

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 for the transcription factor prediction of HOXA11-AS with the threshold.

Chromatin immunoprecipitation (ChIP)-qPCR

The ChIP-qPCR assay was performed to verify the effect of HIF-1α and C/EBPβ on the HOXA11-AS promoter region. The experiment was carried out following a method described previously [21]. In brief, DNA-protein complexes were cross-linked with 1% formaldehyde for 15 min. Immune complexes were

formed with control (IgG) or antibodies against HIF-1α or C/EBPβ. DNA was eluted and purified from complexes, followed by PCR amplification, and qPCR detection. The fold-enrichment has calculated the ratio of amplification efficiency of HIF-1α or C/EBPβ to IgG.

Dual-luciferase reporter assay

Dual-luciferase reporter assay was firstly used to detect the transcription factors binding region of HOXA11-AS promoter. The truncated fragments of HOXA11-AS promoter were amplified and inserted into the pGL3-Basic luciferase reporter (Promega, USA) using restriction enzymes *Mlu1 I* and *Xho I* (TaKaRa, Japan), and then ligated by T4 DNA ligase (TaKaRa, Japan) for the transfection of WiT49 cells. The vector was transfected into WiT49 cells by Lipofectamine 2000 kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) at 80%–90% confluent. After 48 h transfection, firefly and renilla luciferase activities were measured using Dual Luciferase Reporter Assay System (Promega). Relative luciferase activity was determined with renilla luciferase activity as an internal control.

Next, Dual-luciferase reporter assay was used to confirm the binding of HIF-1α and HOXA11-AS, C/EBPβ and HOXA11-AS. Firstly, the binding sequences of HIF-1α or C/EBPβ on HOXA11-AS were synthesized (WT) and mutated (Mut), then the 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 and Hypoxia. Finally, one Dual-Luciferase Reporter Assay System bought from Promega Corporation (Madison, WI, USA) was employed to detect luciferase activity.

Electrophoretic mobility shift assay (EMSA) and shift western blotting

The oligonucleotide probes specific for the HOXA11-AS-binding site of the HIF-1α (5'-ACGTGG-3') and C/EBPβ gene (5'-GCTCAAT-3') were synthesized (Generay Biotech; Shanghai, China) and detected by employing DIG Gel Shift Kit (Roche, Mannheim, Germany). The HOXA11-AS binding specificity was confirmed by mutant biotin-labeled oligonucleotide probes (Biotin-Mut-probe). Then, the complexes were separated by 6% non-denaturing polyacrylamide gels. After electrophoresis, the gels were transferred to nylon membranes, detected chemiluminescent, and analyzed using an ImageQuant LAS 4000 Scanner of GE Healthcare.

Statistical analysis

GraphPad Prism 8 (San Diego, CA, USA) was used for the statistical analysis and chart drawing. Student's *t*-test and one-way ANONA were performed for comparison analyses depends on the groups. Data are exhibited as mean ± standard deviation (SD). $P<0.05$ was considered a significant difference.

Results

Hypoxia microenvironment promoted the expression of HOXA11-AS and nephroblastoma progression, induced EMT, and activated the Wnt signaling pathway

To investigate the role of HOXA11-AS on nephroblastoma under microenvironment, the dysregulation of HOXA11-AS should be assessed first. The cell viability was significantly promoted under the hypoxia microenvironment compared with the normal microenvironment (P<0.001; **Figure 1A**). The hypoxia microenvironment also significantly elevated the expression of HOXA11-AS (P<0.001; **Figure 1B**). Since the expression of HOXA11-AS in hypoxia 48h was the highest, we chose 48h as hypoxia treatment time to the next experiments. To analysis the expression of HOXA11-AS and HOXA11, they were assessed by FISH or IF. It can be observed that the expression level of HOXA11-AS was significantly upregulated, and HOXA11 was significantly downregulated under the hypoxia microenvironment compared with Control (**Figures 1C, D**). Then, we confirmed the protein expression level of HOXA11 by western blot, which was consistent with that of IF (**Figure 1E**). The EMT-associated biomarkers of E-cad, N-cad, vimentin, as well as the Wnt signaling pathway-related factors of β-catenin and LRP6 were assessed by western blot in Control and Hypoxia. We found that the protein levels of E-cad were significantly decreased, and that of N-cad and vimentin were significantly increased by Hypoxia (**Figure 1E**), which demonstrated that hypoxia microenvironment induced EMT in nephroblastoma. As for Wnt signaling pathway, the protein expression levels of β-catenin and p-LRP6 were significantly increased (**Figure 1E**), revealing an activated Wnt signaling pathway under the hypoxia microenvironment. We also evaluated the migration and invasion abilities of nephroblastoma cells under hypoxia microenvironment, and found that the hypoxia significantly promoted migration and invasion (P<0.001; **Figures 1F-H**). Taken together, we summarized that the hypoxia microenvironment promoted the expression of HOXA11-AS, elevated cell viability and EMT, also activated the Wnt signaling pathway.

Combined hypoxia and inflammation have a stronger effect than single hypoxia or inflammation on nephroblastoma EMT

To further investigate the combined effect of hypoxia and inflammatory on nephroblastoma, THP-1 cell was induced to macrophage and co-culture with WiT49 cell to induce inflammation. The inflammation microenvironment was confirmed by detecting inflammatory cytokines. As showed in **Figure 2A**, the levels of inflammatory cytokines (IL-1β, IFN-γ, and TNF-α) were higher in Hypoxia and Co-culture groups, and highest in Co-culture+Hypoxia group superior to Control (P<0.001). We found that HOXA11-AS was significantly upregulated under the Co-culture microenvironment (P<0.05; **Figure 2B**). It was worth noting that co-hypoxia and inflammatory microenvironment significantly promoted the expression level of HOXA11-AS compared with single hypoxia or inflammatory microenvironment (P<0.001; **Figure 2B**). This expression level of the HOXA11-AS detected by FISH analysis was consistent with qRT-PCR (**Figure 2C**). As for HOXA11, Co-culture+Hypoxia exhibited a stronger inhibitor role on the expression HOXA11, followed by Hypoxia and Co-culture (**Figure 2D**). Then, we verified the expression level of HOXA11 by western blot (**Figure 3A**), which was agreed with that of IF analysis. As showed in **Figure 3A**, the expression of E-cad was the lowest in Co-culture+Hypoxia group, while the expression of N-cad and vimentin were the highest. The activation of Wnt signaling pathway was promoted by Co-culture+Hypoxia compared with Hypoxia or Co-culture (**Figure 3A**). The EMT was also confirmed by Transwell and wound-healing assay. The migration and invasion cells were significantly increased by Hypoxia or Co-culture compared with Control (P<0.001; **Figures 3B-E**). Moreover, treatment with Co-culture+Hypoxia induced

more migration and invasion cells than single treatment of hypoxia or co-culture (P<0.001; **Figures 3B-E**). Taken together, we demonstrated that the microenvironments combined with hypoxia and inflammatory have a stronger effect than single hypoxia or inflammation on nephroblastoma cells EMT.

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

Next, we predicted and verified the transcription factors of HOXA11-AS. HIF-1α and C/EBPβ were identified to be the transcription factors of HOXA11-AS responding to hypoxia and inflammation microenvironments by databases, and their expressions on hypoxia and inflammation microenvironments were assessed to verify the response effect. As expected, the expression level of HIF-1α treated with Co-culture+Hypoxia exhibited a higher expression than Co-culture, but not Hypoxia, revealing that HIF-1α is more sensitive to Hypoxia but not Co-culture (**Figure 4A**). While, the expression level of C/EBPβ is different from that of HIF-1α. The expression level of C/EBPβ was significantly upregulated in Hypoxia or Co-culture compared with Control, and continually significantly upregulated in Co-culture+Hypoxia compared with Hypoxia or Co-culture (**Figure 4A**). Hypoxia and Co-culture treatments have a similar role in regulating the expression of C/EBPβ, and their combination strengthened the influence.

The binding sequences of HIF-1α and C/EBPβ on HOXA11-AS promoter were also predicted by databases, in which sequence from -468 to -462 was predicted as C/EBPβ's binding region and sequence from -155 to -150 as HIF-1α's. To confirm that, the truncated fragments of the HOXA11-AS promoters were amplified and inserted into the pGL3-Basic luciferase reporter, and were transfected into WiT49 cells for luciferase activity detection. The results showed that the luciferase activity of pGL3 containing HOXA11-AS promoter ranged from -516 to -417 and -237 to -128 were the highest (**Figure 4B**). To further confirm the binding of HIF-1α and HOXA11-AS, C/EBPβ and HOXA11-AS, the luciferase reporter containing WT and Mut promoter were designed and constructed. As showed in **Figures 4C** and **4D**, the fluorescence activities of WT were obviously higher than MUT in hypoxia environment, both C/EBPβ and HIF-1α. These results confirmed the binding of HIF-1α and HOXA11-AS, C/EBPβ and HOXA11-AS, and further revealed the change of HIF-1α and C/EBPβ transcriptional regulatory ability responding to hypoxia. We also carried out CHIP-QPCR assays and EMSA experiment to further validate whether HIF-1α and C/EBPβ were bound to HOXA11-AS. As shown in **Figures 4E, F**, CHIP-QPCR assays revealed that the fold-enrichment of anti-HIF-1α and anti-C/EBPβ binding to promoter site was higher than IgG under Hypoxia treatment (P<0.001). And EMSA experiment showed that, with the mutant unlabeled competition probe (Mut Competitor), the binding of HIF-1α and C/EBPβ to the biotinylated probe was affected (band 4 compared with band 3; **Figures 4G, H**), revealing the binding of HIF-1α or C/EBPβ on HOXA11-AS. These results confirmed that HIF-1α and C/EBPβ are two transcription factors of HOXA11-AS.

Hypoxia microenvironment upregulated the expression of HOXA11-AS, promoted EMT and Wnt signaling pathway of nephroblastoma cells by activating HIF-1α

Next, we detect whether hypoxia microenvironment affect nephroblastoma cells by regulating HIF-1α and HOXA11-AS. WiT49 cells were treated in Normoxia/Hypoxia microenvironment and transfected with

siCtrl/siHIF-1a. We found that the expression level of HIF-1α was upregulated by hypoxia and downregulated by siHIF-1α. (P<0.001; **Figure 5A, 5B**). Hypoxia environment significantly upregulated the expression level of HOXA11-AS, which was reversed by siHIF-1α (P<0.001; **Figure 5C**). The expression of HOXA11-AS by FISH was consistent with that of qRT-PCR analysis (**Figure 5D**). The expression level of HOXA11 detected by western blot exhibited opposite expression trend with HOXA11-AS, which is upregulated by silenced HIF-1α (**Figure 5B**), and was consistent with that experimented by IF (**Figure 5E**). These results showed that the silenced HIF-1α downregulated the expression of HOXA11-AS, and upregulated the expression of HOXA11. To further investigate whether hypoxia regulated EMT and Wnt signaling pathway by HIF-1α, their related protein expression levels were assessed. We found that the E-cad was significantly downregulated, and N-cad, Vimentin and Wnt signaling pathway biomarkers were significantly upregulated in Hypoxia environment, while these changes were all reversed by siHIF-1α. (**Figure 5B**). The migration and invasion abilities were also significantly reduced by silenced HIF-1α (P<0.001; **Figures 5F-5I**). These results concluded that the hypoxia microenvironment activated the expression of HIF-1α, which further upregulated HOXA11-AS, thus promoting EMT and Wnt signaling pathway of nephroblastoma cells.

Inflammation microenvironment upregulated the expression of HOXA11-AS, promoted EMT and Wnt signaling pathway of nephroblastoma cells by activating C/EBPβ

After the exploration of HIF-1α, we then further detect whether inflammation microenvironment affect nephroblastoma cells by regulating C/EBPβ and HOXA11-AS. The WiT49 cells were cultured alone or co-cultured with THP-1-source macrophages to induce inflammation, and transfected with siCtrl (Control+siCtrl; Co-culture+siCtrl) or siC/EBPβ (Control+siC/EBPβ; Co-culture+siC/EBPβ). When co-cultured with macrophages, the expression level of C/EBPβ and HOXA11-AS were significantly increased, the expression of HOXA11 were significantly downregulated, the EMT and Wnt signaling pathway were significantly promoted in WiT49 cells, while these effects were all reversed by siC/EBPβ (P<0.001; **Figures 6A-E**). It could be observed from **Figures 6F-H** that, the effect of inflammatory microenvironment on cell migration and invasion were also reversed by siC/EBPβ (P<0.001). Taken together, the inflammatory microenvironment activated the expression of C/EBPβ, which further regulates the HOXA11-AS, thus promoting EMT and Wnt signaling pathwa of nephroblastoma cells.

HIF-1α and C/EBPβ promoted the EMT, Wnt signaling pathway, and nephroblastoma progression by regulating HOXA11-AS unidirectional

Finally, we further confirmed the regulatory relationship between transcription factors and HOXA11-AS. The HOXA11-AS overexpressed vectors were constructed and transfected into WiT49 cells. We found that overexpressed HOXA11-AS do not affect regulate the expression of HIF-1α (P>0.05; **Figure 7A, 7B**). However, silencing HIF-1α significantly downregulated the expression of HOXA11-AS (P<0.001; **Figure 7C**). The results demonstrated that HIF-1α regulates HOXA11-AS unidirectional. Western blot (**Figure 7B**) and IF (**Figure 7D**) analyses showed that the expression level of HOXA11 was upregulated by silenced HIF-1α, and the effect was reversed by overexpressing HOXA11-AS. siHIF-1α induced the expression of E-

cad, and inhibited that of N-cad, Vimentin, β-catenin and p-LRP6, while these changes were reversed by overexpressing HOXA11-AS (**Figure 7B**). The migration and invasion ability were also significantly inhibited by silenced HIF-1α and reversed by overexpressing HOXA11-AS (P<0.001; **Figures 7E-H**).

As for C/EBPβ, it could be observed from **Figures 8A-8H** that the effect of siC/EBPβ on HOXA11 and Wnt-related proteins expression, migration and invasion ability were all reversed by overexpressing HOXA11-AS, which was similar to HIF-1α. These results further confirmed that HIF-1α and C/EBPβ were the transcription factors of HOXA11-AS, and regulated nephroblastoma cell EMT, Wnt pathway, nephroblastoma progression by HOXA11-AS.

Discussion

Hypoxia and inflammation are two essential reasons for cancer. However, the molecular mechanism of hypoxia and inflammation microenvironments regulating HOXA11-AS on nephroblastoma has been rarely studied. In the present study, a series of experiments investigating the molecular mechanism of HOXA11-AS on nephroblastoma was performed. Through this study, we found that hypoxia and inflammation microenvironments upregulated the expression of HOXA11-AS, downregulated HOXA11, promoted nephroblastoma progression, induced EMT, and activated the Wnt signaling pathway.

Hypoxia and inflammation are typical microenvironments feature in nearly all solid tumors. As one of the biomarkers of hypoxia, HIF-1α was detected to overexpress in 93–100% of human nephroblastoma samples. Silencing HIF-1α could inhibit nephroblastoma growth and angiogenesis in vivo[22]. Liu *et al.* demonstrated that echinomycin could target to HIF-1α, block the growth and metastasis of recurrent anaplastic nephroblastoma by reducing IGF1-AKT signaling[23]. Except for hypoxia, inflammation is also one essential factor that induces cancer. The inflammatory signaling profile promotes the proliferation of tumor cells, which is consistent with our present study. It also promotes microenvironment rich in growth factors, activated inflammatory cells and factors that support angiogenesis, migration and invasion [24].

Although inflammation has been widely investigated in many cancers, there are little about nephroblastoma[25]. Our study found that combined treatment with hypoxia and inflammation have a severe promoting role in upregulating the expression of HOXA11-AS and nephroblastoma cell EMT. Then, the mechanism of hypoxia and inflammation regulating nephroblastoma was further studied.

To further reveal the mechanism of hypoxia and inflammation microenvironments regulating HOXA11-AS, we searched the transcription factors of HOXA11-AS, and HIF-1α and C/EBPβ were identified. We found that C/EBPβ and HIF-1α regulate the expression of HOXA11-AS, induces EMT, activates Wnt signaling pathway, thereby promotes nephroblastoma progression. The role of transcription factor HIF-1α in cancers have been deeply studied. The differential of normal cells and tumors cells differed in adapting to and surviving in an oxygen-deprived environment. In this process, HIF-1α activation generated the Warburg effect through multiple mechanisms, including enhanced glucose uptake, transcription of glycolytic enzymes, and downregulation of mitochondrial activity [6, 26]. It has been shown that HIF-1α regulates the switch from pyruvate catabolism and oxidative phosphorylation to glycolysis in both

hypoxia and normoxic cells [27]. The studies of Wang *et al*. [28] and Chen *et al*. [29] demonstrated that a higher expression level of HIF-1α is associated with the renal cell carcinoma progression.

C/EBPβ is one transcription regulator involves in the cell cycle of G2/M [30] that regulates cellular proliferation, differentiation, apoptosis, and tumorigenesis. It has been studied in several cancers, including ovarian cancer [31], breast cancer [32], gastric cancer [33]. Yang *et al*. [34] revealed that TNIP1 inhibited the renal cell carcinoma progression by targeting C/EBPβ. Zahid *et al*. [35] demonstrated that knockdown C/EBPβ reduces inflammation, which is consistent with our present study. Inflammation-induced miR-155 and inhibits the self-renewal of neural stem cells through suppressing the expression of C/EBPβ [36]. These studies revealed the relationship between inflammation and C/EBPβ, as well as demonstrated the importance of non-coding RNA on regulating process through C/EBPβ. In the present study, we found that inflammation-induced C/EBPβ upregulates the expression of HOXA11-AS, and suppressed EMT and Wnt signaling pathway, thus inhibiting the migration and invasion of nephroblastoma cells. Our present study provided novel sights on hypoxia/inflammation-induced lncRNA on nephroblastoma progression, which might be essential for the nephroblastoma therapeutic.

In summary, our present study revealed the combined hypoxia and inflammation molecular mechanism on nephroblastoma for the first time. Hypoxia and inflammation activated the expression of HIF-1α and C/EBPβ, which promoted the transcription of HOXA11-AS, thus downregulated HOXA11, promoted EMT and Wnt signaling pathway in nephroblastoma. The present study might provide novel sights into the therapeutic biomarkers for nephroblastoma. However, these results need to be further confirmed in clinical sample and *in vivo* experiments.

Declarations

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Authors' contributions: SZ, WJ designed the research; SZ, SL, XT did the experiments and collected the data; SZ, ZZ analyzed and interpreted the data; SZ, WJ drafted or revised the article:

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Data Availability Statement: The data generated in this study are available within the article.

Consent to participate: Not applicable

Consent to publish: Not applicable

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Tables

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	

Figures

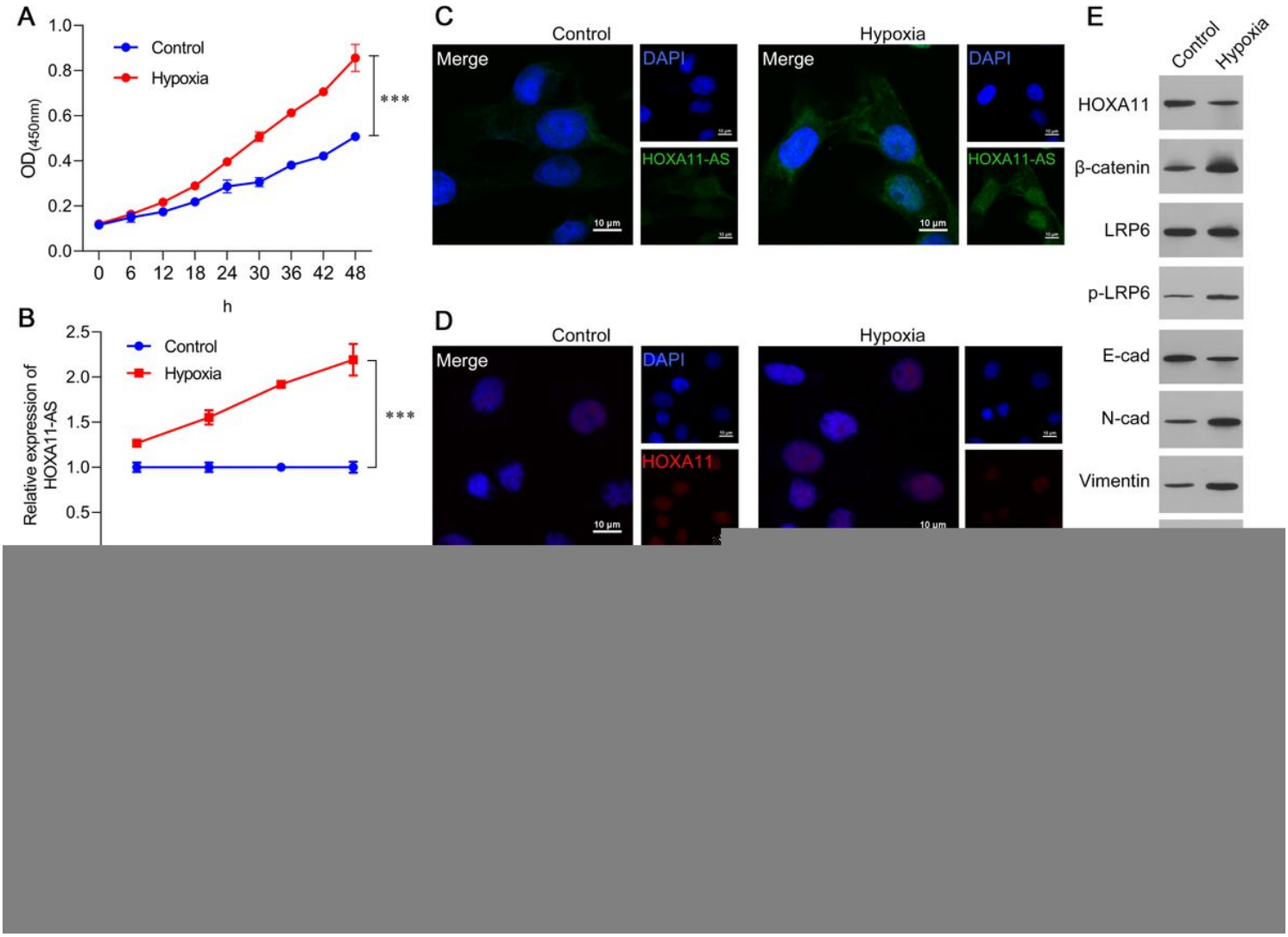

Figure 1

Hypoxia microenvironment promoted the expression of HOXA11-AS, migration, invasion, and EMT, as well as activated the Wnt signaling pathway. (A) The cell viability was evaluated every 6h until 48h by CCK-8 assay. (B) The expression level of HOXA11-AS was assessed in Control and Hypoxia in WiT49 at 6h, 12h, 24h, and 48h by qRT-PCR. (C, D) FISH and IF staining for HOXA11-AS and HOXA11 respective in WiT49 cells treated with Control and Hypoxia are shown here (HOXA11-AS, green; HOXA11, red; DAPI, blue). (E) The protein expression 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 blot, using β-actin as the housekeeping gene. (F, G) Transwell assays on measuring the migration and invasion of WiT49 cells treated with Control and Hypoxia. (H) The wound-healing assay was used for assessing the invasion ability of cells under control and hypoxia microenvironments. *** P<0.001 compared with Control.

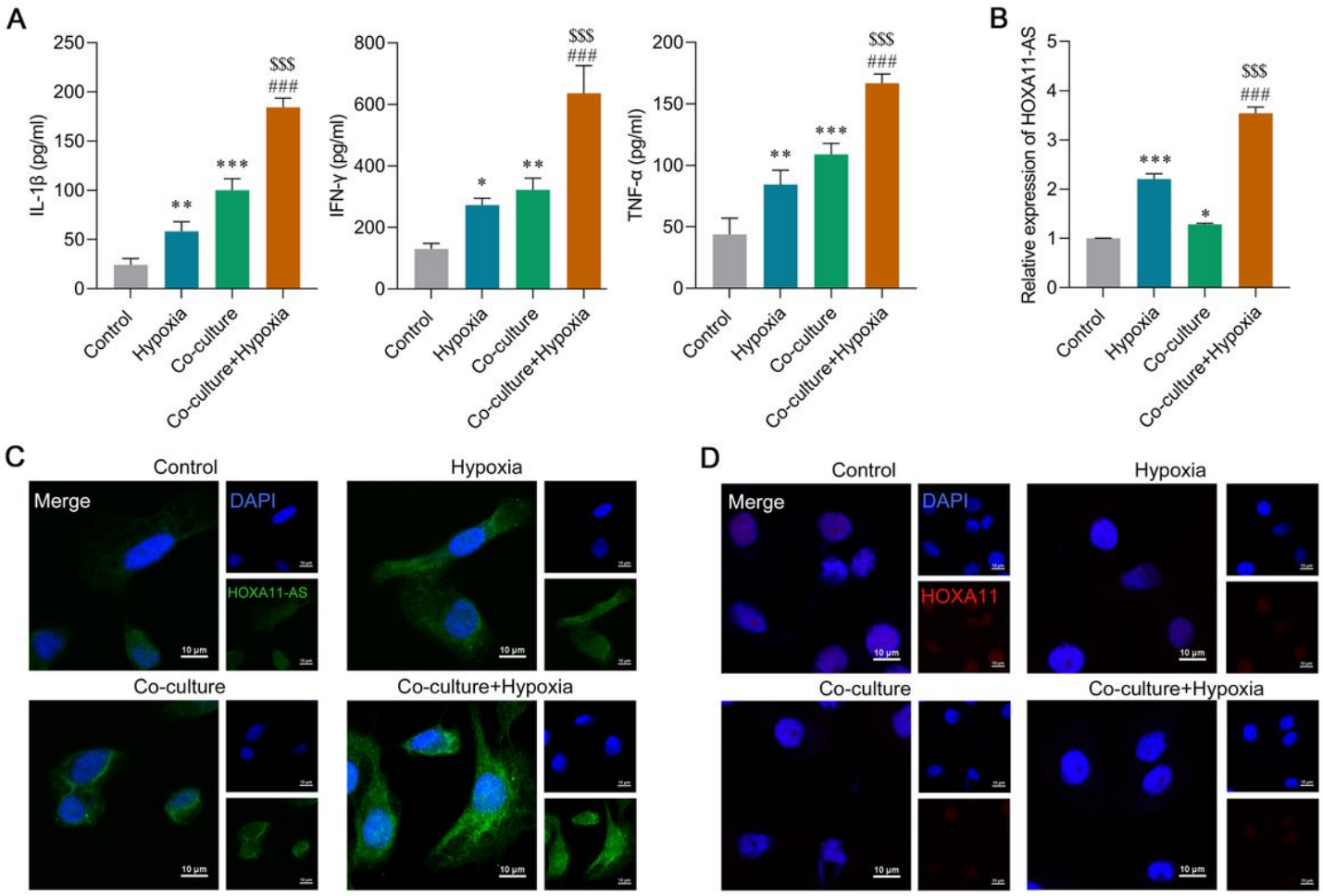

Figure 2

Combined hypoxia and inflammation have a stronger effect than single hypoxia or inflammation on HOXA11-AS and HOXA11 expression. (A) The levels of inflammatory cytokines of IL-1β, IFN-γ, and TNF-α were measured by ELISA assays. (B) The expression level of HOXA11-AS was evaluated by qRT-PCR in Control, Hypoxia, Co-culture, and Hypoxia+Co-culture groups. (C, D) FISH and IF staining for HOXA11-AS and HOXA11 respective 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.

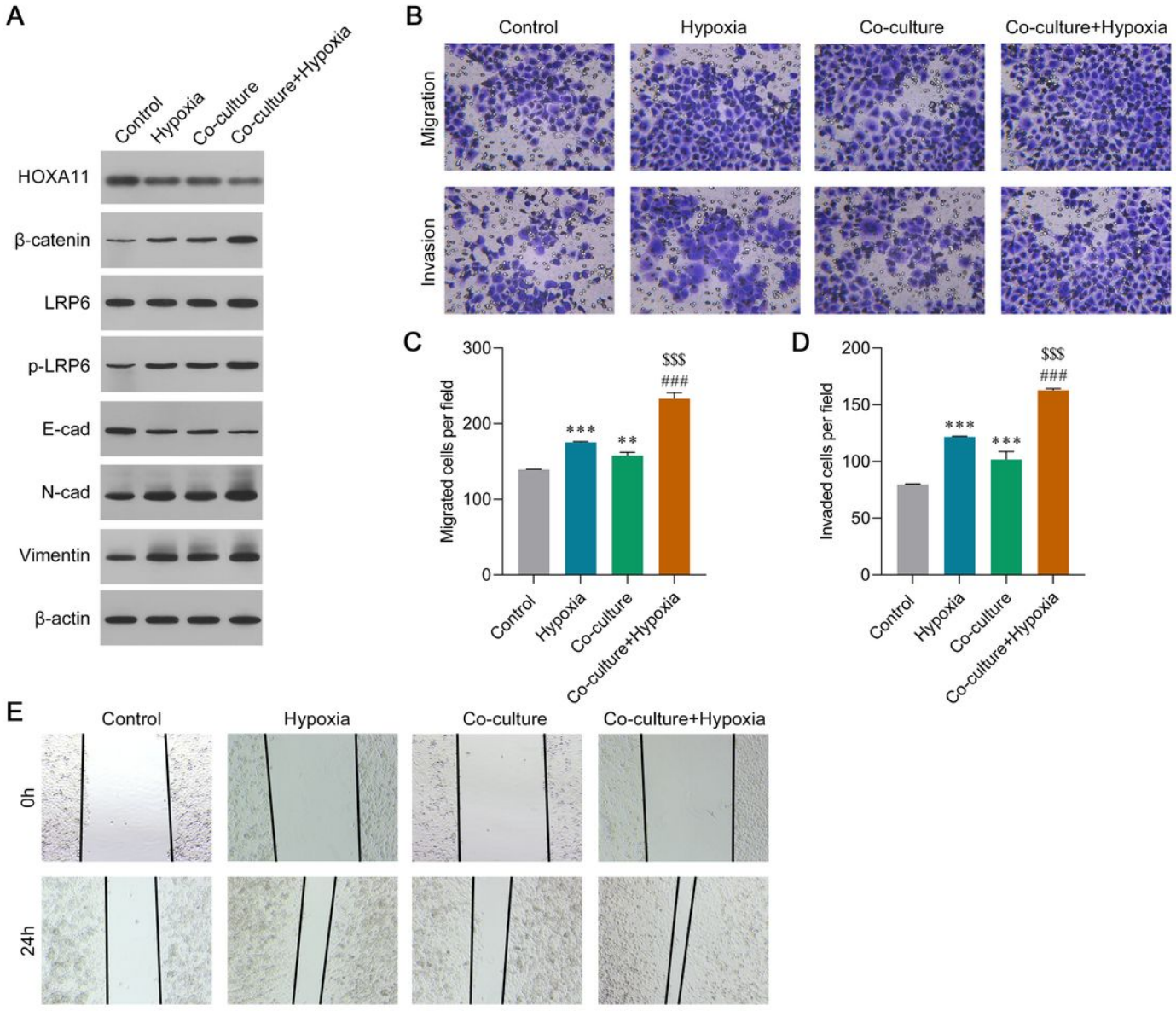

Figure 3

Combined hypoxia and inflammation have a stronger effect than single hypoxia or inflammation on nephroblastoma EMT. (A) The protein expression 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 blot, using β-actin as the housekeeping gene (B-D) Transwell assays on measuring the migration and invasion of WiT49 cells treated with Control, Hypoxia, Co-culture, and Co-culture+Hypoxia. (E) The wound-healing assay was used for assessing the invasion ability of cells under Control, Hypoxia, Co-culture, and Co-culture+Hypoxia microenvironments. *** $P<0.001$ *vs* Control. $$$ $P<0.001$ *vs* Hypoxia. ### $P<0.001$ *vs* Co-culture.

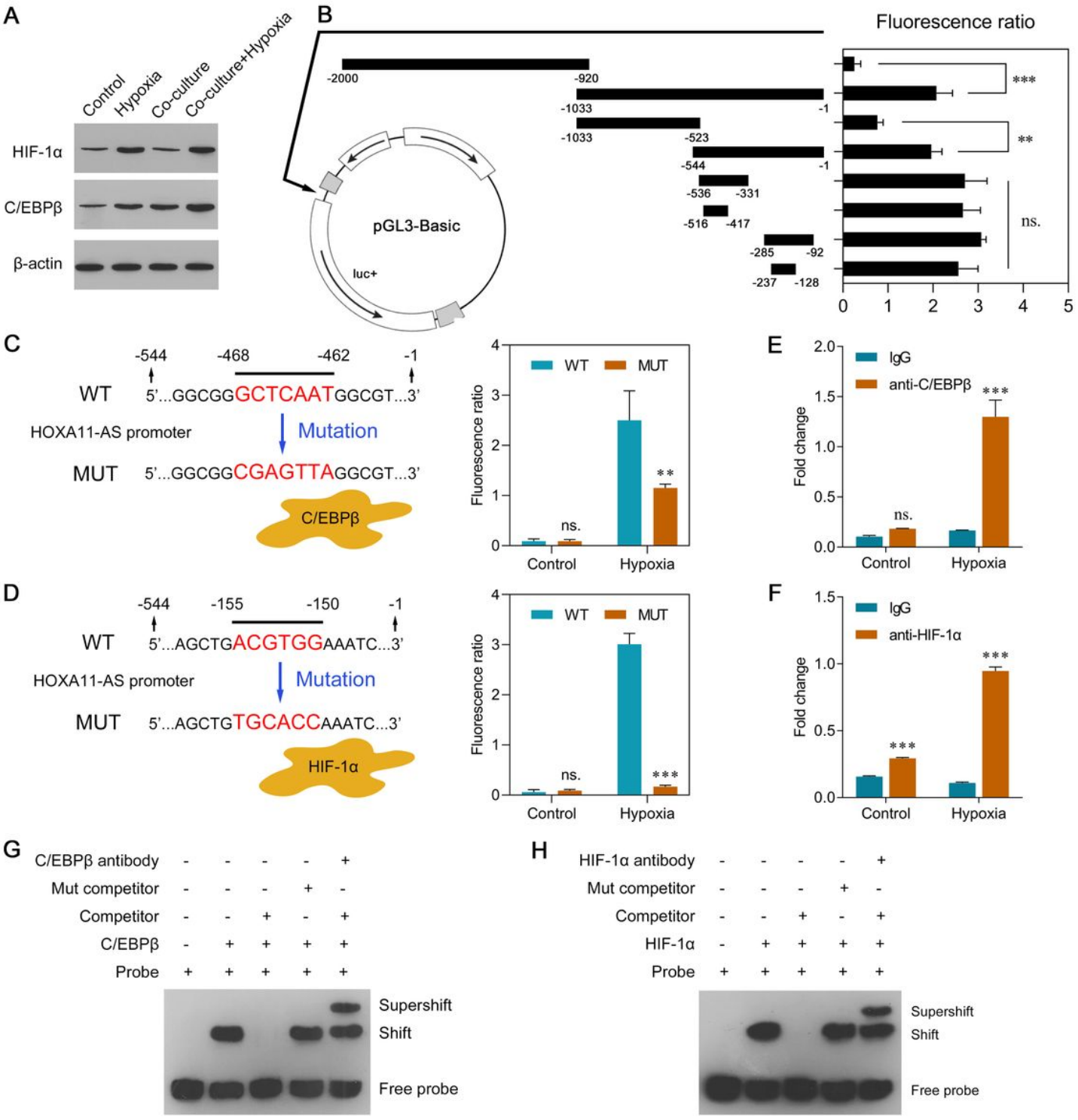

Figure 4

Prediction and verification of HOXA11-AS transcription factors (HIF-1α and C/EBPβ). (A) The expression level of HIF-1α and C/EBPβ 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 carried out to evaluate the binding relationship between HOXA11-AS and its transcription factors of 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 assay by 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.

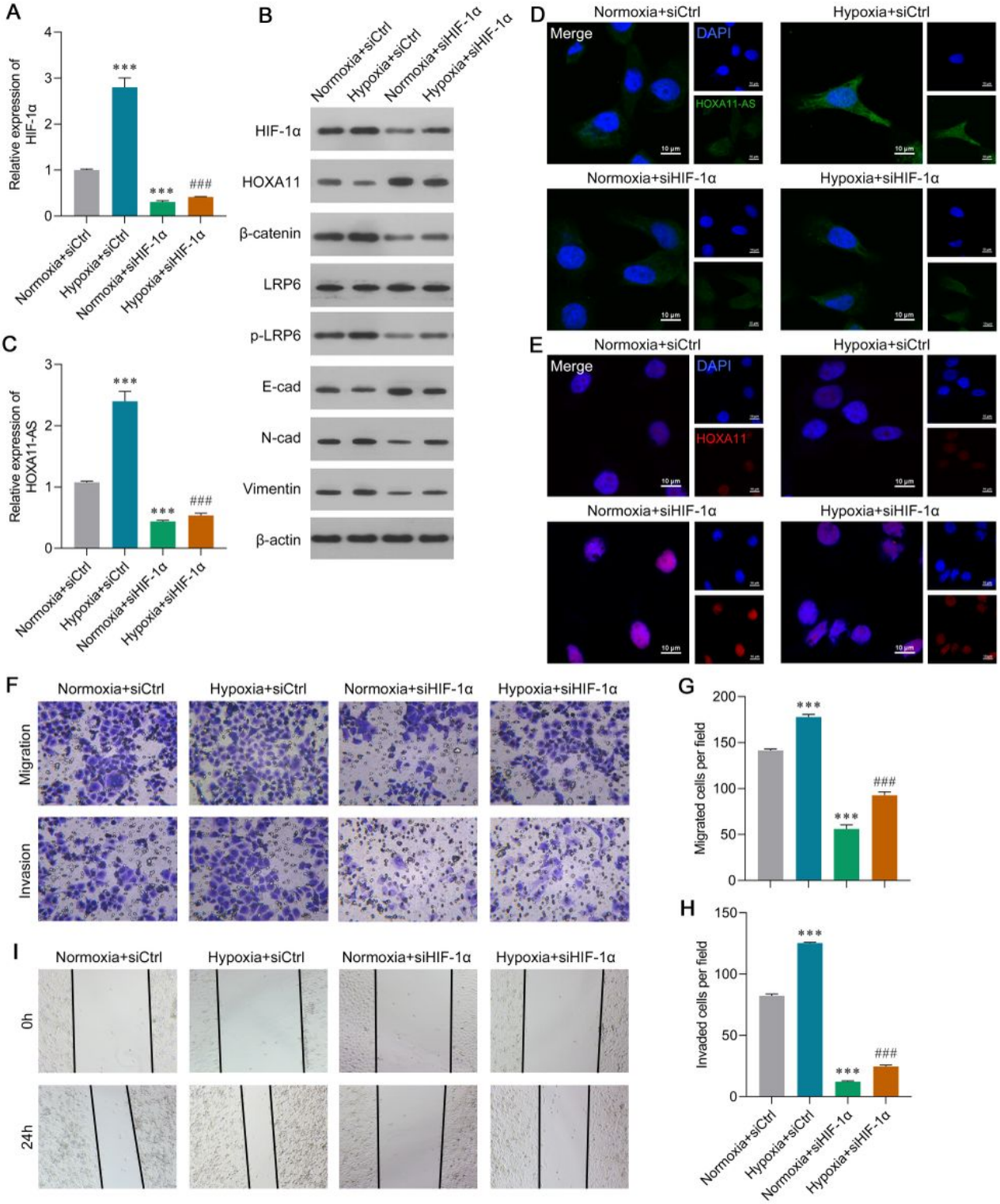

Figure 5

Silencing HIF-1α downregulated the expression of HOXA11-AS, suppressed EMT and Wnt signaling pathway, and inhibited the migration and invasion of nephroblastoma cells in hypoxia microenvironments. (A) The expression level of HIF-1α was evaluated by qRT-PCR in transfected cells. (B) Western blot on assessing the expression level 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, using β-actin as the housekeeping gene. (C) The expression level of HOXA11-AS was evaluated by qRT-PCR in transfected cells (D, E) FISH and IF staining for HIF-1α and HOXA11-AS respective in WiT49 cells (HOXA11-AS, green; HOXA11, red; DAPI, blue). (F-H) Transwell assays on measuring the migration and invasion in transfections. (I) The wound-healing assay was used for assessing the invasion ability of transfected cells. *** P<0.001 *vs* Normoxia+siControl. ### P<0.001 *vs* Hypoxia+siCtrl.

Figure 6

Silencing C/EBPβ downregulated the expression of HOXA11-AS, suppressed EMT and Wnt signaling pathway, and inhibited the migration and invasion of nephroblastoma cells under inflammation microenvironment. (A) The expression level of C/EBPβ was evaluated by qRT-PCR in transfected cells. (B) Western blot on assessing the expression level 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, using β-actin as the housekeeping gene. (C) The expression level of HOXA11-AS was evaluated by qRT-PCR in transfected cells. (D, E) FISH and IF staining for C/EBPβ and HOXA11-AS respective in WiT49 cells (HOXA11-AS, green; HOXA11, red; DAPI, blue). (F-H) Transwell assays on measuring the migration and invasion in transfections. (I) The wound-healing assay was used for assessing the invasion ability of transfected cells. *** P<0.001 *vs* Normoxia+siControl. ### P<0.001 *vs* Hypoxia+siCtrl.

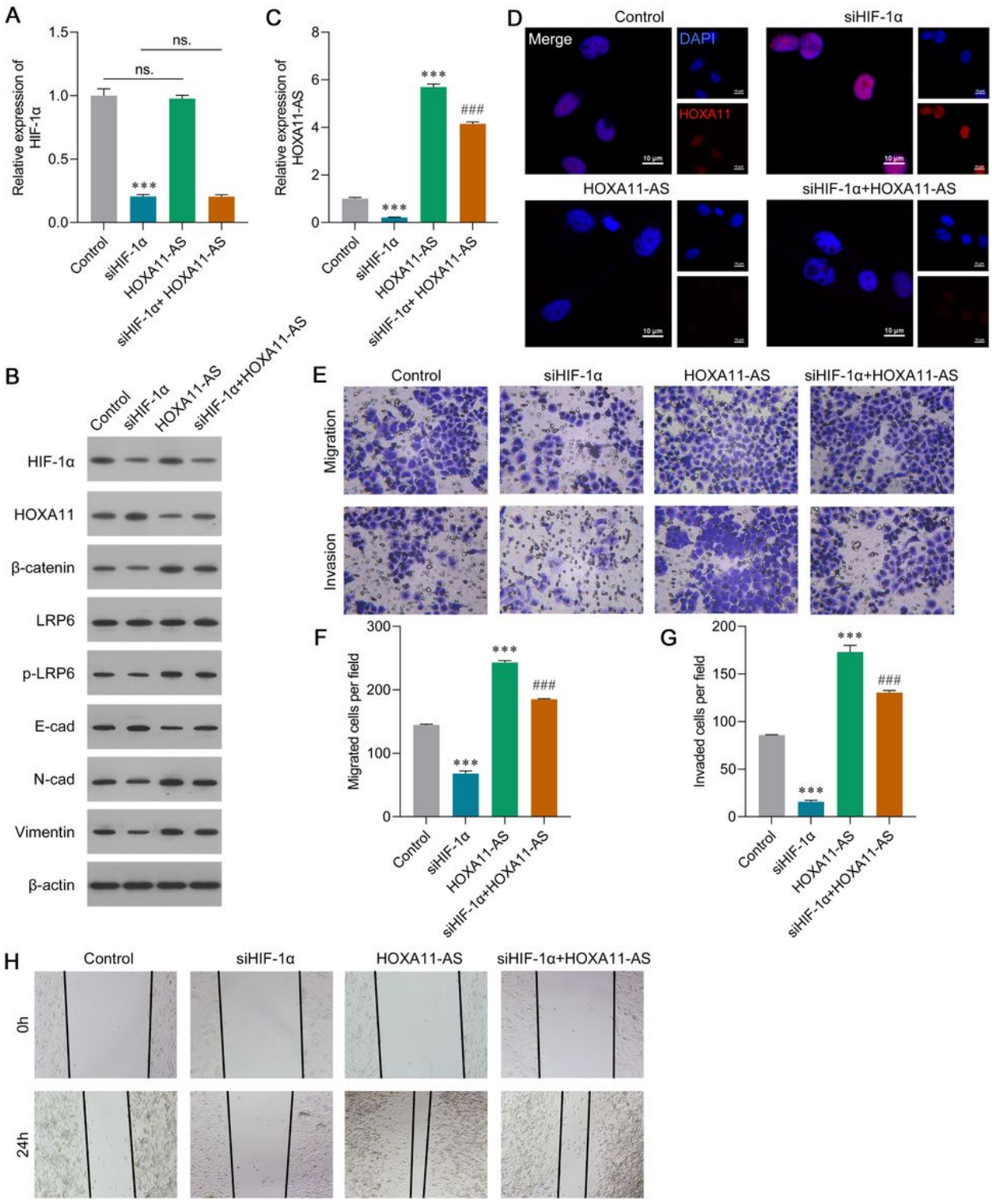

Figure 7

HIF-1α influence the EMT, Wnt signaling pathway, and nephroblastoma progression by regulating HOXA11-AS unidirectional. (A) The silencer and overexpressed transfections were constructed and the expression levels of HIF-1α were assessed by qRT-PCR. (B) Western blot analysis assessed the protein expression levels of HIF-1α, HOXA11, EMT and Wnt signaling pathway proteins. (C) The expression levels of HOXA11-AS were assessed by qRT-PCR. (D) IF staining for HOXA11 in transfections are shown here

(HOXA11, red; DAPI, blue). (E-G) Transwell assays on measuring the migration and invasion of transfected cells. (H) The wound-healing assay was used for assessing the invasion ability of transfected cells. *** *P*<0.001 *vs* Control. ### *P*<0.001 *vs* siHIF-1α.

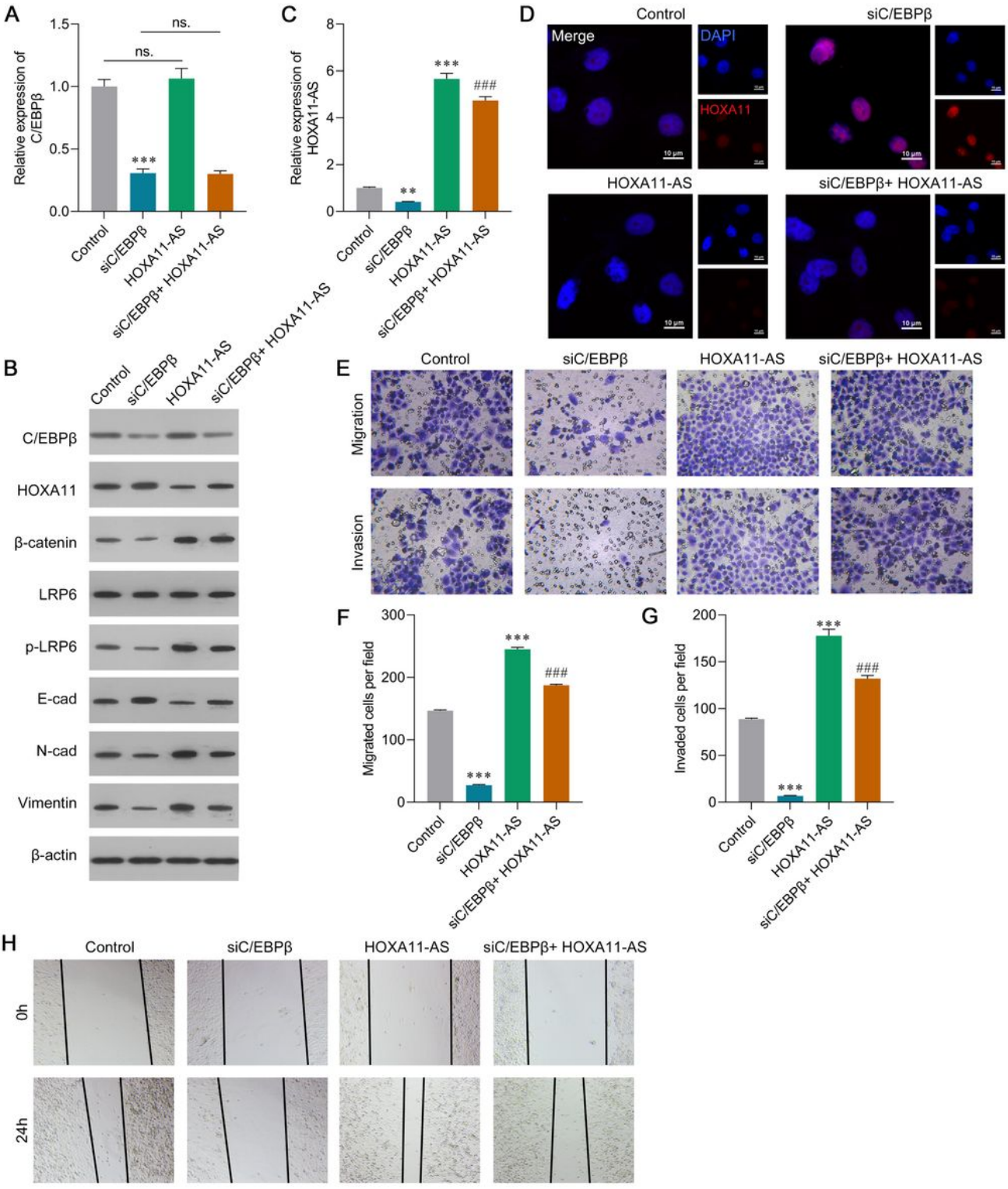

Figure 8

C/EBPβ influence the EMT, Wnt signaling pathway, and nephroblastoma progression by regulating HOXA11-AS unidirectional. (A) The silencer and overexpressed transfections were constructed and the expression levels of C/EBPβ were assessed by qRT-PCR. (B) Western blot analysis assessed the protein expression levels of C/EBPβ, HOXA11, EMT, and Wnt signaling pathway proteins. (C) The expression levels of HOXA11-AS were assessed by qRT-PCR. (D) IF staining for HOXA11 in transfections are shown here (HOXA11, red; DAPI, blue). (E-G) Transwell assays on measuring the migration and invasion of transfected cells. (H) The wound-healing assay was used for assessing the invasion ability of transfected cells. *** $P<0.001$ *vs* Control. ### $P<0.001$ *vs* siC/EBPβ.