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Hypoxia-induced fibroblast growth factor 11 stimulates capillary-like endothelial tube formation

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Abstract. Low oxygen or hypoxia can be observed in the central region of solid tumors. Hypoxia is a strong stimulus for new blood vessel formation or angiogenesis, which is essential for tumor growth and progression. Fibroblast growth factor 11 (FGF11) is an intracellular non-secretory FGF whose function has not yet been fully characterized. In the present study, we demonstrated that FGF11 expression is upregulated under hypoxic conditions in human umbilical vein endothelial cells (HUVECs). FGF11 overexpression stimulated capillary-like tube formation, yet did not affect cell migration. Notably, FGF11 markedly increased the levels of tight junction proteins including occludin, zonula occludens-1 (ZO-1) and claudin-5 in HUVECs. The FGF11 promoter contains hypoxia response elements (HREs), and hypoxia-inducible factor-1 (HIF-1) binds to HREs to activate hypoxia-related genes. We demonstrated that hypoxia or HIF-1 expression under normoxic conditions increased the luciferase activity driven by the FGF11 promoter. However, deletion of the HREs from the FGF11 promoter rendered reporter gene activity unresponsive to hypoxia or HIF-1. Taken together, we propose that FGF11 may be involved in the stabilization of capillary-like tube structures associated with angiogenesis and may act as a modulator of hypoxia-induced pathological processes such as tumorigenesis.

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Introduction

Oxygen is an essential nutrient for cellular respiration and organisms must closely monitor fluctuations in oxygen concentration to maintain homeostasis. Changes in oxygen concentration can signal events in embryonic development (1), determine stem cell fate (2,3) and contribute to pathological conditions (4,5). Inadequate oxygen supply to a tissue, or hypoxia, triggers an adaptive response mediated by hypoxia-inducible factors (HIFs) (6). Hypoxia is a common feature of malignant tumors and can be observed in central regions of solid tumors (7,8). When tumor size reaches 1-2 mm^3, the center of the tumor becomes hypoxic due to the lack of adequate blood supply, hindering tumor growth. The growth of rich, new vasculature or angiogenesis, is triggered by hypoxia and supports the growing tumor by providing nutrients and oxygen (9,10). Cellular responses to hypoxia are diverse and include changes in metabolism, antioxidant gene expression, cell proliferation, apoptosis and angiogenesis (6,8,9).

Angiogenesis is essential for tumor growth and progression (11), meaning that tumor growth could be effectively inhibited when angiogenesis is blocked (12). Angiogenesis is a multistep process that begins when quiescent endothelial cells are activated by signals from ischemic tissue or a hypoxic solid tumor. Activated endothelial cells degrade the extracellular matrix, proliferate and migrate toward the source of the stimuli, forming an immature vascular network. The newly formed network undergoes a process of maturation and stabilization that includes the recruitment of supporting mural cells, association with mural cells and placement of a new basement membrane (9,10). The angiogenic process is tightly regulated through angiogenic and anti-angiogenic factors (9).

Several factors containing heparin-binding domains, including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF/FGF-2), acidic FGF (aFGF/FGF-1) and heparin-binding EGF-like growth factor (HB-EGF) have angiogenic functions (13-15). The hypoxia-inducible factor-1 (HIF-1) transcription factor is

a key regulator of hypoxia-induced angiogenesis (6,16). HIF-1 regulates genes affecting vessel formation such as VEGF, placental growth factor and bFGF (16). We are interested in identifying and characterizing angiogenesis-related factors that are sensitive to hypoxia; in the present study we focused on the fibroblast growth factor (FGF) gene family containing heparin-binding domains.

Twenty-two members of the FGF family (FGF1-FGF23) have been reported in humans and rodents (17). Human FGF19 is the ortholog of rodent FGF15. Fibroblast growth factors (FGFs) can be classified as secretory (FGF1-FGF10 and FGF15-FGF23) or intracellular and non-secretory (FGF11-FGF14) (17,18). Most secretory FGFs and their surface FGF receptors have been well characterized and carry out defined biological roles in cell growth, differentiation and multiple developmental processes. The functions of intracellular FGFs, also referred to as FGF homologous factors (FHFs; FGF11-FGF14), remain to be explored.

In the present study, we described how FGF11 is upregulated in endothelial cells in response to hypoxia. FGF11 overexpression stimulated the formation of capillary-like tube structures in human endothelial cells and increased the levels of tight junction (TJ) proteins. The promoter region of FGF11 contains hypoxia response elements (HREs), which orchestrate FGF11 upregulation. Our results should facilitate the design of new cancer therapeutics aimed at FGF11.

Materials and methods

Cell culture and hypoxic condition. Human umbilical vein endothelial cells (HUVECs) (passages 5-8; Lonza) were cultured in M199 (Gibco) containing 20% fetal bovine serum (FBS) (Lonza), bFGF (3 ng/ml; Invitrogen), heparin (5 U/ml) and 1% penicillin/streptomycin (both from Gibco) (5). HEK293a cells (ATCC CRL-1573) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco) containing 10% FBS. For hypoxic condition, cells were incubated in a Forma hypoxia chamber (Forma Scientific), which is an anaerobic system that strictly regulates oxygen levels; cells were maintained at low oxygen tension (1% O_2, 5% CO_2 and balanced with N_2) to simulate hypoxia.

Real-time PCR and end-point PCR. Total RNA was isolated using the QIAshredder and RNeasyPlus Mini kits (Qiagen Inc.). The PrimeScript™ First Strand cDNA Synthesis kit (Takara) was used to synthesize cDNA from 1 μg of total RNA according to the manufacturer's instructions. Real-time PCR was performed using the SYBR-Green PCR Master Mix (Roche), using primers for human FGF11 as follows: forward, 5'-TGTCGCTTTAAGGAGTGCGT-3' and reverse, 5'-AGAGAAGGCTCCCGGTACAT-3'. Real-time PCR data were acquired using an ABI PRISM-7500 sequence detection system (Applied Biosystems). The 18S rRNA gene was used as a positive control and for normalization. End-point PCR for FGF11 was also performed. GAPDH was used for normalization.

Oligonucleotide primers for PCR were designed as follows: FGF11 forward, 5'-GTCACCATCCAGAGTGCCAA-3' and FGF11 reverse, 5'-CACTGTGGAGAGAAGGCTCC-3'; GAPDH forward, 5'-CATGACAACTTTGGCATTGTG-3' and GAPDH reverse, 5'-GTTGAAGTCGCAGGAGACAAC-3'. The PCR products were analyzed using a 1.2 % agarose gel.

Plasmid cloning, transfection and western blot analysis. Full-length human FGF11 was synthesized by PCR and cloned into the pcDNA3.1/HA vector (Invitrogen). Transfection was carried out using Metafectene Pro (Biontex). For western blotting, cells were harvested and lysed with lysis buffer containing protease inhibitors (Roche). Total protein (20-30 μg) was immunoblotted with antibodies specific to FGF11 (R&D Systems), zonula occludens-1 (ZO-1) (Invitrogen), occludin (Invitrogen) or claudin-5 (Abcam). α-tubulin (Calbiochem) was used as an internal control. Quantification of band intensity was analyzed using ImageJ (NIH).

Tube formation assay. The tube formation assay was performed as previously described (19). Briefly, 200 μl of growth factor-reduced Matrigel (BD Biosciences) was pipetted into a well of a 24-well culture plate and polymerized for 30 min at 37˚C. After transfection, HUVECs (1×10^4 cells/well) were seeded onto polymerized Matrigel and incubated in M199 containing 2% FBS and heparin (10 U/ml). Every hour up to 16 h, the cultures were photographed with an Olympus TH4-200 microscope. Capillary-like tube networks were observed and the branch point number was counted.

Endothelial cell migration assay. HUVECs were transfected with an FGF11-overexpression plasmid or control mock plasmid. After one day, cells were plated on 60-mm culture dishes and the migration assay was performed as previously described (20). Briefly, confluent HUVECs were wounded and incubated in M199 media with 2% FBS and 1 mM thymidine. After 16 h, HUVECs were fixed with absolute methanol for 2 min and stained with Giemsa solution for 3 min. Migration activity was quantitated by counting the number of cells that moved beyond the reference line (20).

Promoter luciferase assay. A partial genomic DNA sequence encompassing the human FGF11 promoter region bearing putative HREs was amplified by PCR and cloned into the luciferase pGL3 promoter vector (Promega). Primer information was as follows: forward, 5'-CTGCTAGCCCAACCTCTCCTTCCTACC-3' (pGL3-FGF11-HREs); forward, 5'-GTGCTAGCGGGGCTGGTTAGATTGGAG-3' (pGL3-FGF11-ΔHREs); and reverse, 5'-ATAGATCTACTAGGGCATGCTCTTGACG-3'. HEK293a cells were plated at a density of 2×10^5 cells/well of a 6-well plate and transfected with various combinations of effector plasmids. Luciferase assays were performed using the luciferase assay system kit with a GloMax luminometer (both from Promega), according to the manufacturer's instructions. Relative luciferase activity was normalized to relative light units and β-galactosidase activity.

Statistical analysis. The data are expressed as means ± standard deviations (SD). The statistical differences between the groups were compared using the unpaired t-test or the one-way analysis of variance (ANOVA). P-values ≤0.05 were considered to indicate statistically significant results.

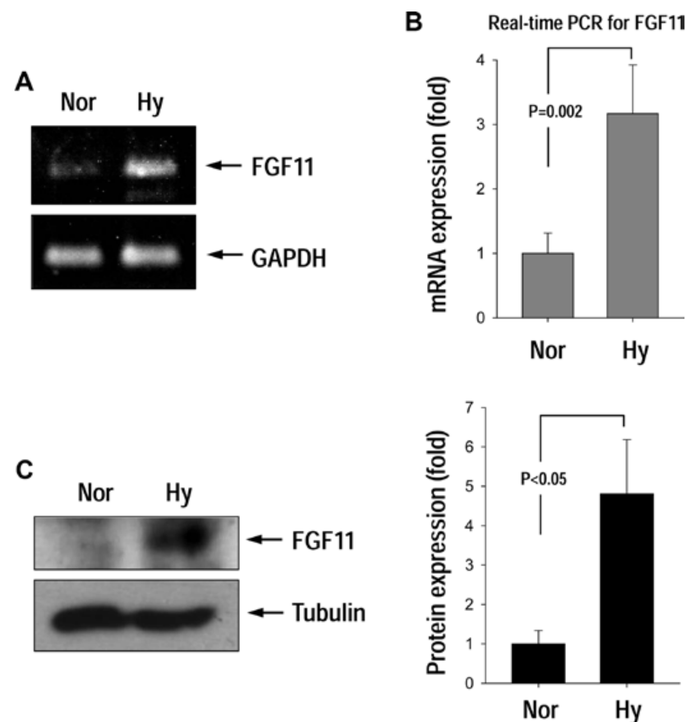

Figure 1. Hypoxia upregulates the expression of FGF11 in HUVECs. (A and B) Upregulation of FGF11 mRNA after hypoxic exposure. (A) End-point PCR for FGF11 was performed and the PCR fragments were separated by agarose gel electrophoresis. (B) Real-time PCR analysis of FGF11 mRNA expression in HUVECs cultured under hypoxic conditions (1% O_2 for 16 h, n=3). Nor, normoxia (20% O_2); Hy, hypoxia. (C) Western blotting of FGF11 after 16 h of hypoxia (left). Quantification graph (right) (n=4). FGF11, fibroblast growth factor 11; HUVECs, human umbilical vein endothelial cells.

Results

Hypoxia-induced FGF11 expression in endothelial cells. Solid tumor angiogenesis is initiated by hypoxic conditions that serve as a strong stimulus for new vessel formation (9,10). Since we are interested in identifying hypoxia-induced genes, we first investigated the mRNA expression of FGF homologous factors (FHFs; FGF11-FGF14) in HUVECs after exposure to hypoxia (1% O_2). FGF14 mRNA was not detected in HUVECs by real-time PCR. FGF12 and FGF13 expression increased slightly under hypoxia, yet their expression level was very low in HUVECs. Whereas FGF11 mRNA expression was relatively high in comparison to FGF12 and FGF13 expression (data not shown). FGF11 mRNA expression was significantly increased in response to hypoxic conditions (Fig. 1A and B). Western blotting results for the FGF11 protein suggests that the protein level was significantly increased under hypoxia (Fig. 1C).

FGF11 overexpression in HUVECs increases capillary-like tube formation. To investigate the effect of FGF11 expression on angiogenesis, tube formation and cell migration were examined for HUVECs transfected with pFGF11 (Fig. 2A). FGF11 overexpression significantly stimulated tube formation compared to the control cells (Fig. 2B and C), yet did not stimulate migration activity (Fig. 2D). HUVECs treated with basic FGF (bFGF) as a positive migration control (14) migrated normally, thus we concluded that FGF11-overexpression did not affect endothelial migration activity. Instead, FGF11 may be involved in stabilizing capillary-like tube structures.

FGF11 overexpression increases the expression of TJ proteins. Since capillary tube formation in HUVECs was increased with FGF11 overexpression, we examined whether FGF11 overexpression in endothelial cells affects the expression of TJ proteins by western blotting (Fig. 3). TJ proteins play a role in stabilizing capillary structure by maintaining adhesive cell-cell interactions (21). We found that FGF11 overexpression markedly increased the levels of the TJ proteins, such as occludin, ZO-1, and claudin-5 (Fig. 3).

Hypoxia increases the FGF11 promoter activity through HIF-1α. We further examined the novel finding that FGF11 expression was upregulated in response to hypoxic conditions by determining the mechanism through which hypoxia stimulates FGF11 expression, focusing on the FGF11 promoter. HIF-1α is a key transcription factor that activates genes involved in the hypoxic response by binding to HREs in the gene promoter region (2,16). Notably, the FGF11 promoter contains two HREs (5'-ACGTG-3') (Fig. 4A).

We determined the effects of HIF-1 on the FGF11 promoter containing two HREs (FGF11-HREs; Fig. 4B and C) using a promoter luciferase assay. Reporter gene activity was significantly increased in response to hypoxia for the cells transfected with FGF11-HREs (WT). In contrast, reporter gene activity was not changed by hypoxia for cells transfected with the HRE-deletion-fragment (ΔHRE) (Fig. 4B), suggesting that the HREs in the FGF11 promoter region are sensitive to hypoxia. To determine whether FGF11-HREs are responsive to hypoxia via the HIF-1α transcription factor, we co-transfected cells with both WT FGF11-HREs and HIF-1α under normoxic

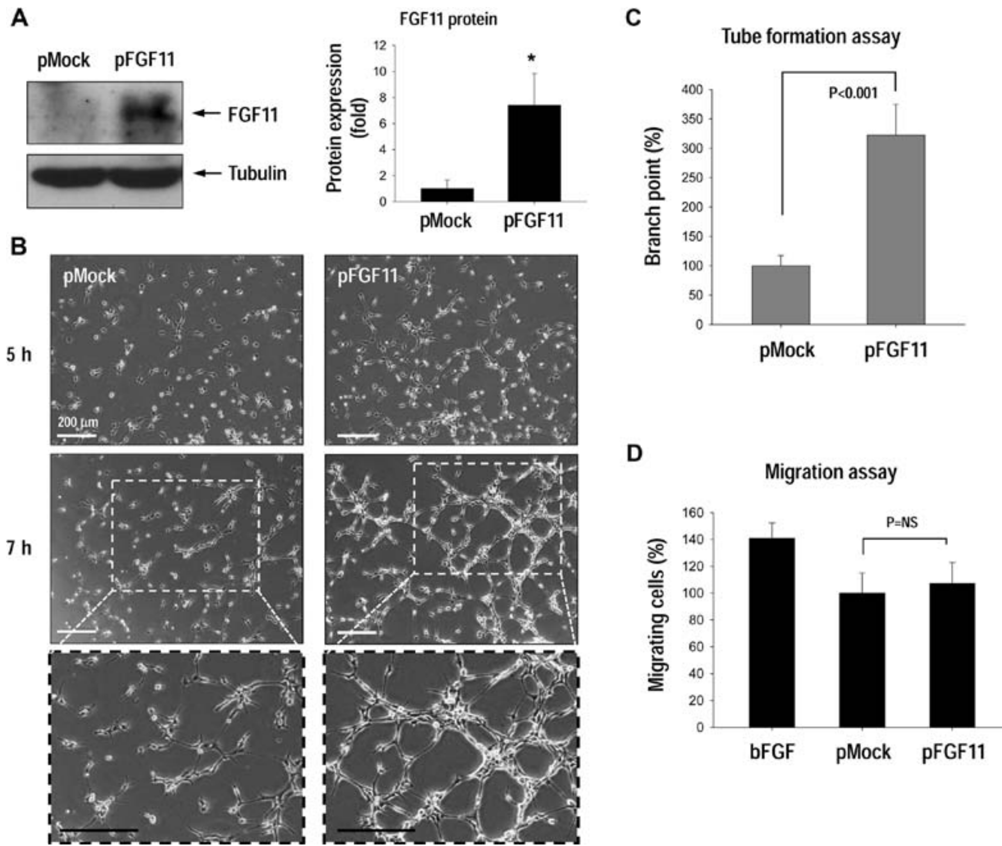

Figure 2. FGF11 overexpression increases capillary-like tube formation in HUVECs. (A) HUVECs were transfected with pFGF11 for 24 h and FGF11 overexpression was observed by western blotting (left). Quantification graph (right) (n=4, *P<0.05). (B) HUVECs transfected with pFGF11 had enhanced tube formation on Matrigel. Magnification, x100. (C) Tube formation was observed (5 h) and the branch point number was counted (n=5, P<0.001). (D) Endothelial cell migration assay. FGF11 overexpression in HUVECs did not affect migration (n=4) (NS, not significant). FGF11, fibroblast growth factor 11; HUVECs, human umbilical vein endothelial cells.

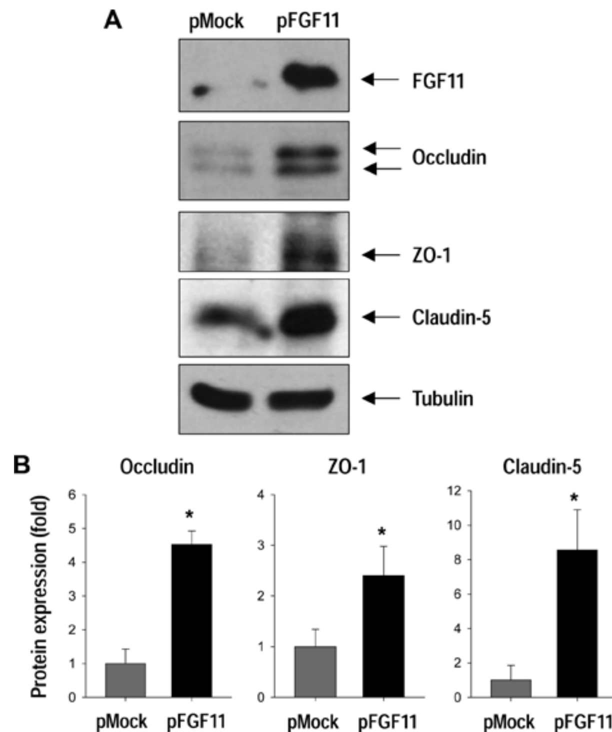

Figure 3. FGF11 regulates the expression of tight junction proteins in HUVECs. (A) HUVECs were transfected with pFGF11 for 48 h. FGF11 overexpression increased the tight junction proteins occludin, ZO-1 and claudin-5. (B) Western blotting quantification (n=4 each, *P<0.05). FGF11, fibroblast growth factor 11; HUVECs, human umbilical vein endothelial cells; ZO-1, zonula occludens-1.

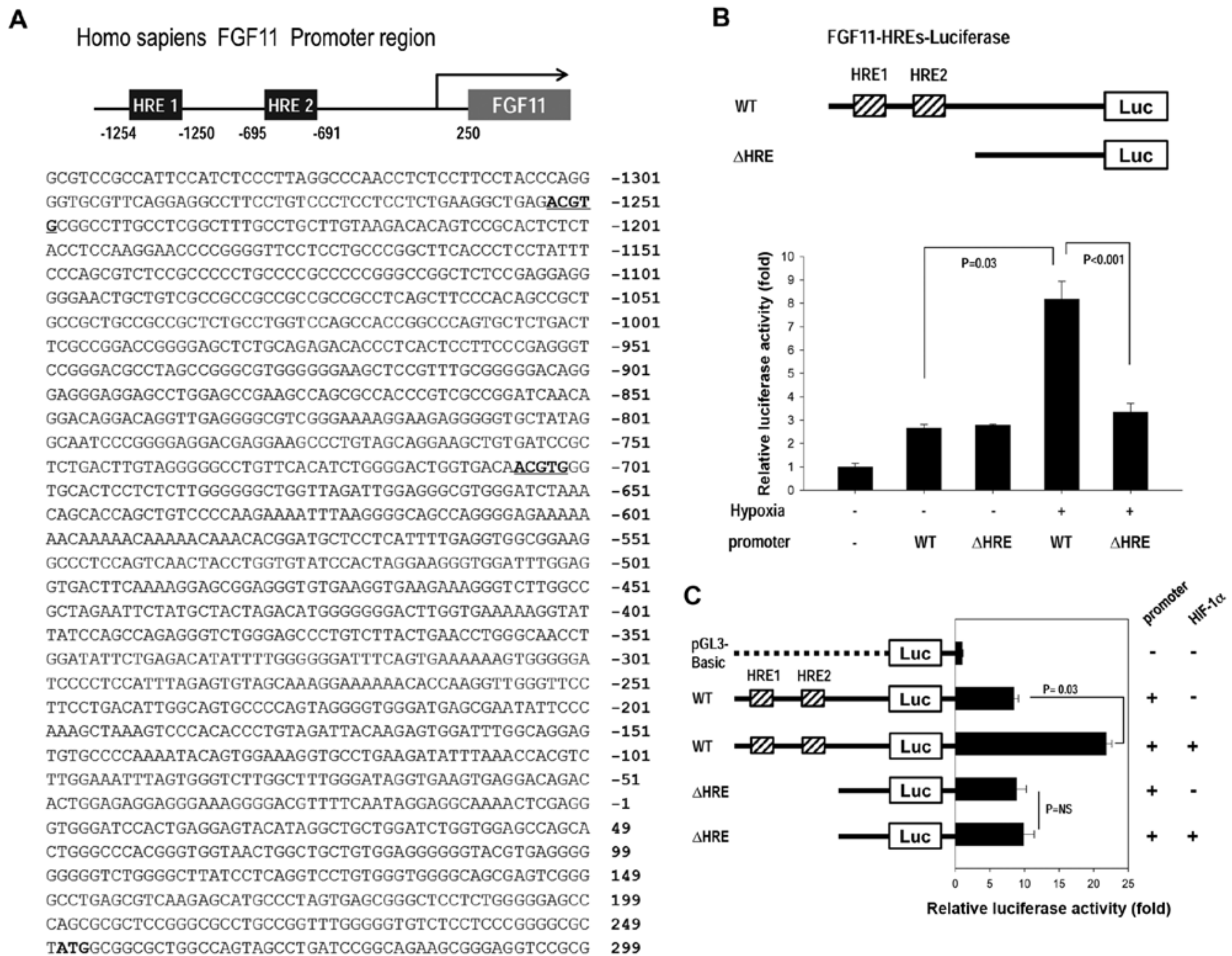

Figure 4. Hypoxia- or HIF-1α-stimulated FGF11 promoter activity. (A) Promoter sequence of the human FGF11 promoter (Transcriptional Regulatory Element Database accession no. 11767). Nucleotides are numbered relative to the transcription start site and the two HREs (5'-ACGTG-3') are underlined. (B) HEK293a cells were co-transfected with pCMV-β-gal, the full promoter sequence containing pGL3-FGF11-HREs (WT) or HRE-deletion-fragment pGL3-FGF11-ΔHREs (ΔHRE) for 24 h and incubated for 16 h under hypoxic conditions (n=4). (C) Cells were co-transfected with pGL3-FGF11-HRE (WT) or pGL3-FGF11-ΔHREs (ΔHRE), pCMV-β-gal, pEGFP-HIF-1α and pEGFP-HIF-1β plasmids under normoxic conditions, and then reporter gene activity was quantified using the luciferase assay (n=4). HIF-1α, hypoxia-inducible factor-1α; FGF11, fibroblast growth factor 11.

conditions (Fig. 4C). To promote assembly of the functional HIF1 complex, cells were also co-transfected with the partner of HIF-1α, HIF-1β. With HIF-1α overexpression, reporter gene activity was high even under normoxic conditions, whereas the HRE-deletion-fragment (ΔHRE) was unaffected by HIF-1α overexpression (Fig. 4C), which indicates that FGF11 promoter induction occurs via HIF-1.

Discussion

Tumor growth is strongly limited by oxygen availability; tumorigenesis is dependent on angiogenesis for the formation of rich vasculature around the tumor that delivers oxygen and nutrients (11). Under hypoxic conditions, the transcription factor HIF-1 binds to HREs in the promoter regions of hypoxia-induced genes, which then orchestrate hypoxia adaptations and promote angiogenesis (6). We identified a member of the fibroblast growth factor family, intracellular FGF11 whose expression was upregulated in response to hypoxic conditions (Fig. 1). Furthermore, FGF11 has been reported to play a role in tumorigenesis, particularly in mitogenic and cell-survival activities that are related to tumor invasion and growth (22). Infiltrating T cells enhanced prostate cancer growth through regulation of FGF11-mediated MMP9 signaling (23). Microarray-based expression profiles for oral cancer cells indicated that increased FGF11 expression is associated with increased cell proliferation, resistance to apoptosis and enhanced capillary-like structures (24).

Since FGF11 is associated with tumorigenesis (22-24) and its expression was upregulated under hypoxic conditions in endothelial cells (Fig. 1), we examined whether FGF11 is involved in angiogenesis which is essential for tumorigenesis (9-11). FGF11 overexpression in HUVECs stimulated capillary tube formation (Fig. 2); however, FGF11 overexpression did not affect endothelial migration. Notably, the expression of tight junction (TJ) proteins including occludin, ZO-1 and claudin-5 increased by FGF11 overexpression (Fig. 3). TJ complexes are composed of occludins, claudins and junctional adhesion molecules (JAMs), which are stabilized by ZO scaffold proteins (21). TJs in epithelial and endothelial cells establish

a barrier to diffusion through the paracellular pathway and block diffusion of membrane proteins between the apical and basal regions of the cell (19,21,25).

Numerous studies have reported TJ protein downregulation in multiple types of cancer. However, upregulation of TJ proteins has been observed in various types of cancers, indicating that there is an emerging role for TJ proteins in cancer cell proliferation, transformation and metastasis (21,26). Occludin and claudin-5 expression were upregulated in human hepatocellular carcinoma tissue in comparison to non-neoplastic liver or normal control tissues (27). Claudin-5 expression was elevated in borderline ovarian tumors and was implicated in malignant transformation (28). Strong claudin-5 expression is a biomarker for elevated risk of pancreatic adenocarcinoma and breast cancer (29,30). Increased ZO-1 expression and altered localization were observed in primary and metastatic pancreatic cancers (31). Clearly, the role of TJ proteins in tumor initiation and development is more complicated than originally understood and a more systematic examination is warranted for clarification. Since FGF11 overexpression is associated with increased occludin, ZO-1 and claudin-5 expression, it is reasonable to expect that FGF11 modulates tumorigenesis.

HIF-1 is a master transcription factor that regulates genes involved in the adaptive response to hypoxia through binding to *cis*-acting HREs (2,16,30) and it regulates genes affecting cell survival, metabolism and tumor vessel formation such as VEGF, erythropoietin, placental growth factor and bFGF (6,16,32). In the present study, we found that FGF11 expression can be induced through HIF-1 binding sites in its promoter region (Fig. 4). Based on our results, we suggest that FGF11 acts as a novel modulator of hypoxia-induced pathological processes such as tumor progression. Future studies focusing on the role of FGF11 in human tumors, as well as a more systematic examination of FGF11 biology, may facilitate the development of new cancer therapeutics.

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