

Hypoxia-inducible factor 1 α regulates branching morphogenesis during kidney development.

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Abstract

The kidneys are exposed to hypoxic conditions during development. Hypoxia-inducible factor (HIF), an important mediator of the response to hypoxia, is believed to have an important role in development. However, the relationship between HIF and branching morphogenesis has not been elucidated clearly.

In this study, we examined whether HIF regulates kidney development. We harvested kidneys from day 13 rat embryos (E13Ks) and cultured the organs under normoxic (20% O₂ / 5% CO₂) or hypoxic (5% O₂ / 5% CO₂) conditions. We evaluated the kidneys based on morphology and gene expression. E13Ks cultured under hypoxic conditions had significantly more ureteric bud (UB) branching than the E13Ks cultured under normoxic conditions. In addition, the mRNA levels of GDNF and GDNF receptor (GFR- α 1), increased under hypoxic conditions in E13Ks. When we cultured E13Ks with the HIF-1 α inhibitor digoxin or with siRNA targeting HIF-1 α under hypoxic conditions, we did not observe increased UB branching. In addition, the expression of GDNF and GFR- α 1 was inhibited under hypoxic conditions when the kidneys were treated with siRNA targeting HIF-1 α . We also elucidated that hypoxia inhibited UB cell apoptosis and promoted the expression of FGF7 mRNA levels in metanephric mesenchymal (MM) cells *in vitro*.

These findings suggest that hypoxic condition has important roles in inducing branching morphogenesis during kidney development. Hypoxia might mediate branching morphogenesis via not only GDNF/Ret but also FGF signaling pathway.

Key word: HIF-1 α , Kidney development, Hypoxia, ureteric bud branching

1. Introduction

It is known that embryos are exposed to low levels of oxygen (O_2) during development [1]. In general, the plasma O_2 (pO_2) is thought to be in the range of 2–9% (14.4-64.8 mm Hg), falling to even lower levels in tissues such as the kidney medulla, bone marrow and thymus [1]. This hypoxia is believed to have an important role in embryonic development [1]. For example, O_2 controls branching during tracheal development [2].

Hypoxia inducible factor (HIF), the major responder to hypoxia, regulates the expression of genes related to energy metabolism, angiogenesis, erythropoiesis, cell proliferation, apoptosis and other functions [3]. In the developing kidney, HIF-1 α is expressed weakly in the outer cortex and strongly in some tubular and collecting duct epithelial cells, whereas HIF-2 α is localized to the glomeruli [3]. A recent report based on HIF-2 α knockout mice showed that HIF-2 α may have no role in the formation of the renal vasculature and glomeruli, which implies that HIF-1 α may play an important role in kidney development [4].

Although many studies have demonstrated that GDNF signaling via Ret and GFR- α 1 is a central and important pathway involved in both UB outgrowth from the WD and UB branching morphogenesis [5-10], other pathways are also involved. For example, Costantini et al. reported that the FGF10/FGFR2 pathway and the HGF/MET pathway are involved [11], while Maeshima et al. reported that the fibroblast growth factor 7 (FGF7)/follistatin pathway is involved [12]. Recently, wnt signaling pathway has reported to be important for correct development of the kidneys [13]. Of these, wnt9b is required for the development of mesonephric and metanephric tubules and the caudal extension of the Mullerian duct whereas wnt11 regulates UB branching reciprocally dependent of GDNF/Ret signaling [13]. In addition, the planar cell polarity gene, vangl2, has recently reported to control UB branching morphogenesis [14]. The detailed mechanisms of UB branching morphogenesis are still being investigated, but many complex mechanisms may be involved.

Previous research has shown that low ambient O_2 enhances ureteric bud branching in vitro [15], but the detailed mechanism is unknown. We investigated the effect of hypoxia on kidney development.

2. Materials and methods

2.1. Animals

Timed-pregnant Sprague-Dawley rats (gestational day 13) were purchased (CLEA, JAPAN). The experimental protocol was approved by the Ethics Review Committees for Animal Experimentation of Okayama University Graduate School (OKU-2013169).

2.2. Organ culture

We surgically isolated embryonic kidneys from pregnant SD rats on day 13 under a microscope. The isolated kidneys were transferred to polyester membrane filters with a 0.4 μm pore size and a diameter of 6.5 mm (Corning, USA) in 400 μl of DMEM/F12 medium (Gibco, USA). The E13Ks were cultured and divided into the normoxic group (n=10) and the hypoxic group (n=10). The normoxic group was incubated at 37°C with 20% O₂ and 5% CO₂ (MCO-19AIC(UV), Sanyo, Japan). The hypoxic group was incubated at 37°C with 5% O₂ and 5% CO₂ (MCO-5M(UV), Sanyo, Japan). The organs were cultured for 96 hours. We used digoxin (Sigma, USA) at a concentration of 100 nmol/l as an HIF-1 α inhibitor (n=10) and HIF-1 α siRNA (Ambion) to inhibit HIF-1 α expression (n=10).

2.3. Establishment of ureteric bud (UB) cells and metanephric mesenchymal (MM) cells

Ureteric bud and metanephric mesenchymal tissues were dissected from the embryonic kidneys of timed-pregnant SD rats (CLEA) on day 13 of gestation with fine forceps. The MM cells were cultured in DMEM/F12 medium (Gibco) containing 10% FCS. The UB cells were cultured on type IV collagen (BD Biosciences, USA) and maintained in a 1:1 mixture of culture supernatant (DMEM containing 10% FCS) from mouse mesenchymal cells (MCS) and modified K1 medium (1:1 mixture of DMEM and Ham's F12 medium supplemented with 10% FCS, 5 $\mu\text{g/ml}$ insulin, 2.75 $\mu\text{g/ml}$ transferrin, 3.35 ng/ml sodium selenous acid (GIBCO), 50 nM hydrocortisone (Sigma), 25 ng/ml hepatocyte growth factor (Sigma) and 2.5 mM nicotinamide (Sigma) at 37°C and 5% CO₂/100% humidity. Outgrowing cells were observed after 7 days. We harvested the outgrowing cells and divided them to obtain single cells using the limiting dilution method. Finally, we harvested the UB cells. We used the cells after 4-8 passages for this study.

For quantitative PCR analysis, the UB and MM cells were cultured under normoxia (incubated at 37°C with 20% O₂ and 5% CO₂) or hypoxia (incubated at 37°C with 5% O₂ and 5% CO₂). The cells were cultured for 96 hours and total RNA was prepared.

2.4. Real-Time RT-PCR

Total RNA was prepared from embryonic kidneys after 4 days of organ culture (n=5 for each condition) using an RNA extraction kit (QIAGEN, USA). cDNA was synthesized using an oligo(dT) primer. Real-time PCR was performed on a StepOnePlus™ Real-Time PCR system (Applied Biosystems) with the Taqman Gene Expression Master Mix. The amplification conditions were as follows: 20 s of enzyme activation at 95°C and 40 cycles of denaturation at 95°C for 1 s, annealing at 60°C for 20 s. The comparative threshold cycle (Ct) method was used to calculate the fold amplification. All samples were measured in triplicate and normalized to GAPDH. Each experiment was evaluated with three PCR reactions, and each experiment was repeated three times. The data are presented as the mean±SD.

2.5. Immunofluorescence staining

After 4 days of culture, E13Ks were fixed with 10% formalin for 1 hour. The E13Ks were then washed with PBS and incubated with primary antibodies against fluorescein-labeled Dolichos biflorus agglutinin (DBA) (Vector Laboratories, USA) and rhodamine-labeled peanut agglutinin (PNA) (Vector Laboratories) for 1 hour. The E13Ks were washed with PBS for 10 min and then viewed under an FSX100 microscope (Olympus Corporation, Japan) to evaluate UB branching.

2.6. RNA interference (siRNA)

The transient expression of small interfering siRNA targeting HIF-1 α was performed by transfection using Lipofectamine 2000 (Invitrogen). The siRNA for HIF-1 α was purchased from Ambion. siRNA (6 pmol) solution was diluted in 100 μ l of K1 medium (DMEM/F12 medium, Gibco) without serum. Then, 4 μ l of Lipofectamine RNAiMAX (Invitrogen) and the siRNA solution were mixed and incubated for 10 min, and then, 500 μ l of K1 medium was added. Isolated E13Ks were cultured in 2000 μ l of the siRNA and Lipofectamine complexes for 2 hours. After 2 hours of incubation, the E13Ks were transferred onto filters floating in 200 μ l of the siRNA and Lipofectamine complexes and 200 μ l of K-1 modified medium, which consisted of K1 medium supplemented with 10% FCS.

2.7. Electric cell-substrate impedance sensing (ECIS)

The resistance and capacitance of cells in culture were measured by ECIS. All tested cell lines were grown on type IV collagen-coated dishes. Cell adhesion was assessed

based on the changes in the ratios of resistance and capacitance to the current flow applied to the electrode arrays at different frequencies (Applied Biophysics, USA) [16]. A frequency scan was performed to determine the frequency at which the greatest difference in R_{ep} was obtained between the cell-covered and cell-free electrodes. The baseline was established using culture medium ($400 \mu\text{L}\cdot\text{well}^{-1}$) alone and was compared with the values obtained using electrodes covered with a monolayer of cells in $400 \mu\text{L}$ of medium. The optimal frequency for studying resistance appeared to be 500 Hz, whereas that for studying capacitance appeared to be 64 kHz. For the resistance and capacitance measurements, the cells were inoculated at 1.5×10^4 cells $\cdot\text{well}^{-1}$ into 8-chambered electrode wells (8W10E⁺ slide).

2.8. Western blotting

Western blotting was performed as described previously [17]. Briefly, after 4 days culture, E13Ks (n=3 in each group) were homogenized in RIPA buffer at 4°C. The samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the proteins were electrotransferred onto a nitrocellulose membrane (Amersham, USA). The membranes were blocked with 5% non-fat dry milk in 1X Tris-buffered saline containing 0.1% Tween-20 for 1 hour and then incubated for 1 hour with a polyclonal rabbit anti-rat phospho-Akt antibody (1:1000 dilution; Cell Signaling Technology Inc., USA) or an anti- β -actin antibody (1:1000 dilution; Cell Signaling Technology Inc.) at 4°C. After the membranes had been incubated with horseradish peroxidase-labeled anti-rabbit IgG antibodies (1:1000 dilution, Bio-Rad) for 1 hour, the signals were detected using an enhanced chemiluminescence system (Amersham) (ImageQuant LAS 4000 mini, GE). The density of each band was determined using NIH Image software and was expressed as a value relative to the density of the corresponding band obtained from the actin immunoblot.

2.9. Statistical analysis

The data are expressed as the mean \pm SE. Comparisons between groups were evaluated by Student's *t* test. A *P* value of <0.05 denotes a statistically significant difference.

3. Results

3.1. *The number of ureteric bud branches depends on the level of cell oxygenation.*

We tried to culture E13Ks under strong hypoxia (O₂ 3%) and hypoxia (O₂ 5%). Under strong hypoxia, E13Ks grew with a smaller number of UB branches and with a shorter length of major axis (Figure S1 A-E). These results indicate that the strict control of the oxygen concentration is important for appropriate UB branching morphogenesis and that strong hypoxia due to ischemia, for example, has negative effects on branching morphogenesis. We defined to compare the morphogenesis between normoxic condition and hypoxic condition.

We isolated embryonic kidneys from pregnant SD rats on day 13 and cultured E13Ks under normoxic (20% O₂ and 5% CO₂) or hypoxic (5% O₂ and 5% CO₂) conditions. We observed the kidneys under a light microscope and performed immunofluorescence staining to evaluate UB branching. Each image for the hypoxic conditions indicated that there was more UB branching than under the normoxic conditions (Figure 1 A-H). When we evaluated UB branching based on the number of ureteric end buds in E13Ks after 4 days of culture under normoxic or hypoxic conditions, we could indicate that hypoxia significantly increased the number of ureteric end buds [Hypoxic conditions vs. normoxic conditions; 57.7±12.5 v.s. 40.6±6.6, $p < 0.01$] (Figure 1 I). These results suggest that hypoxia activates UB branching.

3.2. *Hypoxia leads to the expression of HIF-1 α and GDNF/Ret signaling.*

Based on previous data, we next examined whether hypoxia could regulate GDNF/Ret signaling, which is related to UB branching. We evaluated the expression levels of GDNF, Ret, HIF-1 α and GFR- α 1 in E13Ks cultured under hypoxic and normoxic conditions by quantitative RT-PCR. Quantitative RT-PCR revealed that the GDNF and GFR- α 1 mRNA levels under hypoxic conditions were significantly greater than those under normoxic condition (Figure 1J). ($p < 0.01$) This data suggested that hypoxia induced the expression of HIF-1 α and GDNF/Ret signaling. Interestingly, quantitative RT-PCR also revealed that the FGF10 mRNA level was higher under hypoxic conditions than under normoxic conditions (Figure 1J).

3.3. *Inhibition of HIF-1 α signaling decreases ureteric bud branching.*

To determine whether HIF-1 α signaling is related to UB branching, we treated E13Ks with digoxin, an inhibitor of HIF-1 α signaling, during culture under hypoxic conditions [18]. Digoxin treatment significantly decreased UB branching in cultured

E13Ks (Figure 2A,B,D). The number of ureteric end buds was significantly lower than the number under hypoxic conditions without digoxin [41.5 ± 7.5 (with digoxin) vs. 57.7 ± 12.5 (without digoxin), $p < 0.01$]. There was significant difference in the length of the major axis of the cultured kidneys between those cultured under hypoxic with or without digoxin treatment (Figure S1E). These data suggest that the effect of HIF-1 α signaling on UB branching is important.

We directly inhibited HIF-1 α expression using siRNA. We cultured E13Ks under hypoxic conditions with / without HIF-1 α siRNA. The siRNA treatment significantly decreased ureteric bud branching (Figure 2A,C,D). The number of ureteric end buds was significantly lower than the number under hypoxic conditions without HIF-1 α siRNA [39.0 ± 4.76 (with HIF-1 α siRNA) vs. 57.7 ± 12.5 (without HIF-1 α siRNA), $p < 0.01$]. There was also significant difference in the length of the major axis of the cultured kidneys between those cultured under hypoxic with or without HIF-1 α siRNA treatment (Figure S1E). We examined the expression levels of HIF-1 α , GDNF, Ret, and GFR- α 1 mRNA. Quantitative RT-PCR revealed that the inhibition of HIF-1 α with siRNA during culture under hypoxic conditions significantly decreased the HIF-1 α , GDNF and GFR- α 1 mRNA expression levels ($p < 0.01$), which suggested that HIF-1 α regulates GDNF and GFR- α 1 gene expression (Figure 2E). Taken together, these results revealed that HIF-1 α regulates GDNF and GFR- α 1 expression, which activates ureteric bud branching.

We next tried to determine whether the enhanced GDNF level due to stimulation by HIF-1 α affected UB branching. GDNF has been reported to activate phospho-Akt (p-Akt) [19]. We examined p-Akt expression in the cultured E13Ks. We observed that the E13Ks cultured under hypoxic conditions expressed higher levels of p-Akt than under normoxic conditions (Figure 2F). The E13Ks cultured with digoxin under hypoxic conditions had lower p-Akt levels than E13Ks cultured under hypoxic condition without digoxin (Figure 2F). These results indicate that the enhanced GDNF expression stimulated by HIF-1 α has an important role in UB branching.

Next, we examined whether knockdown of HIF-1 α affect the expression of Wnt9b, Wnt11 and beta-catenin. Quantitative RT-PCR revealed that HIF-1 α targeting siRNA treatment decreased the expression of Wnt11 mRNA levels (Figure S2A). Quantitative RT-PCR revealed that hypoxic conditions significantly decreased the vangl2 mRNA expression levels regardless of the presence of the inhibition of HIF-1 α (Figure S2B). The data suggested that other mechanisms, not for vangl2 involving responders to tissue hypoxia other than HIF-1 α might affect UB branching.

3.4. Hypoxic conditions inhibit caspase-3 mRNA levels in UB cells.

Hypoxia may activate UB cell proliferation, which may result in UB branching. To determine whether UB branching is the result of hypoxia-induced cell proliferation, we examined the cell proliferation and attachment ability of the UB cells using the electrical cell-substrate impedance sensing (ECIS) method. In this method, high resistance reflects greater cell adhesion and greater coverage of the electrode, and low capacitance reflects active cell proliferation. We cultured UB cells under normoxic (20% O₂ and 5% CO₂) or hypoxic (5% O₂ and 5% CO₂) conditions. The capacitance (64 kHz) of UB cells cultured under normoxic conditions (O₂ 20%) was significantly lower than that of ureteric bud cells cultured under hypoxic conditions, indicating that the level of cell proliferation was lower under hypoxic conditions than under normoxic conditions (Figure 3A). In addition, the resistance (500 Hz) of UB cells grown under normoxic conditions (O₂ 20%) were significantly higher than those of ureteric bud cells grown under hypoxic conditions, indicating that the levels of cell adhesion and cell proliferation were lower under hypoxic conditions than under normoxic conditions (Figure 3B). These results revealed that hypoxia does not activate UB cell proliferation. We also examined the cell migration ability which revealed that UB cell migration was not activated under hypoxic conditions (data not shown)

We next examined whether hypoxia affects apoptosis for the UB cells. Quantitative RT-PCR revealed that caspase-3 mRNA levels of UB cells cultured under hypoxic conditions were significantly lower than those under normoxic condition (Figure 3C).

MM cells secreted FGF7 in Hypoxic conditions.

UB branching is largely influenced by MM. We examined the factors from MM cells which influence UB branching. Quantitative PCR revealed that FGF7, not FGF10 mRNA levels of MM cells cultured under hypoxic conditions were significantly higher than those under normoxic conditions (Figure 3D). These results suggested that hypoxia might mediate branching morphogenesis not only GDNF/Ret but also FGF signaling pathway.

4. Discussion

Oxygen is known to control cell differentiation and development in many tissues [1]. For example, recent research has demonstrated that O₂ controls branching during tracheal development [2] and that mammalian cardiovascular morphogenesis is regulated by HIF [1]. Another study showed that oxygen tension regulates pancreatic beta-cell differentiation [20]. There are some mechanisms involving responders to tissue hypoxia, including HIFs; environmental sensing by the mammalian target of rapamycin (mTOR); and the endoplasmic reticulum (ER) [1]. HIF has recently been suggested to participate in embryonic development and is thought to be the main responder to hypoxia.

Regarding kidney development, there is little information on the role of hypoxia in the regulation of embryonic kidney development because most previous kidney organ cultures were cultured at 20% oxygen tension, which is not physiological. Recent research has shown that HIF might control embryonic vascular development [21]. When we cultured embryonic kidneys under hypoxic conditions, the embryonic kidneys produced more UB branches than when grown under normoxic conditions, which indicates that hypoxia has an effect on kidney development. We investigated the effect of hypoxia on kidney development. We hypothesized that HIF-1 α affects UB branching morphogenesis, and we sought to determine whether kidney development is regulated by HIF.

Our study revealed that the culture of kidneys under hypoxic conditions (O₂ 5%), which seems to be physiological, stimulated UB branching, which is related to the nephron number. Regarding gene expression, hypoxia induced the expression of GDNF, and GFR- α 1, as well as HIF-1 α , at the mRNA level. In addition, we sought to determine whether the inhibition of HIF1- α can abrogate the UB branching stimulated by hypoxia. Because recent research has shown that digoxin inhibits HIF-1 α -dependent gene transcription [18], we used digoxin as an HIF-1 α inhibitor. Under hypoxic conditions, HIF1- α inhibition using digoxin or HIF-1 α siRNA down-regulates the stimulated UB branching. In addition, HIF-1 α targeting siRNA treatment decreased GDNF and GFR- α 1 mRNA expression, which indicates that HIF-1 α regulates UB branching morphogenesis via GDNF/Ret signaling. We also compared the growth of these cultured kidneys to normal kidney growth by harvesting kidneys from 17-day rat embryos (E17Ks). The E17Ks were larger than the E13Ks cultured under hypoxic conditions for 4 days (The data not shown). These differences may result from the vascularization of normal growth.

In our data, hypoxia induced FGF10 mRNA expression in Organ culture analysis,

whereas the expression of FGF7 and follistatin mRNA was not induced (data not shown). These results indicate that hypoxia activates UB branching not only via the GDNF/Ret pathway but also via the FGF10/FGFR2 pathway (Figure 4). In our data, HIF-1 α targeting siRNA treatment decreased the expression of Wnt11. This data suggested that HIF-1 α controls wnt11. As wnt11 functions with GDNF/Ret signaling to promote collecting duct development [22], hypoxia might promote UB branching via wnt11-GDNF/Ret signaling pathway.

We also examined the effects of hypoxia on UB cell proliferation, migration and apoptosis *in vitro*. In our study, hypoxia inhibited UB cell proliferation. However, hypoxia didn't activate cell migration and inhibited UB cells caspase-3 mRNA levels which are activated during apoptosis [23]. These results indicated that UB branching might be promoted by hypoxia through the down-regulation of UB cell apoptosis without the activation of the UB cell proliferation or migration.

In addition, we elucidated that hypoxia promoted the FGF7 mRNA expression of MM cells. However, we could not observe FGF10 mRNA expressed significantly increased in MM cells under hypoxic condition *In Vitro* analysis. These differences may be resulted from the presence of the interaction between MM and UB cells under development. It might be because that various factors such as FGF7, FGF10 and Wnt11 involved UB morphogenesis orchestrally under hypoxic condition.

In conclusion, we demonstrated that hypoxic conditions has important roles in inducing branching morphogenesis during kidney development and that the effects of hypoxia might be mediated by HIF-1 α to some extent via GDNF/Ret signaling and FGF signaling pathway.

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Figure Legends

Figure 1

Representative images and gene expressions of cultured E13Ks.

E13Ks were cultured under normoxic (20% O₂ and 5% CO₂) or hypoxic (5% O₂ and 5% CO₂) conditions. Normoxic conditions: day 0 (A), day 4 (C), and day 7 (E) assessed using light microscopy and day 4 (G) assessed using fluorescence microscopy (red; PNA, green; DBA). Hypoxic conditions: day 0 (B), day 4 (D), and day 7 (F) assessed using light microscopy and day 4 (H) assessed using fluorescence microscopy (Red; PNA, Green; DBA). (I) The number of ureteric end buds of cultured E13Ks under normoxic or hypoxic conditions (J) Gene expression in kidneys cultured for 4 days was determined by quantitative RT-PCR. **: $p < 0.01$. Each point represents the mean \pm SE.

Figure 2

The effect of the inhibition of HIF-1 α .

Representative images of cultured E13Ks are shown (A-C). E13Ks were cultured under hypoxic conditions (5% O₂ and 5% CO₂) or hypoxic condition with digoxin (100 nM) or hypoxic conditions with HIF-1 α siRNA treatment. Hypoxic conditions: day 4 (A), hypoxia with digoxin: day 4 (B), and hypoxia with HIF-1 α siRNA: day 4 (C), assessed using fluorescence microscopy (Red; PNA, Green; DBA). (D) The number of end buds under hypoxic conditions, hypoxic conditions with digoxin and hypoxic conditions with HIF-1 α siRNA treatment. (E) Gene expression was analyzed by quantitative RT-PCR in cultured E13Ks under hypoxic conditions (5% O₂ and 5% CO₂) or hypoxic conditions with HIF-1 α siRNA treatment. (F) P-Akt level in cultured E13Ks evaluated by western blotting. E13Ks were cultured under normoxic (20% O₂ and 5% CO₂) or hypoxic (5% O₂ and 5% CO₂) conditions or hypoxic conditions with digoxin (100 nM). The bands were scanned and analyzed by densitometry. The intensity of the p-Akt protein band relative to that of the β -actin band is shown. **: $p < 0.01$. Each point represents the mean \pm SE.

Figure 3

Evaluation of cell proliferation and cell attachment using the electrical cell-substrate impedance sensing (ECIS).

UB cells were cultured under normoxic (20% O₂ and 5% CO₂) or hypoxic (5% O₂ and 5% CO₂) conditions. Resistances and capacitances were normalized to the starting point at the uncovered electrode. Capacitance (A: 64 kHz) and resistance (B: 500Hz) of UB cells grown under normoxic conditions (green, n=10), hypoxic conditions (blue, n=10) and control (red: n=2, PBS without UB cells).

(C) Gene expression was analyzed by quantitative RT-PCR in UB cells (C) or MM cells (D) cultured under normoxic (20% O₂ and 5% CO₂) or hypoxic (5% O₂ and 5% CO₂) conditions. **: $p < 0.01$, *: $p < 0.05$. Each point represents the mean \pm SE.

Figure 4

The schema for UB branching morphogenesis under hypoxia condition.

HIF-1 α activation by hypoxic conditions activates GDNF/Ret signaling, FGF10 signaling and Wnt11.

Figure 1

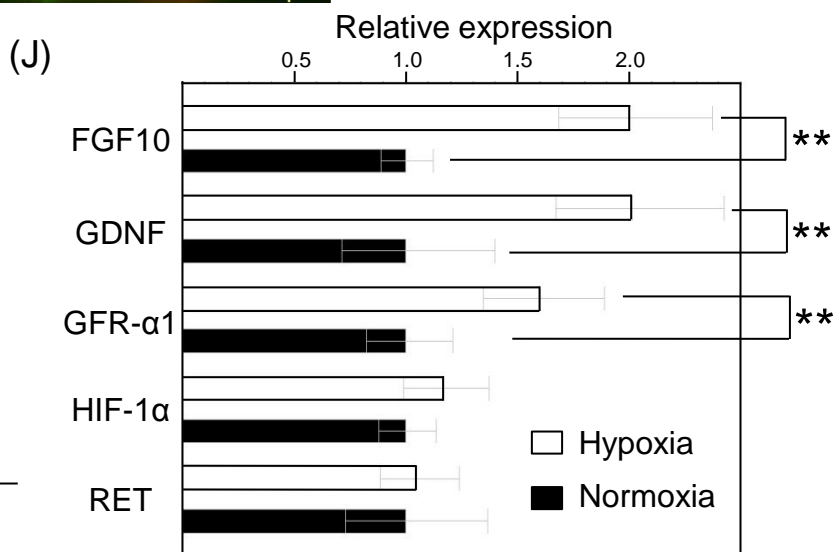
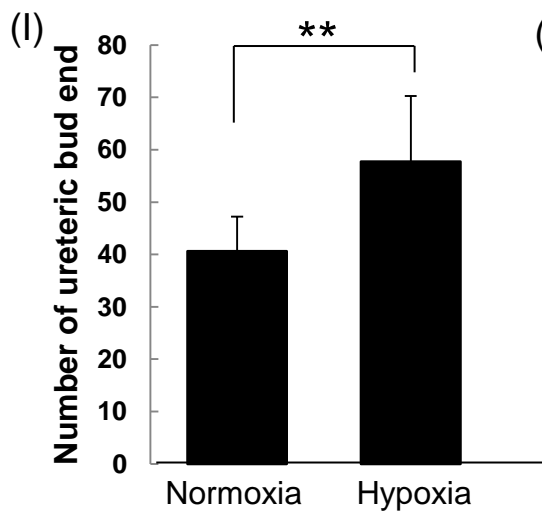
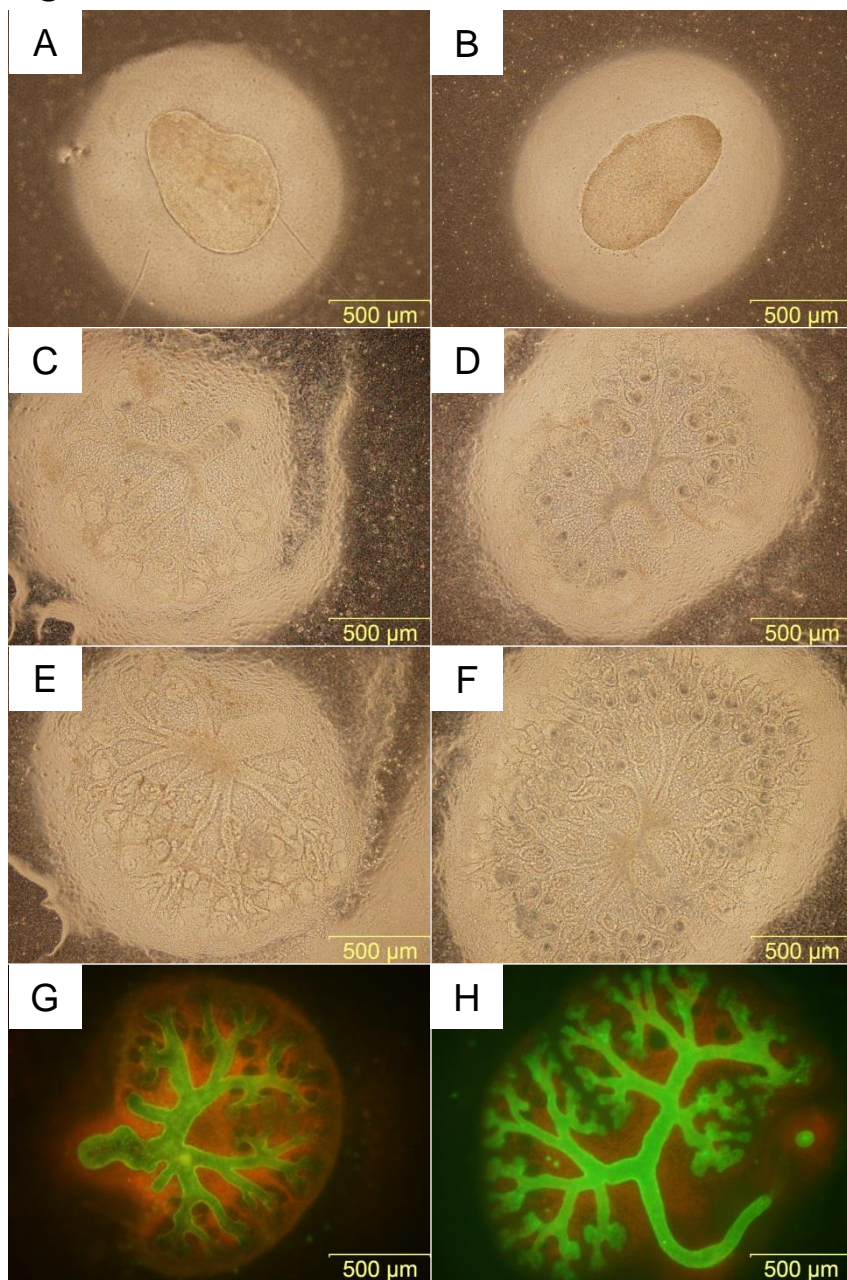


Figure 2

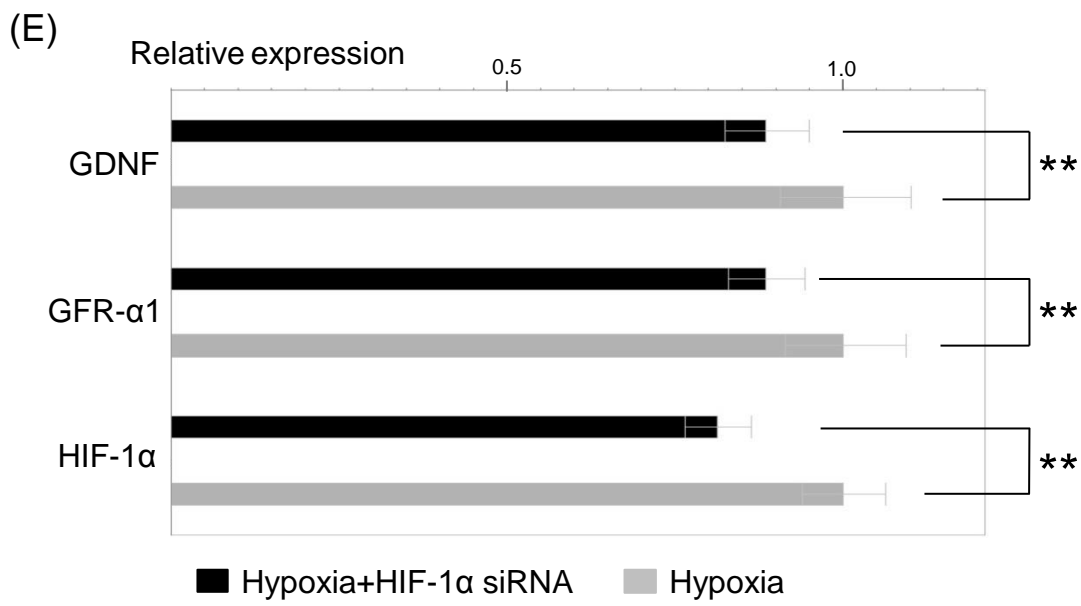
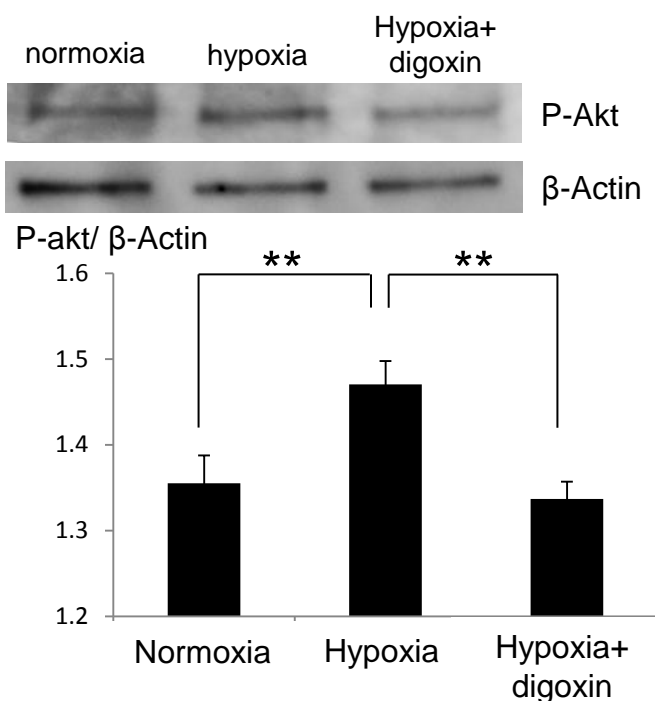
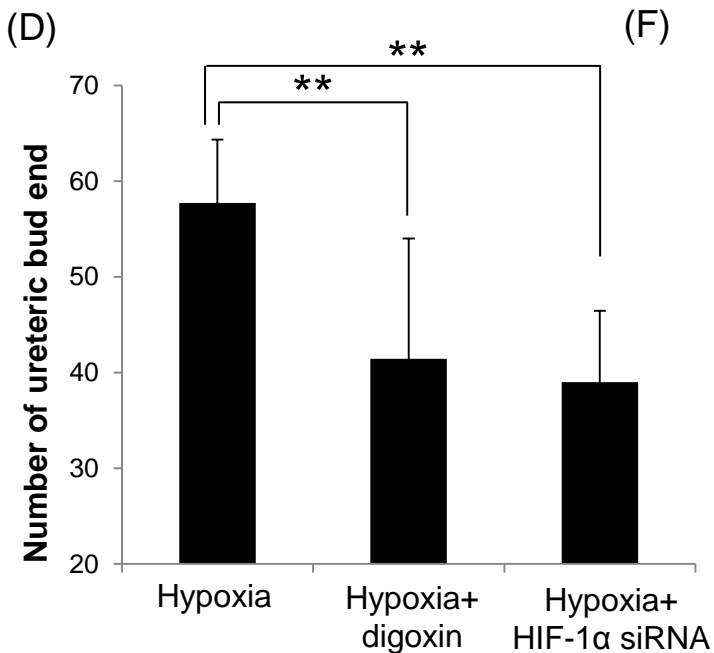
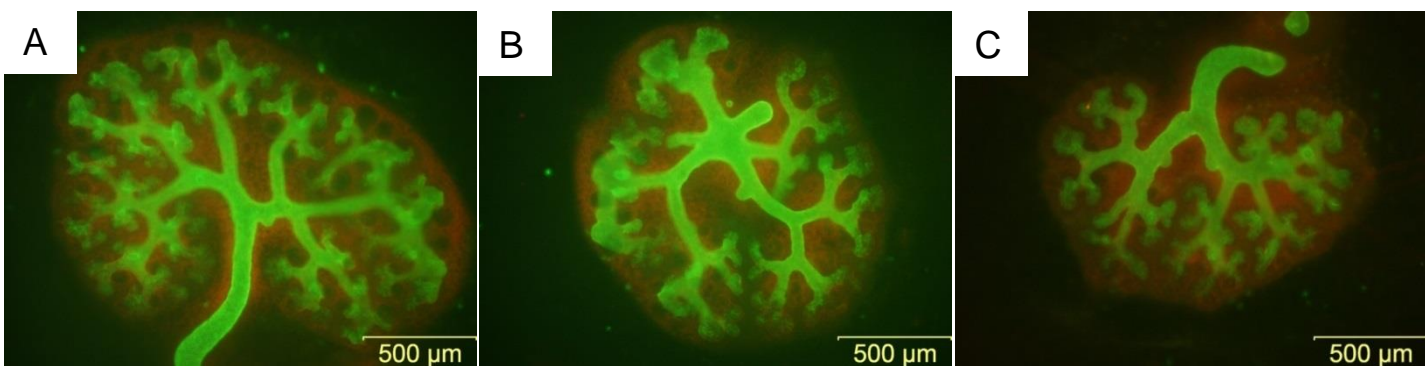
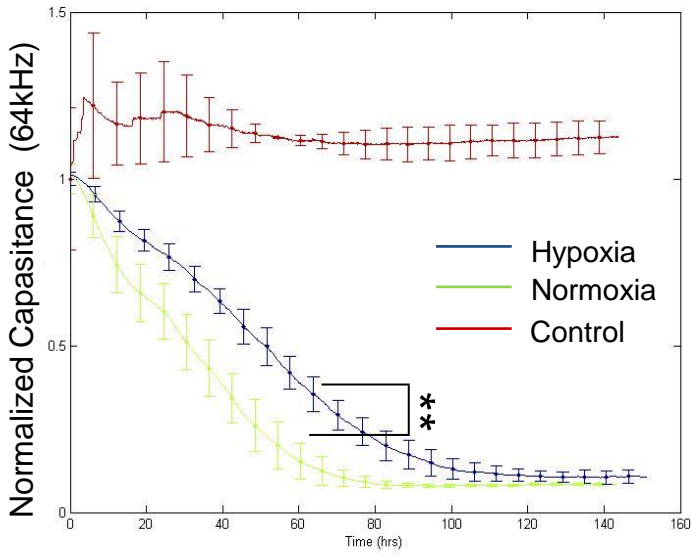
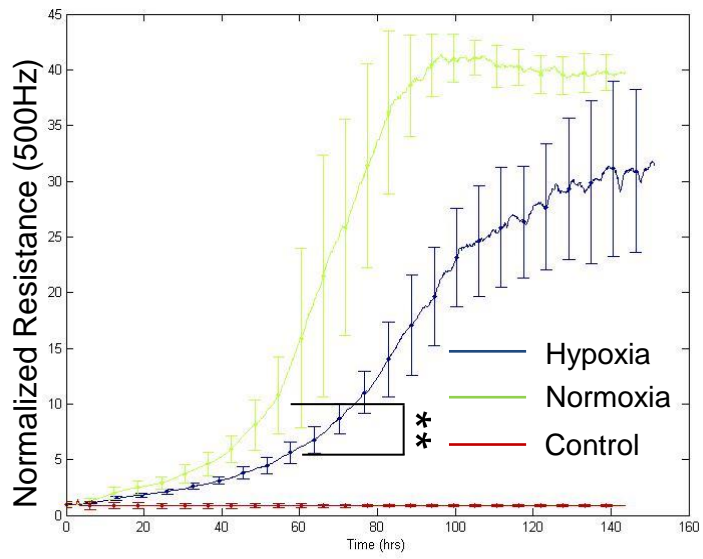


Figure 3

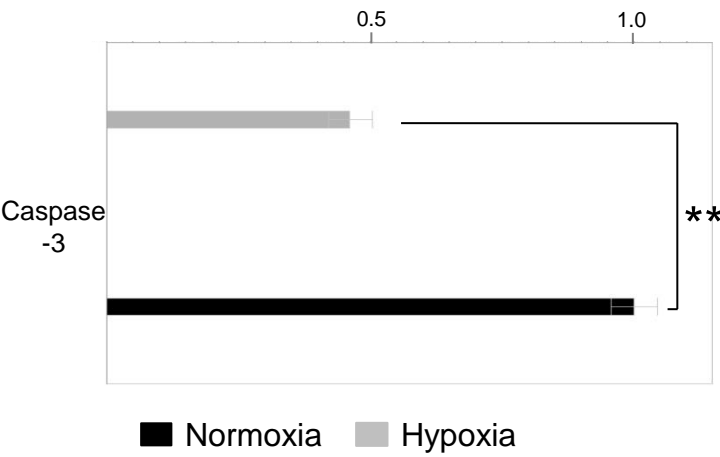
(A)



(B)



(C) Relative expression



(D)

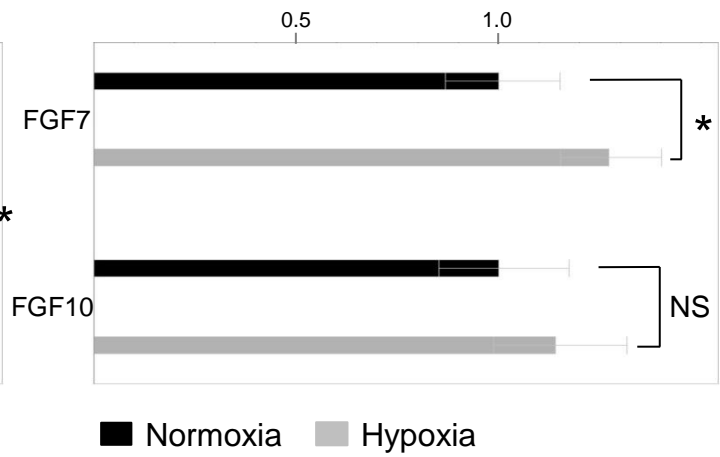


Figure 4

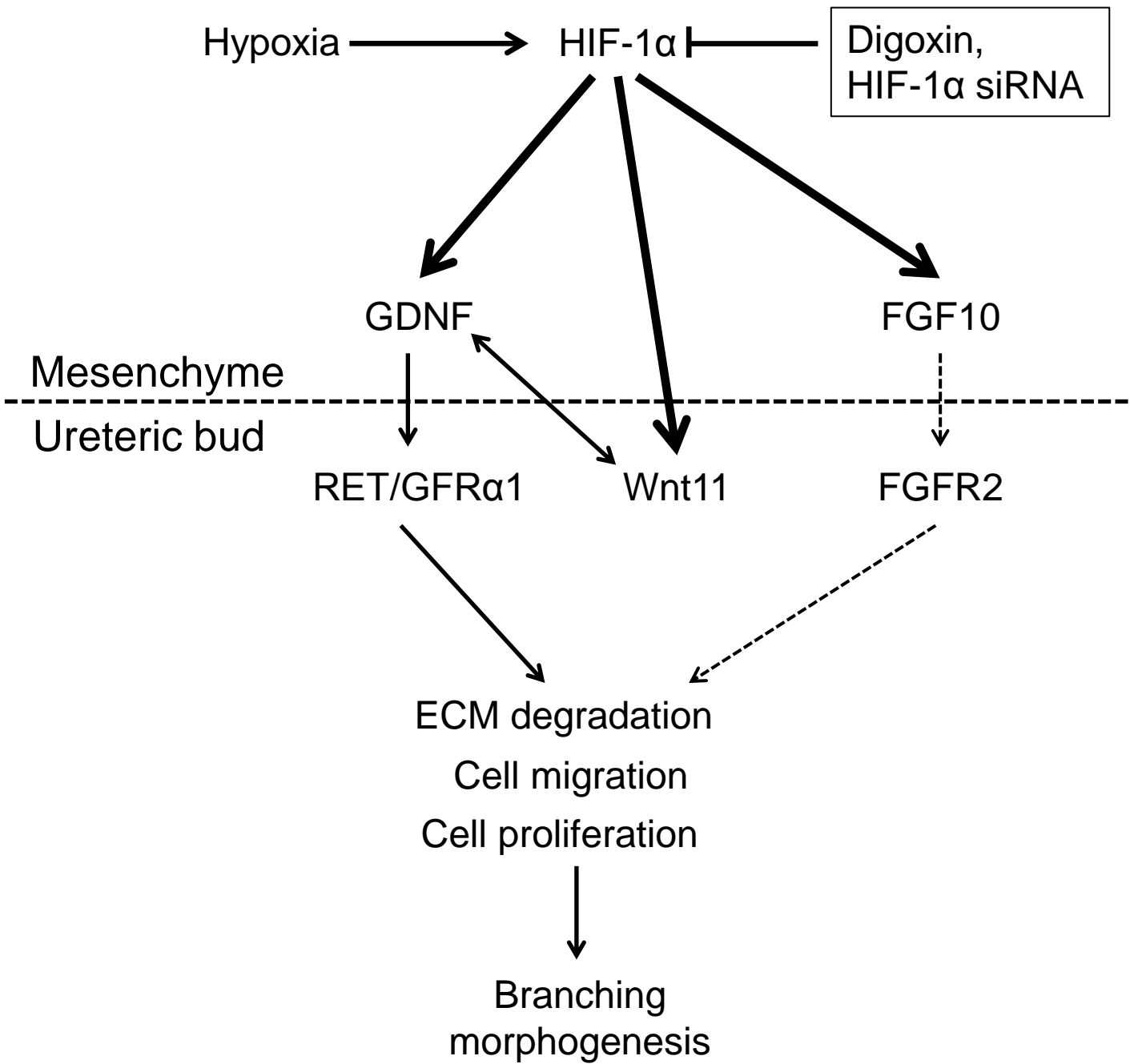


Figure S1

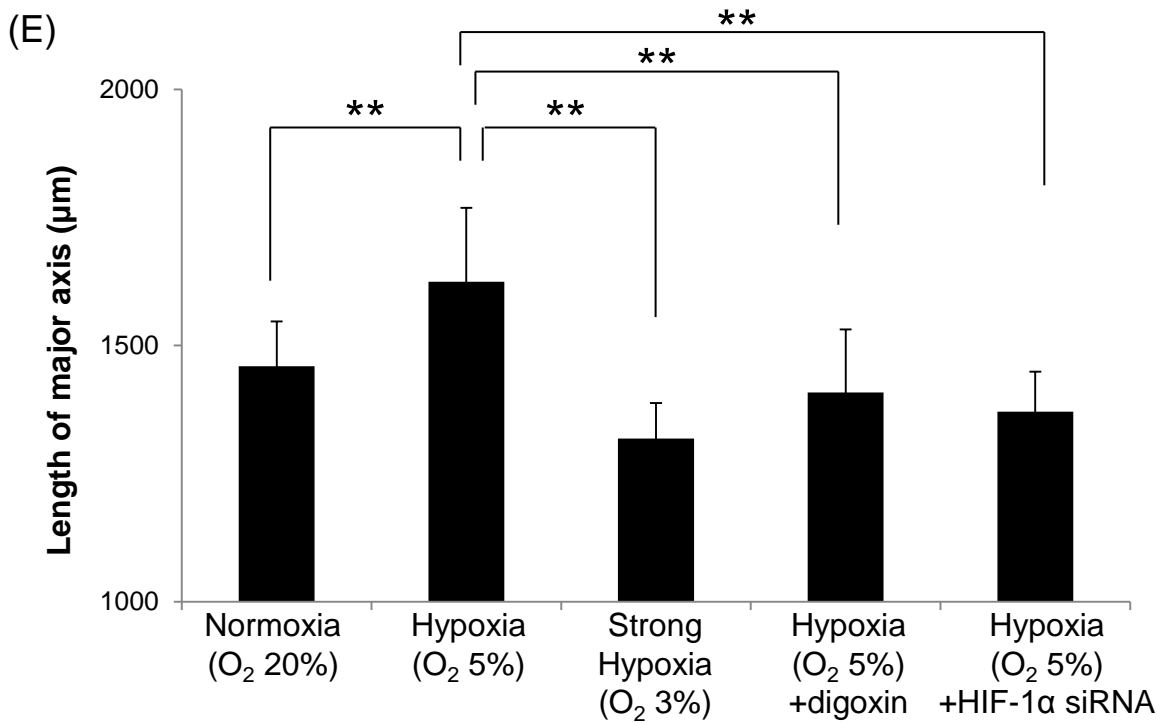
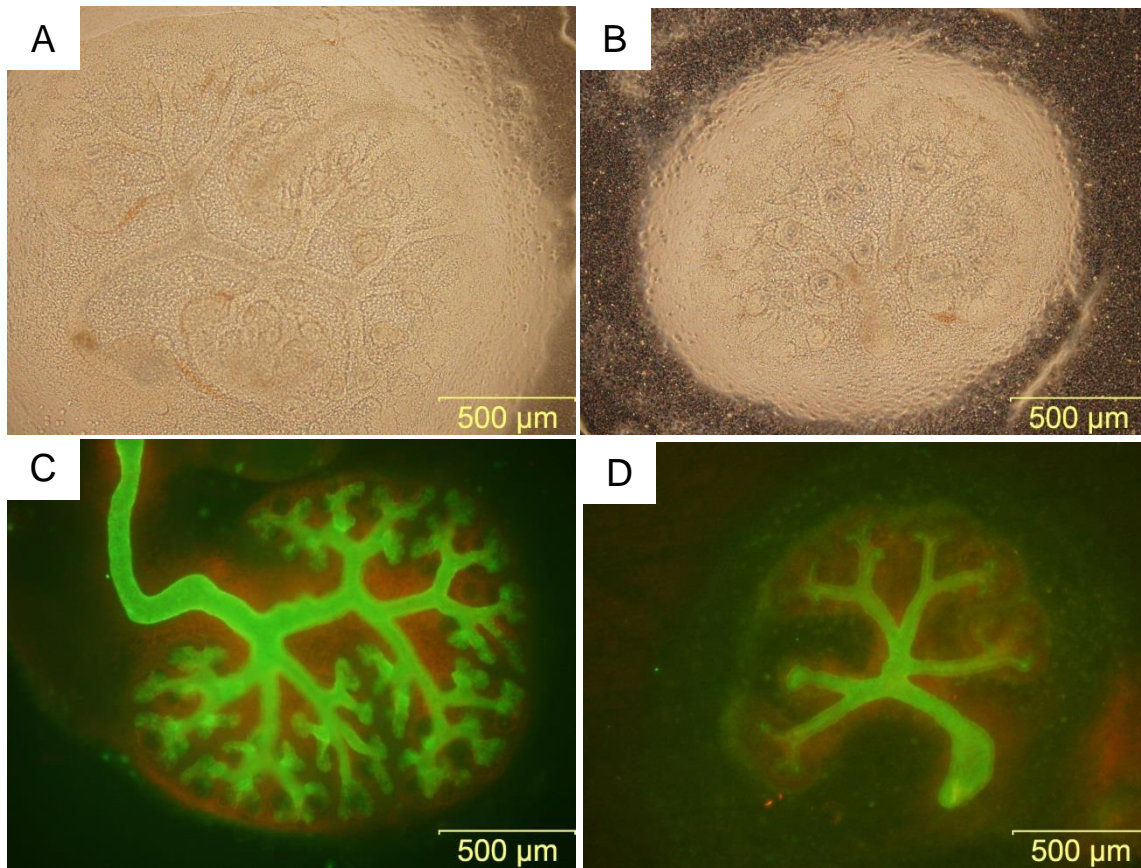
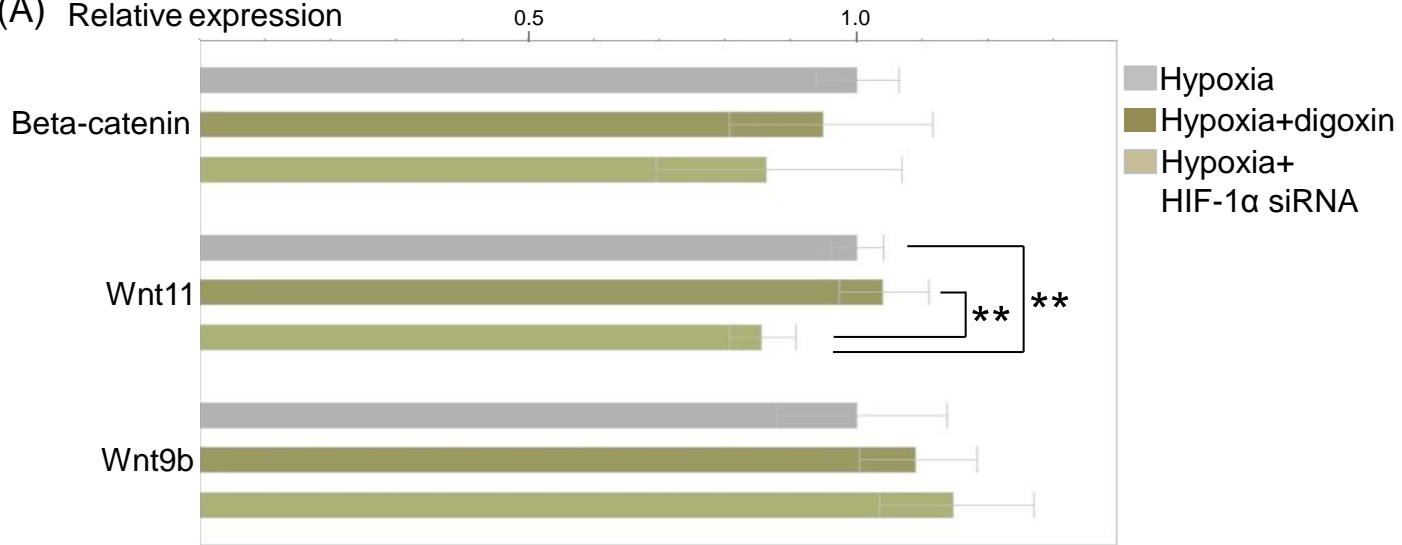


Figure S2

(A) Relative expression



(B) Relative expression: Vangl2

