

# Overexpression and Knockdown of Hypoxia-Inducible Factor 1 Disrupt the Expression of Steroidogenic Enzyme Genes and Early Embryonic Development in Zebrafish

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Tianfeng Tan<sup>1,2,3</sup>, Richard Man Kit Yu<sup>4</sup>, Rudolf Shiu Sun Wu<sup>1,5</sup>  
and Richard Yuen Chong Kong<sup>1,3</sup>

<sup>1</sup>State Key Laboratory in Marine Pollution, City University of Hong Kong, Hong Kong SAR. <sup>2</sup>Shenzhen Key Laboratory for the Sustainable Use of Marine Biodiversity, Research Centre for the Oceans and Human Health, City University of Hong Kong Shenzhen Research Institute, Shenzhen, China. <sup>3</sup>Department of Biology and Chemistry, City University of Hong Kong, Kowloon, Hong Kong SAR. <sup>4</sup>School of Environmental and Life Sciences, The University of Newcastle, Callaghan, NSW, Australia. <sup>5</sup>Department of Science and Environmental Studies, The Hong Kong Institute of Education, Tai Po, Hong Kong SAR.

**ABSTRACT:** Hypoxia is an important environmental stressor leading to endocrine disruption and reproductive impairment in fish. Although the hypoxia-inducible factor 1 (HIF-1) is known to regulate the transcription of various genes mediating oxygen homeostasis, its role in modulating steroidogenesis-related gene expression remains poorly understood. In this study, the regulatory effect of HIF-1 on the expression of 9 steroidogenic enzyme genes was investigated in zebrafish embryos using a “gain-of-function and loss-of-function” approach. Eight of the genes, *CYP11a*, *CYP11b2*, *3β-HSD*, *HMGCR*, *CYP17a1*, *17β-HSD2*, *CYP19a*, and *CYP19b*, were found to be differentially upregulated at 24 and 48 hpf following zHIF-1α-ΔODD overexpression (a mutant zebrafish HIF-1α protein with proline-414 and proline-557 deleted). Knockdown of zHIF-1α also affected the expression pattern of the steroidogenic enzyme genes. Overexpression of zHIF-1α and hypoxia exposure resulted in downregulated *StAR* expression but upregulated *CYP11a* and *3β-HSD* expression in zebrafish embryos. Conversely, the expression patterns of these 3 genes were reversed in embryos in which zHIF-1α was knocked down under normoxia, suggesting that these 3 genes are regulated by HIF-1. Overall, the findings from this study indicate that HIF-1-mediated mechanisms are likely involved in the regulation of specific steroidogenic genes.

**KEYWORDS:** HIF-1, hypoxia, steroidogenic enzyme genes, zebrafish, steroid hormones

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**CORRESPONDING AUTHOR:** Richard Yuen Chong Kong, Department of Biology and Chemistry, City University of Hong Kong, Tat Chee Avenue, Kowloon, Hong Kong SAR. Email: bhrkong@cityu.edu.hk

## Introduction

Hypoxia caused by aquatic ecosystem enrichment with nutrients and organic matter is a major global threat that is predicted to worsen as climate change progresses. The adverse impacts of aquatic hypoxia include significant reductions in fisheries production, fish growth, alteration of species composition, and mass fish mortality.<sup>1,2</sup> Hypoxia impairs fish reproduction by inhibiting testicular and ovarian development, reducing sperm and egg quality, affecting fertilization, and hatching and influencing the survival of larvae and juvenile fitness.<sup>3</sup> Ovarian and testicular growth, gametogenesis, and oocyte and sperm production were significantly reduced in Atlantic croakers in hypoxic regions of the Mississippi River<sup>4,5</sup> and in the northern Gulf of Mexico.<sup>6</sup>

In fish, the inhibition of key reproductive processes by hypoxia is associated not only with reduced metabolism but also with the repression of specific hormones and hormone receptors along the hypothalamus-pituitary-gonad (HPG) axis.<sup>3,7</sup> Numerous *in vivo* studies have shown that fish reproduction is affected by hypoxia via alteration of the steroidogenesis pathway.<sup>7–11</sup> Steroid hormones biosynthesis is a

fundamental process for reproduction in vertebrates, and all steroid hormones are derived from cholesterol. The rate-limiting step in steroid production is the delivery of cholesterol from the outer to the inner mitochondrial membrane, which is mediated first by the steroidogenic acute regulatory (StAR) protein<sup>12</sup> followed by the conversion of cholesterol to pregnenolone by the cytochrome P450 cholesterol side-chain cleavage (P450<sub>scc</sub>) enzyme encoded by *CYP11a1*.<sup>13</sup> Pregnenolone is then metabolized to progesterone by 3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase (encoded by *3β-HSD*)<sup>14</sup> or hydroxylated to 17α-hydroxyprogesterone by cytochrome P450 17α-hydroxylase (encoded by *CYP17a1*). Progesterone and 17α-hydroxyprogesterone are then converted through a series of steps to androgen and estrogen by enzymes such as 17β-hydroxysteroid dehydrogenase (17β-HSD) and P450 aromatase (CYP19).<sup>15</sup> Estradiol and testosterone are steroid hormones that are important for sexual and reproductive development in animals.<sup>16,17</sup> Molecular oxygen is essential for the synthesis of sex steroid hormones,<sup>18</sup> and recent studies revealed that the expression of certain steroidogenic enzyme



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genes, such as *StAR*, *CYP19*, and *CYP17A1* (which specifies 17 $\alpha$ -monooxygenase), may be regulated by hypoxia.<sup>19</sup>

Hypoxia-inducible factor 1 is a heterodimeric protein consisting of an oxygen-labile HIF-1 $\alpha$  subunit and a constitutively expressed HIF-1 $\beta$  subunit.<sup>20</sup> Due to the oxygen-sensitive oxygen-dependent degradation domain (ODD) in the HIF-1 $\alpha$  subunit, HIF-1 is rapidly degraded under normoxia.<sup>21</sup> HIF-1 $\alpha$  is stabilized under hypoxia and is transported into the nucleus where it dimerizes with HIF-1 $\beta$ <sup>22</sup> to form a heterodimeric complex that binds to the hypoxia-responsive elements (HREs) in the promoters of HIF-target genes.<sup>23</sup> HIF-1 has been shown to negatively regulate *StAR* expression and steroidogenesis in granulosa cells under hypoxia.<sup>24</sup>

Although more than 100 different genes are now known to be controlled by HIF-1, whether steroidogenic enzyme genes are regulated under hypoxia through HIF-1 remains poorly understood. Zebrafish (*Danio rerio*) have 2 zHIF-1 $\alpha$  paralogs: zHIF-1aA and zHIF-1aB. Recent studies found that zHIF-1aB was more sensitive to oxygen than zHIF-1aA in zebrafish.<sup>25,26</sup> Using a gain-of-function and loss-of-function approach, we describe here the effects of zHIF-1 $\alpha$  (zHIF-1aB) on the expression of 9 selected steroidogenic genes and embryonic development in zebrafish (*D. rerio*).

## Materials and Methods

### *Zebrafish maintenance*

Wild-type adult zebrafish were maintained under a constant 14 hours:10 hours light:dark cycle at 28°C. Flow through systems were set up in the laboratory to provide a constant temperature and normoxic (7.0 $\pm$ 0.2 mg O<sub>2</sub> L<sup>-1</sup>) or hypoxic (1.0 $\pm$ 0.2 mg O<sub>2</sub> L<sup>-1</sup>) environments during experimental periods as described previously by Shang et al.<sup>10</sup> Embryos were obtained by natural spawning and cultured in zebrafish E3 embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl<sub>2</sub>, 0.33 mM MgSO<sub>4</sub>, and 5% methylene blue) at 28.5°C and staged according to Kimmel et al.<sup>27</sup>

### *Plasmid construction*

The full-length zHIF-1 $\alpha$  complementary DNA (cDNA) was amplified by reverse transcription polymerase chain reaction (RT-PCR) from zebrafish embryos and cloned into the pGEM-T Easy Vector (Promega, Madison, WI, USA) to form pGEMT-zHIF-1 $\alpha$ . Due to the short half-life of the wild-type HIF-1 $\alpha$  protein under normoxia,<sup>28</sup> a zHIF-1 $\alpha$  mutant construct (pGEMT-zHIF-1 $\alpha$ - $\Delta$ ODD) was synthesized to increase the stability of the zHIF-1 $\alpha$  protein during its overexpression in normoxic embryos. To form pGEMT-zHIF-1 $\alpha$ - $\Delta$ ODD, 2 sites containing proline residues (proline-414 and proline-557) in wild-type ODD (at nucleotide positions 1234-1251: TTG GCA CCT GCA GGC and at nucleotide positions 1659-1680: ATG CTG GCT CCT TAC ATC CCA) were deleted from pGEMT-zHIF-1 $\alpha$  using the Gene Tailor Site-Directed Mutagenesis Kit (Invitrogen, Carlsbad, CA USA).

To monitor the overexpression of zHIF-1 $\alpha$  in developing zebrafish embryos, a zHIF-1 $\alpha$ -eGFP fusion expression vector was constructed. The open reading frame (ORF) of enhanced green fluorescent protein (eGFP) was amplified from the pEGFP-N1 (Clontech Laboratories, Mountain View, CA, USA) plasmid using the gene-specific primers EGFP-F-Sma: 5'-TCC CCC GGG GGA ATG GTG AGC AAG GGC GA-3' and EGFP-R-Not: 5'-TTG CCG CCG CAA TTA CTT GTA CAG CTC-3', which contained built-in *Sma*I and *Not*I sites (underlined), respectively. *Sma*I-digested and *Not*I-digested eGFP PCR products and the pCMV-TNT vector (Promega) were purified and ligated overnight at 16°C. The plasmid for which the sequence was verified, pCMV-eGFP, was subsequently used in the subcloning experiment. The zHIF-1 $\alpha$ - $\Delta$ ODD ORF was amplified from pGEMT-zHIF-1 $\alpha$ - $\Delta$ ODD with the specific primers HIF-1 $\alpha$ -F-Kpn: 5'-CGG GGT ACC CCG ATG GAT ACT GGA GTT GTC AC-3' and HIF-1 $\alpha$ -R-Sal: 5'-ACG CGT CGA CGT CGG TTG ACT TGG TCC AGA GCA C-3', which contained built-in *Kpn*I and *Sal*I sites, respectively. The reverse primer was designed to eliminate the stop codon by converting TGA (stop codon) to CGA (arginine). The *Kpn*I-digested and *Sal*I-digested pCMV-eGFP plasmid and zHIF-1 $\alpha$ - $\Delta$ ODD PCR products were ligated with T4 ligase (Invitrogen) to produce pCMV-zHIF-1 $\alpha$ - $\Delta$ ODD-eGFP. The sequence of this construct was verified by sequencing the DNA.

### *Hypoxia exposure experiment*

Zebrafish embryos were divided into 2 groups: 1 group was reared in a hypoxic system and the other in a normoxic system. Tanks used for hypoxia exposure were set up as previously described.<sup>10</sup> Dissolved O<sub>2</sub> concentrations were monitored continuously using dissolved oxygen meters (Cole-Parmer 01972-00, Vernon Hills, IL, USA) and polarographic probes (Cole-Parmer 5643-00), and adjustment was made using a dissolved oxygen controller (Cole-Parmer 01972-00). Fertilized embryos (4 hours postfertilization [hpf]) were placed in net cages and allowed to develop to desired stages of development (24, 48, or 72 hpf). Each treatment consisted of 5 replicates of 60 embryos each. At the end of the experiments, the embryos were immersed in 200  $\mu$ L of TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) and stored at -80°C until RNA was extracted. The embryos used for hormone analysis were snap-frozen in liquid nitrogen and stored at -80°C. Animal care and experiments were conducted in accordance with the City University of Hong Kong animal care guidelines.

### *RNA isolation and first-strand cDNA synthesis*

Total RNA was extracted using TRI Reagent (Molecular Research Center) according to the manufacturer's instructions. First-strand cDNA was synthesized in a 25- $\mu$ L reaction that contained 0.25  $\mu$ g (0.5  $\mu$ g/ $\mu$ L) of random primer, 5  $\mu$ L of 5 $\times$  reaction buffer, 1.25  $\mu$ L of 10 mM deoxynucleotide

**Table 1.** Sequences of real-time PCR primers.

GENE	PRIMER SEQUENCE (5' → 3') FORWARD	REVERSE
<i>IGFBP-1a</i>	ACTCCAGACAGCCCTTGACAA	TGTACAGGCCGTGTTTGTGCG
<i>β-actin</i>	CCCTGAATCCCAAAGCCAAC	ACAGCCTGGATGGCCACATA
<i>StAR</i>	TCGGCTGAAACACCAGGAAA	ATTCCAGGGTGCTGTGTGGA
<i>CYP11a</i>	AAAATCTGCTGCAGGTCAAGGT	TGCCCACTCCTCTCAATCTGTT
<i>CYP11b2</i>	TGGAGGCGGGACAGTTATCA	CGCACACACTCCTGCACATC
<i>3β-HSD</i>	AAAGGCTTGCAGGGAGTTTCG	CGCCAGCCAATCAGTAGTGC
<i>HMGCR</i>	GTGGTGCGTTTTCCATCTGC	TGAAGCCTTCGGGACTCTCC
<i>cyp17a1</i>	GACACGCGCTGTCACAAATG	ATCCACGATTCCCTGGCTGT
<i>17β-HSD2</i>	GTGAATTCCTCGGCAGTGT	CTTTTGATGCGCCATAACCT
<i>Cyp19a</i>	CCATCAGTCTGTTCTTCATGC	CTTGGACAGATGCGAGTGCTG
<i>cyp19b</i>	ACCATCCGGTGGTGGATTTTC	TGCATGCGTCCAATGTTTCAG
<i>CITED-2</i>	AACCATGGTCTTTCCAGAA	ACTGTCCCATTCCCATCCTG

triphosphates, 0.65 μL of 40U ribonuclease (RNase inhibitor) (Invitrogen), 1 μL of 200U M-MLV Reverse Transcriptase (Promega), and 1 μg of total RNA, which was pretreated with RNase-free deoxyribonuclease I (Invitrogen) to eliminate genomic DNA contamination. cDNA synthesis was performed at 37°C for 1 hour. The cDNAs were kept at -20°C until they were analyzed by real-time PCR.

### Real-time PCR

Real-time PCR, which was used to quantify gene expression, was performed using a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) as described previously.<sup>11</sup> PCR assays were conducted using the SYBR Green-based detection method (#4367659; Applied Biosystems, Warrington, UK) according to the manufacturer's instructions. Sequences of the primers used for real-time PCR are shown in Table 1. Melting curve analysis was performed after the PCR was completed to assess the amplification specificity. The identity of the PCR amplicons was confirmed by DNA sequencing. The cycling conditions were as follows: initial denaturation at 95°C for 10 minutes, followed by 40 cycles performed at 95°C for 15 seconds, and 0°C for 30 seconds. β-Actin was employed as an endogenous control for normalization. All PCR reactions were performed in triplicate. Fold change was calculated according to the following formula: fold change =  $2^{-\Delta(\Delta C_T)}$ , where  $\Delta C_T = C_{T(\text{target})} - C_{T(\beta\text{-actin})}$  and  $\Delta(\Delta C_T) = \Delta C_{T(\text{stimulated})} - \Delta C_{T(\text{control})}$ . All data were expressed as the mean ± SD and  $P \leq .05$  was considered statistically significant.

### Estradiol (E2) and testosterone (T) measurement

In total, 60 zebrafish embryos from each replicate were collected and immediately stored at -80°C for hormone extraction (E2 and T) as described by Yu et al.<sup>11</sup> E2 and T levels were

measured using a commercial competitive enzyme-linked immunosorbent assay (ELISA) kit (Cayman Chemical, Ann Arbor, MI, USA).

### Overexpression of zHIF-1α

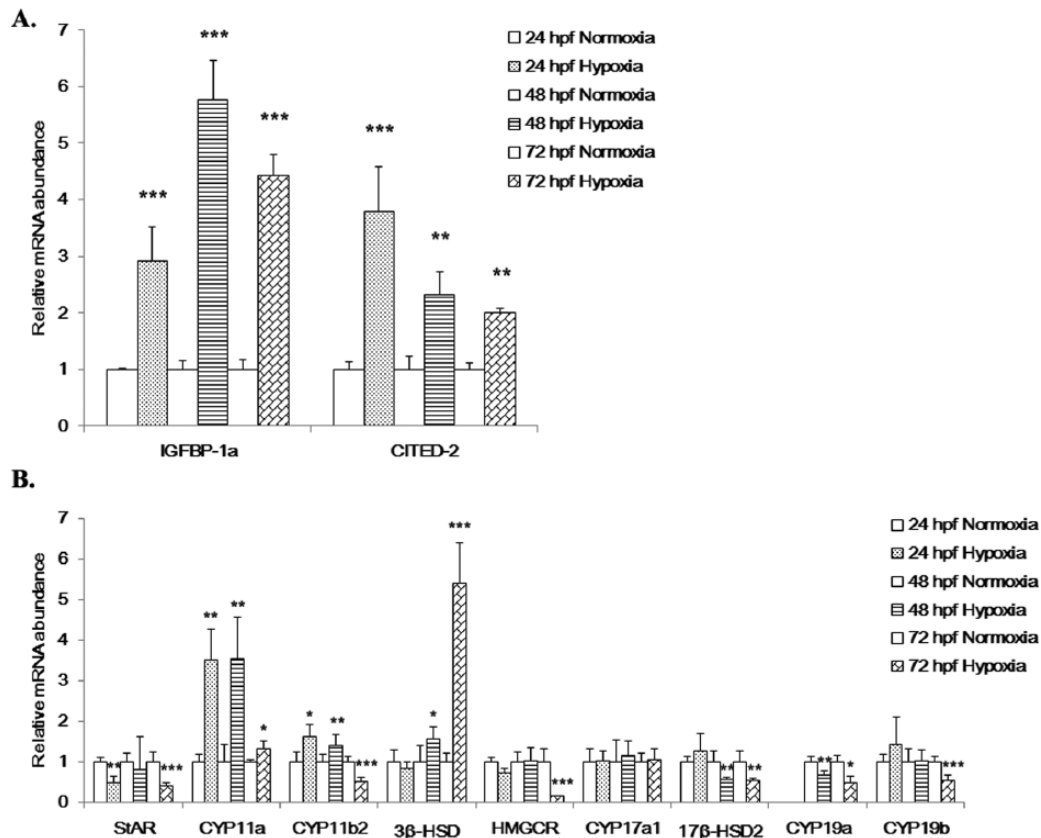
Capped eGFP and zHIF1α-ΔODD-eGFP messenger RNAs (mRNAs) were synthesized from linearized pCMV-eGFP and pCMV-zHIF-1α-ΔODD-eGFP, respectively, using the T7 mMESSAGE mMACHINE Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. mRNAs were microinjected at a concentration of 0.5 μg/μL in Hanks buffer containing 0.15% phenol red, and each embryo was injected with 2 nL mRNA at a 1 to 2 cell stage using a FemtoJet microinjection system (Eppendorf AG, Hamburg, Germany). Five replicates of 60 fertilized embryos each were used for both treatments. Injected embryos were incubated under normoxic conditions and sampled at various developmental stages for analyses.

### zHIF-1α knockdown

Antisense morpholino oligonucleotides (MOs) were obtained from Gene Tools (Philomath, OR, USA). An MO targeted against the translation initiation region of zHIF-1α (zHIF-1α MO: 5'-CAG TGA CAA CTC CAG TAT CCA TTC C-3') was used to knockdown the zHIF-1α protein. The standard control MO (5'-CCT CTT ACC TCA GTT ACA ATT TAT A-3') was used as an injection control. The optimized morpholino amount for zHIF-1α knockdown experiments was 8 ng/embryo. Injected embryos were reared to the desired developmental stages under hypoxic or normoxic conditions as described above.

### Statistical analysis

The Student *t* test was used to test the null hypothesis that there is no significant difference in mean gene expression



**Figure 1.** Effects of hypoxia on the expression of hypoxia markers and steroidogenic genes. Effect of hypoxia on the expression of (A) the HIF-1 target genes, IGFBP-1a and CITED-2 and (B) 9 steroidogenic enzyme genes. Fertilized zebrafish embryos were exposed to normoxic ( $7.0 \pm 0.2 \text{ mg O}_2 \text{ L}^{-1}$ ) or hypoxic ( $1.0 \pm 0.2 \text{ mg O}_2 \text{ L}^{-1}$ ) conditions for 24, 48, or 72 hours. Gene expression was quantified using real-time PCR and normalized against  $\beta$ -actin mRNA. Data are presented as the mean relative fold change  $\pm$  SD with respect to the normoxia control (its expression level was arbitrarily set to 1). Expression levels significantly different from the control are indicated by asterisks (t test,  $n \geq 4$ , \* $P \leq .05$ , \*\* $P \leq .01$ , \*\*\* $P \leq .001$ ). HIF-1 indicates hypoxia-inducible factor 1; mRNA, messenger RNA.

between the treatment and concurrent control groups. Significance was set at  $P \leq .05$ . All statistical analyses were conducted using Prism 3.02 (GraphPad, San Diego, CA, USA).

## Results

### *Hypoxia alters the expression pattern of steroidogenic enzyme genes*

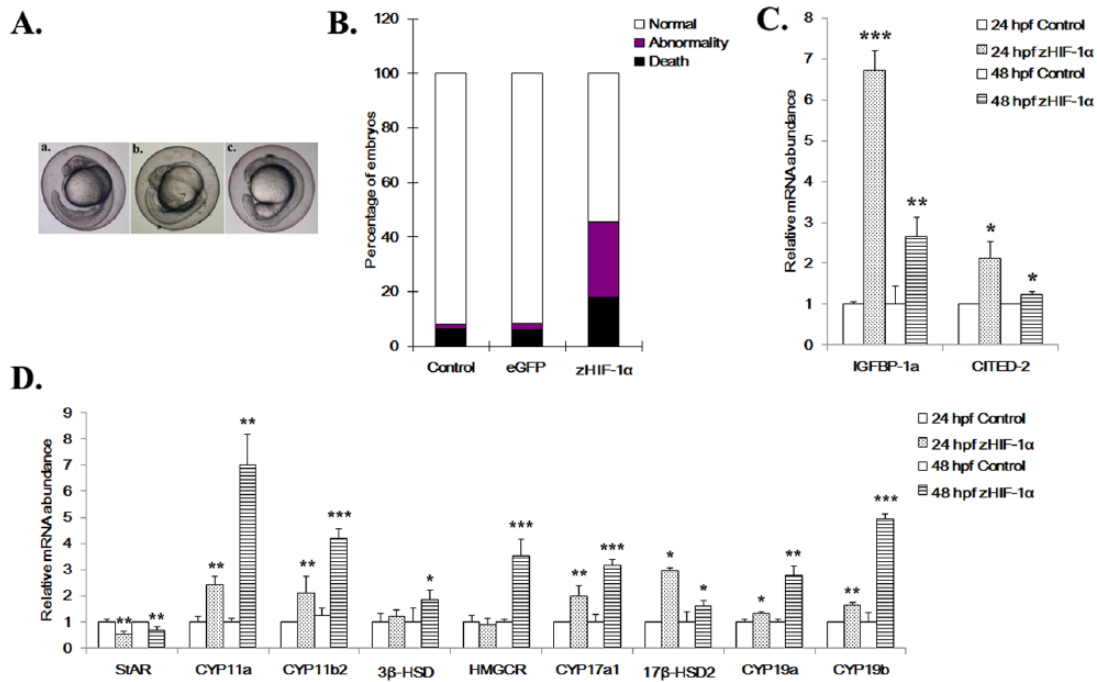
To confirm that HIF-1 signaling was activated in the zebrafish embryos under the experimental hypoxic conditions employed in this study, real-time PCR was performed to measure the expression of 2 HIF-1 target genes, *IGFBP-1a*<sup>29</sup> and *CITED-2*,<sup>30</sup> in embryos at 24, 48, and 72 hpf following exposure to hypoxia. Both the *IGFBP-1a* and *CITED-2* mRNAs were significantly upregulated in embryos under hypoxia at all detected stages (Figure 1A), which indicated that the hypoxic conditions used in the study were appropriate.

The mRNA expression levels for 9 selected steroidogenic genes in embryos exposed to hypoxia were differentially affected (Figure 1B). Five steroidogenic genes were significantly downregulated. *StAR* expression was downregulated by  $2.1 \pm 0.05$  ( $P \leq .01$ ) and  $2.6 \pm 0.2$  ( $P \leq .001$ ) fold at 24 and 72 hpf,

respectively; *HMGCR* was reduced by  $1.4 \pm 0.02$  ( $P \leq .01$ ) and  $7.1 \pm 2.78$  ( $P \leq .001$ ) fold at 24 and 72 hpf, respectively; *17β-HSD2* was reduced by  $1.8 \pm 0.19$  ( $P \leq .01$ ) and  $1.9 \pm 0.2$  ( $P \leq .01$ ) fold at 48 and 72 hpf, respectively; *CYP19a* was downregulated by  $1.5 \pm 0.01$  ( $P \leq .01$ ) and  $2.1 \pm 0.02$  ( $P \leq .05$ ) fold at 48 and 72 hpf, respectively; and *CYP19b* was downregulated by  $0.5 \pm 0.01$  ( $P \leq .001$ ) fold at 72 hpf. In contrast, 3 genes were significantly upregulated in response to hypoxia. *CYP11a* was upregulated by  $3.5 \pm 0.62$  ( $P \leq .01$ ),  $3.6 \pm 0.8$  ( $P \leq .01$ ), and  $1.3 \pm 0.19$  ( $P \leq .05$ ) fold at 24, 48, and 72 hpf, respectively; *CYP11b2* was upregulated by  $1.6 \pm 0.11$  ( $P \leq .05$ ) and  $1.4 \pm 0.09$  ( $P \leq .01$ ) fold at 24 and 48 hpf, respectively; and *3β-HSD* was increased by  $1.6 \pm 0.11$  ( $P \leq .05$ ) and  $5.4 \pm 1.04$  ( $P \leq .001$ ) fold at 48 and 72 hpf, respectively. *CYP17a1* mRNA expression showed no significant changes in hypoxic embryos in any of the 3 developmental stages.

### *Overexpression of zHIF-1α mRNA delays embryonic development and the expression pattern of steroidogenic genes*

To investigate the relationship between zHIF-1α expression and steroidogenesis in zebrafish, overexpression and knock-



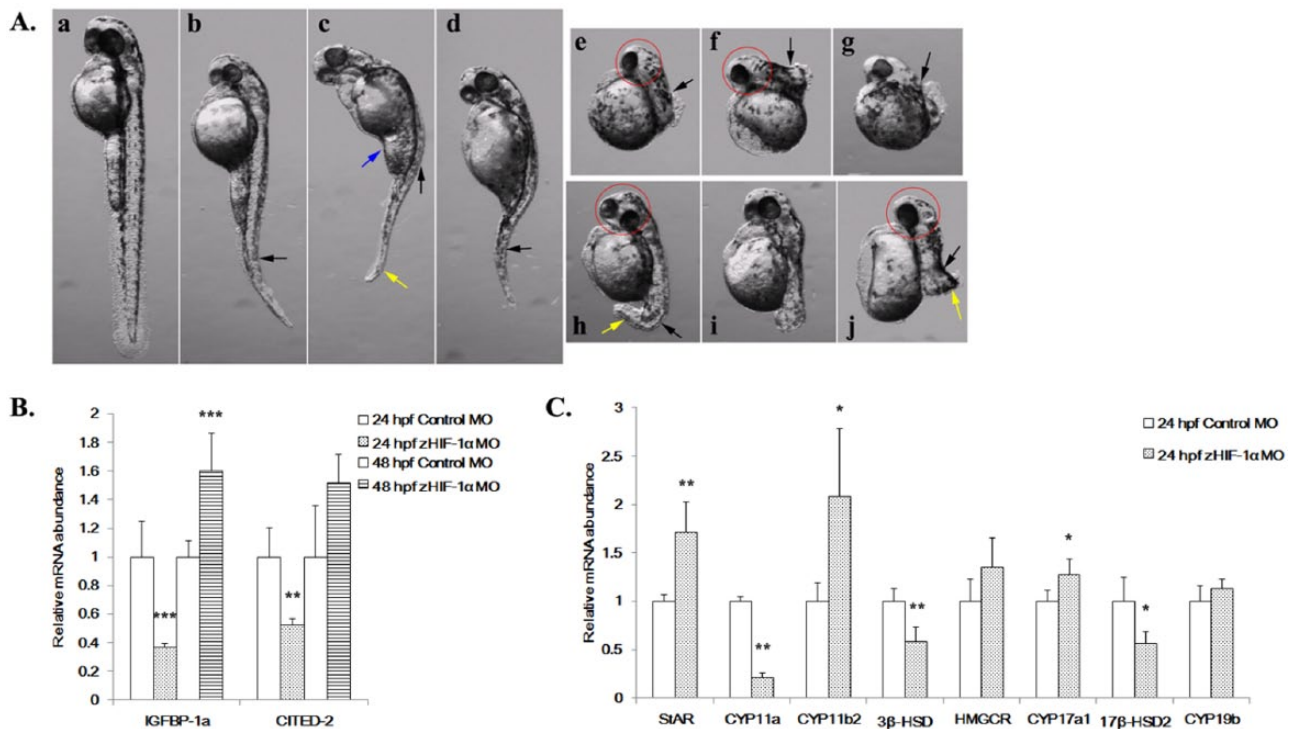
**Figure 2.** Effects of zHIF-1 $\alpha$  overexpression on embryonic development and the expression of hypoxia markers and steroidogenic genes. (A) Development of zebrafish embryos at 24 hpf. a, uninjected control embryo; b, embryo microinjected with eGFP mRNA shows normal development (Prim-6 stage); c, embryo microinjected with zHIF-1 $\alpha$ - $\Delta$ ODD-eGFP mRNA shows developmental retardation (at approximately 18-somite stage). (B) Percentage of mortality and morphological abnormality in zebrafish embryos (24 hpf) microinjected with zHIF-1 $\alpha$  mRNA. The effect of zHIF-1 $\alpha$  overexpression on steroidogenic gene expression was examined by microinjecting zHIF-1 $\alpha$  mRNA into 1- to 2-cell stage zebrafish embryos and maintained under normoxic conditions. Zebrafish embryos microinjected with eGFP mRNA and maintained under normoxia were used as the control. Because no statistically significant difference was observed between the data sets of the microinjected control and the no-injection control (One-way analysis of variance with a  $P < .05$  threshold), the latter data set is presented here. Control: uninjected embryos ( $n = 373$ , dead = 31, abnormal = 4); eGFP: embryos microinjected with eGFP mRNA ( $n = 719$ , dead = 48, abnormal = 16); and zHIF-1 $\alpha$ : embryos microinjected with zHIF-1 $\alpha$ - $\Delta$ ODD-eGFP mRNA ( $n = 1081$ , dead = 193, abnormal = 297). (C) Effects of zHIF-1 $\alpha$  overexpression on IGFBP-1a and CITED-2 expression. (D) Effects of zHIF-1 $\alpha$  overexpression on steroidogenic gene expression. Gene expression was quantified using real-time PCR and normalized with  $\beta$ -actin mRNA. Data are presented as the mean relative fold change  $\pm$  SD with respect to the gene expression level in the control (its expression level was arbitrarily set to 1) for each experiment. Expression levels significantly different from the control are indicated by asterisks ( $t$  test,  $n \geq 4$ , \* $P \leq .05$ , \*\* $P \leq .01$ , \*\*\* $P \leq .001$ ). eGFP indicates enhanced green fluorescent protein; mRNA, messenger RNA.

down of zHIF-1 $\alpha$  were conducted by microinjection of the in vitro-transcribed zHIF-1 $\alpha$ - $\Delta$ ODD-eGFP mRNAs and zHIF-1 $\alpha$  translation-blocking morpholinos (MO) into embryos, respectively. Five treatment replicates were used in each experiment. The total numbers of embryos injected with the eGFP mRNA (control) and zHIF-1 $\alpha$ - $\Delta$ ODD-eGFP mRNA were 719 and 1081, respectively. Based on the embryo staging criteria described by Westerfield,<sup>31</sup> the development of embryos microinjected with zHIF-1 $\alpha$ - $\Delta$ ODD-eGFP mRNA was found to be delayed by at least 6 hours when compared with that of the eGFP-injected and uninjected controls at 24 hpf (Figure 2A).

At 24 hpf, 17.8% of the embryos microinjected with zHIF-1 $\alpha$ - $\Delta$ ODD-eGFP mRNA were dead compared with the 6.1% dead embryos observed with eGFP mRNA microinjection. The percentage of mortality of the embryos microinjected with zHIF-1 $\alpha$  mRNAs was approximately 3-fold greater than that of the eGFP control (Figure 2B). In addition, the percentage of

abnormal embryos observed in the zHIF-1 $\alpha$  microinjection experiments was 27.5% (297/1081), whereas only 2.2% (16/719) of abnormal embryos were observed in the eGFP control group. Following microinjection with the zHIF-1 $\alpha$ - $\Delta$ ODD-eGFP mRNAs, IGFBP-1a and CITED-2 were significantly upregulated in embryos at 24 and 48 hpf (Figure 2C), which suggested successful overexpression of zHIF-1 $\alpha$  in the embryos up until 48 hpf.

Following microinjection of the zHIF-1 $\alpha$  mRNAs, the expression patterns of all 9 steroidogenic genes were found to be affected in zebrafish embryos at 24 and 48 hpf (Figure 2D). With the exception of *SIAR* which was downregulated by  $1.82 \pm 0.12$  ( $P \leq .01$ ) and  $1.43 \pm 0.12$  ( $P \leq .01$ ) fold at 24 and 48 hpf, respectively, all other steroidogenic genes were upregulated by the overexpression of zHIF-1 $\alpha$ . *CYP11a* was upregulated by  $2.4 \pm 0.04$  ( $P \leq .01$ ) and  $7.0 \pm 1.2$  ( $P \leq .01$ ) fold at 24 and 48 hpf, respectively; *CYP11b2* was upregulated by  $2.1 \pm 0.6$  ( $P \leq .01$ ) and  $3.4 \pm 0.13$  ( $P \leq .001$ ) fold at 24 and 48 hpf,



**Figure 3.** Effects of zHIF-1 $\alpha$  knockdown on zebrafish embryonic development and the expression of hypoxia markers and steroidogenic genes under normoxia. (A) Morphological abnormalities of the zHIF-1 $\alpha$  knockdown embryos at 48 hpf under normoxia. The effect of zHIF-1 $\alpha$  knockdown on steroidogenic gene expression was examined by microinjecting zHIF-1 $\alpha$  MOs into 1- to 2-cell stage zebrafish embryos and maintained under either normoxia or hypoxia. Zebrafish embryos microinjected with a standard control MO were used as controls. All morphants were imaged at the left lateral view. a, Control: embryo injected with control MO; b-j, embryos microinjected with zHIF-1 $\alpha$  MO; b, c, and d, morphants with decreased body length, bended notochords (black arrows), and distorted abdomens (blue arrows); e-j, morphants with bended notochords, small head circles (red circles), and short curved tail (yellow arrows). (B) Effects of zHIF-1 $\alpha$  knockdown on IGFBP-1a and CITED-2 expression under normoxia. (C) Effects of zHIF-1 $\alpha$  knockdown on steroidogenic gene expression under normoxia. Gene expression was quantified by real-time PCR and normalized against  $\beta$ -actin mRNA. Data are presented as the mean relative fold change  $\pm$  SD with respect to the gene expression level in the control (its expression level was arbitrarily set to 1) for each experiment. Expression levels significantly different from the control are indicated by asterisks ( $t$  test,  $n \geq 4$ , \* $P \leq .05$ , \*\* $P \leq .01$ , \*\*\* $P \leq .001$ ). mRNA indicates messenger RNA; MO, morpholino oligonucleotide.

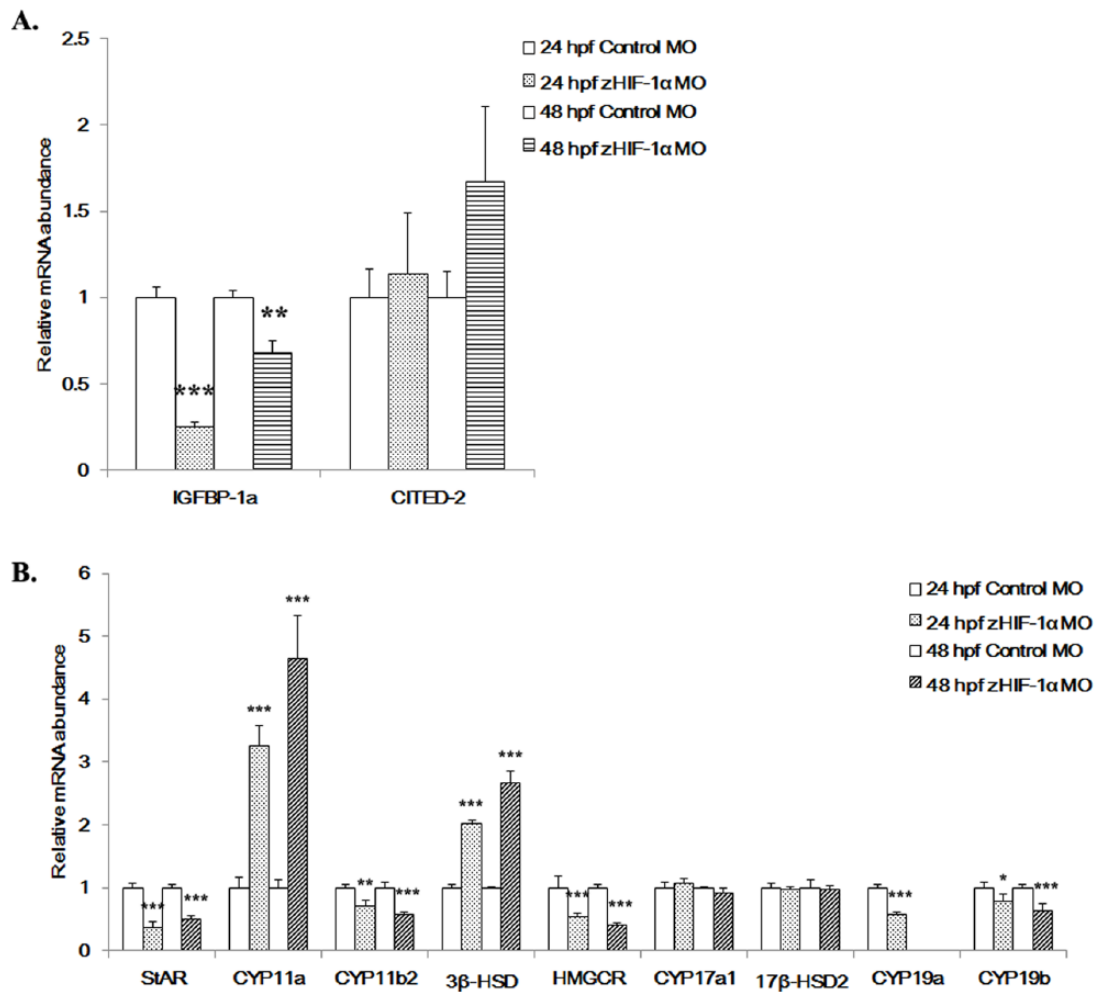
respectively; *CYP17a1* was upregulated by  $2.0 \pm 0.3$  ( $P \leq .01$ ) and  $3.2 \pm 0.06$  ( $P \leq .001$ ) fold at 24 and 48 hpf, respectively; *17 $\beta$ -HSD2* was upregulated by  $2.98 \pm 0.1$  ( $P \leq .05$ ) and  $1.6 \pm 0.39$  ( $P \leq .05$ ) fold at 24 and 48 hpf, respectively; *CYP19a* was upregulated by  $1.34 \pm 0.05$  ( $P \leq .05$ ) and  $2.8 \pm 0.27$  ( $P \leq .01$ ) fold at 24 and 48 hpf, respectively; *CYP19b* was upregulated by  $1.6 \pm 0.11$  ( $P \leq .01$ ) and  $4.9 \pm 0.21$  ( $P \leq .001$ ) fold at 24 and 48 hpf, respectively; and *3 $\beta$ -HSD* and *HMGCR* were upregulated by  $1.9 \pm 0.4$  ( $P \leq .05$ ) and  $3.5 \pm 0.4$  ( $P \leq .001$ ) fold, respectively, at 48 hpf.

#### Knockdown of zHIF-1 $\alpha$ alters expression of steroidogenic genes

The experiment in which zHIF-1 $\alpha$  was knocked down with a morpholino was conducted to complement the study in which zHIF-1 $\alpha$  was overexpressed. Following zHIF-1 $\alpha$  knockdown, morphological abnormalities were observed in morphant embryos at 48 hpf under normoxia (Figure 3A). The observed deformities included small head circumference,

bent notochords, deformed hearts, distorted abdomens, and short curved tails. Knocking down zHIF-1 $\alpha$  also resulted in significantly shorter zebrafish embryo body length at the same developmental stage (Figure 3A). In morphant embryos at 24 hpf, the expression levels of *IGFBP-1a* and *CITED-2* were significantly reduced by  $2.7 \pm 0.48$  ( $P \leq .001$ ) and  $1.9 \pm 0.14$  ( $P \leq .01$ ) fold, respectively, compared with those of the control. However, *IGFBP-1a* was elevated by  $1.6 \pm 0.18$  ( $P \leq .001$ ) fold in morphants at 48 hpf, whereas *CITED-2* expression was unaffected.

The expression patterns of the steroidogenic genes in the zHIF-1 $\alpha$  morphants were different from those of the control embryos (microinjected with control MO) under normoxia (Figure 3C). Three steroidogenic genes were specifically upregulated in the zHIF-1 $\alpha$  morphants. *StAR* was significantly increased by  $1.7 \pm 0.24$  ( $P \leq .01$ ) fold, *CYP11b2* was upregulated by  $2.1 \pm 0.5$  ( $P \leq .05$ ) fold, and *CYP17a1* was upregulated by  $1.3 \pm 0.04$  ( $P \leq .05$ ) fold at 24 hpf. In contrast, *CYP11a* was downregulated by  $4.8 \pm 0.01$  ( $P \leq .01$ ) at 24 hpf, whereas *3 $\beta$ -HSD* and *17 $\beta$ -HSD2* were downregulated by  $1.7 \pm 0.02$



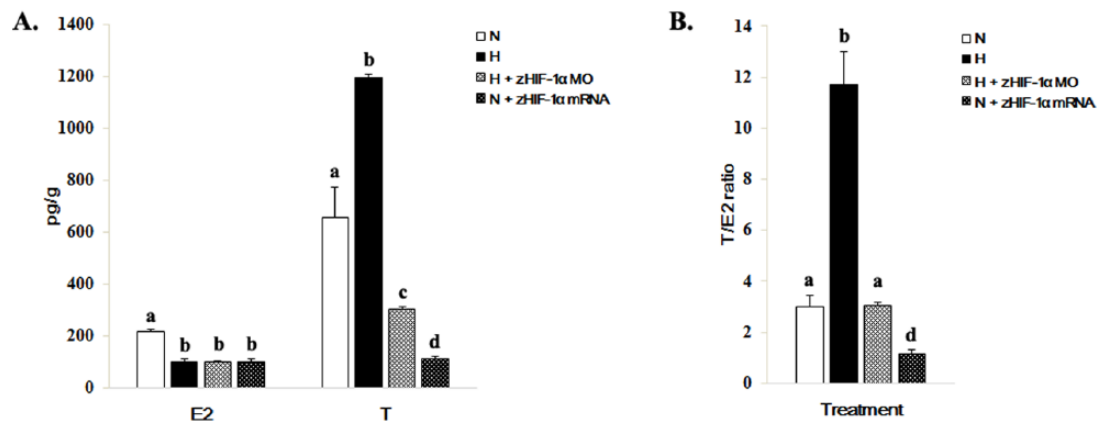
**Figure 4.** Effects of zHIF-1 $\alpha$  knockdown on the expression of hypoxia markers and steroidogenic genes under hypoxia. (A) Effects of zHIF-1 $\alpha$  knockdown on the hypoxia marker genes IGFBP-1a and CITED-2 under hypoxia. Methods for zHIF-1 $\alpha$  knockdown are detailed in Figure 3. (B) Effects of zHIF-1 $\alpha$  knockdown on steroidogenic gene expression under hypoxia. Gene expression was quantified by real-time PCR and normalized against  $\beta$ -actin mRNA. Data are presented as the mean relative fold change  $\pm$  SD with respect to the gene expression level in the hypoxia control (its expression level was arbitrarily set to 1) for each experiment. Expression levels significantly different from the control are indicated by asterisks ( $t$  test,  $n \geq 4$ , \* $P \leq .05$ , \*\* $P \leq .01$ , \*\*\* $P \leq .001$ ). mRNA indicates messenger RNA.

( $P \leq .01$ ) and  $1.8 \pm 0.1$  ( $P \leq .05$ ) fold, respectively, at 24 hpf. No significant changes in *HMGCR* or *CYP19b* expression were observed in embryos in the zHIF-1 $\alpha$  morphants at 24 hpf compared with embryos microinjected with control MO. Among the 9 steroidogenic genes examined, only *StAR*, *CYP11a*, *3 $\beta$ -HSD*, and *17 $\beta$ -HSD2* in the zHIF-1 $\alpha$  morphants exhibited opposite patterns of expression to those of the embryos overexpressing zHIF-1 $\alpha$ . These observations suggest that expressions of these genes might be directly and/or indirectly regulated by HIF-1.

To confirm whether changes in the expression of steroidogenic genes under hypoxia (Figure 1B) were due to regulation by zHIF-1 $\alpha$ , embryos in which zHIF-1 $\alpha$  had been knocked down were exposed to hypoxia. Comparing embryos in which zHIF-1 $\alpha$  had been knocked down with the control MO-injected embryos under hypoxia, *IGFBP-1a* was downregulated by  $4 \pm 0.04$  ( $P \leq .001$ ) and  $1.47 \pm 0.02$  ( $P \leq .01$ ) fold

at 24 and 48 hpf, respectively, whereas *CITED-2* was unaffected at both stages (Figure 4A). These observations suggest that zHIF-1 $\alpha$  was successfully downregulated in the knock-down treatment.

In the zHIF-1 $\alpha$  morphant embryos under hypoxia, *StAR* was significantly downregulated by  $2.8 \pm 0.08$  ( $P \leq .001$ ) and  $2.0 \pm 0.01$  ( $P \leq .001$ ) fold at 24 and 48 hpf, respectively; *CYP11a* was upregulated by  $3.2 \pm 0.26$  ( $P \leq .001$ ) and  $4.7 \pm 0.86$  ( $P \leq .001$ ) fold at 24 and 48 hpf, respectively; *CYP11b2* was downregulated by  $1.4 \pm 0.13$  ( $P \leq .01$ ) and  $1.7 \pm 0.09$  ( $P \leq .001$ ) fold at 24 and 48 hpf, respectively; *3 $\beta$ -HSD* was upregulated by  $2.0 \pm 0.05$  ( $P \leq .001$ ) and  $2.7 \pm 0.34$  ( $P \leq .001$ ) fold at 24 and 48 hpf, respectively; *HMGCR* was downregulated by  $1.8 \pm 0.29$  ( $P \leq .001$ ) and  $2.5 \pm 0.05$  ( $P \leq .001$ ) fold at 24 and 48 hpf, respectively; and *CYP19a* was downregulated by  $1.8 \pm 0.01$  ( $P \leq .001$ ) fold at 24 hpf but was undetectable at 48 hpf; *CYP19b* was downregulated by  $1.3 \pm 0.11$  ( $P \leq .05$ ) and



**Figure 5.** Effects of hypoxia and overexpression of zHIF-1 $\alpha$  on E2 and T levels in zebrafish embryos. (A) Effects of hypoxia and zHIF-1 $\alpha$  expression on E2 and T levels. Methods for hypoxic exposure and zHIF-1 $\alpha$  overexpression and knockdown are detailed in Figures 1 to 3, respectively. Hormone levels in whole zebrafish embryos (5 replicates of 60 pooled embryos each) were measured by the ELISA method. The data are presented as the mean concentration of hormone (in picograms per gram of embryo wet weight)  $\pm$  SD. The different letters above the bars indicate statistically significant differences between the indicated groups for the same hormone ( $P < .05$ , Student-Newman-Keuls). (B) Effects of hypoxia and zHIF-1 $\alpha$  expression on the T/E2 ratios. Data are presented as the mean  $\pm$  SD ( $P < .05$ , Student-Newman-Keuls). The different letters above the bars indicate statistically significant differences between the indicated groups.

1.6  $\pm$  0.17 ( $P \leq .001$ ) fold at 24 and 48 hpf, respectively; and *CYP17a1* and *17 $\beta$ -HSD2* were not affected in the zHIF-1 $\alpha$  morphants at any of the time points.

#### E2 and T levels

The concentrations of E2 and T in whole embryos varied under normoxic and hypoxic conditions and with or without overexpression and knockdown of zHIF-1 $\alpha$  (Figure 5). The concentrations of E2 and T in whole embryos under hypoxia were 2.2-fold less and 1.8-fold greater than those of the normoxic controls (Figure 5A), which resulted in a 3.9-fold greater T/E2 ratio (from  $3.0 \pm 0.42$  (normoxia) to  $11.73 \pm 1.28$  (hypoxia) (Figure 5B). The concentrations of both E2 and T were lower in embryos overexpressing zHIF-1 $\alpha$  under normoxic conditions and in zHIF-1 $\alpha$  knockdown embryos under hypoxic conditions (Figure 5A). The T/E2 ratio in the zHIF-1 $\alpha$  morphants under hypoxia was similar to that of the control embryos under normoxia. However, the T/E2 ratio in embryos overexpressing zHIF-1 $\alpha$  under normoxia was 10.3-fold lower than those maintained under hypoxia (Figure 5B).

#### Discussion

There is increasing evidence that the HIF-1 signaling system plays essential roles in embryonic development in vertebrates through the activation of genes that regulate energy metabolism and survival. Previous studies have reported the expression of HIF-1 $\alpha$  mRNA in the brain, blood vessels, somites, notochord, retina and optic tectum in zebrafish embryos,<sup>32</sup> and HIF-1 $\alpha$ -deficient mouse embryos (HIF-1 $\alpha$ -/-) show defects in the formation of blood vessels and the neural fold as well as malformations in the cardiovascular system.<sup>33,34</sup> HIF-1 has also been

demonstrated to affect the development of zebrafish embryos through the regulated expression of *IGFBP-1*.<sup>29</sup> In this study, zHIF-1 $\alpha$  knockdown using morpholinos resulted in various types of morphological abnormalities in developing zebrafish embryos (Figure 3A) which indicates that the expression of zHIF-1 $\alpha$  is essential for the normal development of zebrafish embryos under normoxic conditions and is consistent with the results from previous studies conducted in mice.<sup>35</sup> Recently, studies have shown that in addition to its canonical role as a transcription factor, HIF-1 $\alpha$  has a nontranscriptional role as a signaling regulator of Notch signaling,<sup>36,37</sup> which is involved in many cellular processes during embryonic development including somitogenesis,<sup>38</sup> muscle tissue formation,<sup>39</sup> blood vessel maturation,<sup>40</sup> and heart formation.<sup>41</sup> It is reasonable to speculate that the varied morphological abnormalities observed in zebrafish embryos could be attributed to the disruption of Notch signaling as a result of ectopic zHIF-1 $\alpha$  expression.

There is ample evidence that hypoxia impairs the sexual and reproductive development of fish through perturbation of the HPG axis at multiple levels, including enzymes that control steroidogenesis.<sup>5,10</sup> A qualitative comparison of the results from this study on the expression patterns of steroidogenic genes in zebrafish embryos exposed to hypoxia, embryos overexpressing zHIF-1 $\alpha$  mRNA, and zHIF-1 $\alpha$  knockdown embryos is provided in Table 2. Changes in the expression of genes encoding steroidogenic enzymes, including *StAR*, *CYP11a*, *CYP11b2*, *3 $\beta$ -HSD*, *HMGCR*, *CYP17a1*, *17 $\beta$ -HSD2*, *CYP19a*, and *CYP19b*, would likely affect the production of steroid hormones, such as testosterone, estradiol, aldosterone, and cortisol. Steroid hormones are crucial substances that play a number of important physiological roles. An important function of steroid hormones is coordinating



**Table 2.** Comparison of steroidogenic gene expression patterns in hypoxic, zHIF-1 $\alpha$  overexpressing, and zHIF-1 $\alpha$  knockdown embryos.

	HYPOXIA			ZHIF-1 $\alpha$ OVEREXPRESSION		ZHIF-1 $\alpha$ MO KNOCKDOWN			
	24 HPF	48 HPF	72 HPF	24 HPF	48 HPF	NORMOXIA		HYPOXIA	
						24 HPF	48 HPF	24 HPF	48 HPF
<i>StAR</i>	↓	=	↓	↓	↓	↑	=	↓	↓
<i>CYP11a</i>	↑	↑	↑	↑	↑	↓	↓	↑	↑
<i>CYP11b2</i>	↑	↑	↓	↑	↑	↑	=	↓	↓
<i>3<math>\beta</math>-HSD</i>	=	↑	↑	=	↑	↓	=	↑	↑
<i>HMGCR</i>	↓	=	↓	=	↑	=	=	↓	↓
<i>CYP17a1</i>	=	=	=	↑	↑	↑	↓	=	=
<i>17<math>\beta</math>-HSD2</i>	=	↓	↓	↑	↑	↓	=	=	=
<i>CYP19a</i>	\	↓	↓	↑	↑	\	\	↓	\
<i>CYP19b</i>	=	=	↓	↑	↑	=	=	↓	↓

Abbreviation: MO, morpholino oligonucleotide.

↓: downregulated; ↑: upregulated; =: unaffected; \: not detected.

the physiological and behavioral responses for specific biological purposes, such as reproduction. Mutations in *StAR* are known to cause a complete lack of steroidogenesis in the adrenals and gonads, which, in turn, leads to classical congenital lipid adrenal hyperplasia in humans.<sup>42</sup> Mutations in the *CYP11a1* gene causes adrenal insufficiency in humans and rabbits,<sup>43,44</sup> whereas *CYP11a1*-null mice are deficient in steroid synthesis ability.<sup>45</sup> In zebrafish embryos in which *CYP11a1* was knocked down, the embryos exhibited a shortened axis and defects in cell movement during epiboly.<sup>46</sup> CYP11B2 catalyzes 3 sequential reactions that convert 11-deoxycorticosterone to aldosterone.<sup>47</sup> Because the biosynthesis of aldosterone is solely catalyzed by CYP11B2,<sup>48</sup> downregulation of *CYP11b2* might result in the reduced production of aldosterone. 3 $\beta$ -HSD deficiency results in lower amounts of adrenal steroids including mineralocorticoids, glucocorticoids, and sex steroids.<sup>14,49</sup> Knockdown studies of CYP17 showed a significant reduction in progesterone formation and de novo synthesis of steroids in mouse tumor Leydig cells.<sup>50</sup> Inactivating mutations in *CYP19* are rare in humans, but affected individuals are unable to synthesize endogenous estrogens, which results in virilization of both the fetus and the mother.<sup>51</sup>

The results of downregulating of *StAR* and *CYP19a* under hypoxia were consistent with the results of previous observations reported in mammalian cells<sup>52</sup> and in the male and female gonads of zebrafish, which also correlated with significant reductions of estradiol in females and testosterone in males.<sup>53</sup> A positive relationship between *StAR* expression and sex steroid production has also been reported in male rainbow trout, where sex steroid hormone secretion was increased by the elevated expression of *StAR*.<sup>54</sup> Repression of *StAR* can lead to the decreased production of sex hormones, as *StAR* is a cholesterol-shuttling protein that facilitates the

translocation of cholesterol from the outer to the inner mitochondrial membrane in endocrine tissues, and this represents a rate-limiting step in steroidogenesis.<sup>55</sup> In rats under hypoxia, *CYP11a* expression was previously reported to be stimulated, whereas *StAR* expression was suppressed.<sup>56–58</sup> Similar expression patterns of *CYP11a* and *StAR* were found in this study. Concentrations of E2 and T in zebrafish embryos were less under all treatment conditions, with the exception of T, which was upregulated under hypoxia. The findings are consistent with the reduced expression of *StAR* under all treatment conditions relative to that of the normoxia control. The increased concentration of T under hypoxia presumably reflects the reduced conversion of T to E2 due to the downregulation of *CYP19a*.

Specifically, we showed that hypoxia and zHIF-1 $\alpha$  overexpression significantly downregulated the expression of *StAR* but upregulated the expression of *CYP11a* and *3 $\beta$ -HSD*. The reversed expression of these 3 genes in the zHIF-1 $\alpha$  knockdown embryos under normoxia strongly suggests that these genes are directly regulated by HIF-1. Moreover, computational analysis of the 5' flanking regions of these 3 genes revealed several putative HREs (5'-A/GCGTG-3'). Within the first 3 kb of the 5' flanking sequence, 4, 3, and 4 putative HREs (forward/reverse) were found in *StAR*, *CYP11a*, and *3 $\beta$ -HSD*, respectively. These results indicate that zHIF-1 $\alpha$  may directly regulate these genes via HRE binding in the promoter region.

*CYP11b2* is the only gene that showed an opposite expression pattern in hypoxia and hypoxic zHIF-1 $\alpha$  knockdown embryos. Considering the unaffected expression pattern of *CYP11b2* under zHIF-1 $\alpha$  overexpression and knockdown treatment under normoxia, these results imply that *CYP11b2* is unlikely to be regulated by zHIF-1 $\alpha$  directly, or the HREs

residing in the *CYP11b2* promoter may be occupied by other HIFs under hypoxia.

## Conclusions

In this study, it is demonstrated that zHIF-1 regulates the expression of several steroidogenic enzyme genes in zebrafish embryos. Specifically, the regulated expression of *StAR*, *CYP11a*, *3 $\beta$ -HSD*, and *CYP19a* likely affects the production levels of the steroid hormones, E2 and T. This study provides evidence that zHIF-1 has a role in the regulation of embryonic development and steroidogenesis in zebrafish.

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## Author Contributions

TT conceived and designed the experiments; collected, analyzed, and interpreted the data; wrote the first draft of the manuscript. RMKY performed literature review, contributed to the writing and made critical revisions of the manuscript. RSSW contributed to the writing of the manuscript and approved the final version. RYCK designed the study and performed a critical review of the manuscript. All authors reviewed and approved the final manuscript.

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