Molecular Mechanisms of Embryonic Growth Regulation in Response to Hypoxia

低酸素下における魚類の初期発生調節機構に関する研究

A Dissertation Submitted in Partial Fulfillment of the Requirements for
The Degree of Doctor of Philosophy

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March, 2006

CONTENTS

ABBREVIATIONS

ANOVA:	analysis of variance
AP-1:	activating protein-1
bp:	base pairs
BrdU:	5'-Bromo-2'-deoxyuridine
cDNA:	complementary DNA
CMV:	cytomegalovirus
CRE:	c-AMP response element
CREB:	CRE-binding protein
DAPI:	4,6-diamidino-2-phenylindole
ENO1:	elonase1
EPO:	erythropoietin
EGFP:	enhanced green fluorescent protein
EMSA:	electrophoretic mobility shift assay
FIH-1:	factor inhibiting HIF-1
GFP:	green fluorescent protein
GH:	growth hormone
HA:	influenza hemagglutin
HAS:	HIF-ancillary sequence
HIF-1:	hypoxia-inducible factor
Hpf:	hour post fertilization
HRE:	hypoxia-responsive element
IGF:	insulin-like growth factor
IGF-1:	insulin-like growth factor-1
IGF-2:	insulin-like growth factor-2
IGF-1R:	insulin-like growth factor-1 receptor

IGFBP:	insulin-like growth factor binding protein
IUGR:	intrauterine growth restriction
KDa:	kilodalton
LDHA:	lactate dehydrogenase A
mRNA:	messenger RNA
mTOR:	mammalian target of rapamycin
MO:	morpholino oligonucleotide
Odc:	ornithinedecarboxylase
ORF:	open reading frame
PCR:	polymerase chain reaction
PDH:	prolyl hydroxylase
PLSD:	protected least significant difference
pVHL:	von Hippel-Lindau tumor suppressor
ROS:	reactive oxygen species
TBE:	tris, bortic acid, EDTA
VEGF:	vascular endothelial growth factor

GENERAL INTRODUCTION

Life is surrounded by a variety of stressful environments. Evolution favors to develop a number of molecular pathways to combat these stressors and maintain homeostasis. Hypoxia, decreased level of oxygen, is one of the environmental changes to which animals are required to coordinate homeostasis for adaptation. Since oxygen is the terminal acceptor of electrons in the respiratory chain and is required for oxidative phosphorylation, this adaptation process demands a number of physiological responses at cellular and organism levels. These include augmentation of red blood cell, angiogenesis, and ventilation through the carotid body to supply enough oxygen into each organ. At the cellular level, hypoxia leads to marked changes in metabolism, switching from aerobic oxidation to anaerobic process such as activating glycolysis for ATP generation (Bruick, 2003; Guillemin and Krasnow, 1997; Semenza, 1999; Wenger, 2002). To achieve these physiological responses, a number of genes are altered at the transcription and post-transcription levels (Gracey et al., 2001; Huang et al., 2004; Seta and Millhorn, 2004).

Hypoxia is especially a severe stress to fetuses. Intrauterine hypoxia, caused by insufficiency of placental oxygen supplied to fetus, is one of the leading causes of intrauterine growth restriction (IUGR) (Giudice, 2002; Pollack et al., 1997; Semenza, 2000; Soothill et al., 1987). Reduced birth weights by low oxygen levels is also observed in high altitude, averaging a 100 g of reduced birth weight per 1000 m elevation gain (Moore, 2003). It has been known that IUGR significantly increases in fetal and neonatal morbidity and mortality, and also in risk of adult diseases, such as cardiovascular disease, type-2 diabetes, obesity, and hypertension (Ong and Dunger, 2002). However, in contrast to the physiological responses in adult stage, knowledge in early development is limited. Emerging question is therefore how the changes in oxygen levels are perceived, integrated, and responded for appropriate modifications of internal cues to maintain homeostasis in early development.

A central step in the hormonal regulatory network of embryonic growth and development is insulin-like growth factor (IGF) system. IGF system is composed of two ligands (IGF-1 and -2),

two types of IGF receptors (type-I and -II receptor) on the cell membrane, and six forms of IGF binding proteins (IGFBPs). IGFs were initially identified as a growth factor that stimulates sulfate uptake in cartilage, as a non-suppressible insulin-like factor that stimulates glucose uptake in adipose tissue, and as a multiplication stimulating activity on the chick embryo fibroblasts. Purification and amino acid sequences identified two distinct, but structurally related 70-amino acid peptide hormones with a high degree of structural similarity to proinsulin, referred to as IGF-1 and IGF-2 (Firth and Baxter, 2002; Le Roith et al., 2001; Wood et al., 2005a). The binding of IGFs to IGF-1 receptor on the cell surface leads to tyrosine phosphorylation of the beta-domain of the receptor, which triggers subsequent signaling cascades such as the mitogen-activated protein kinase (MAPK), phosphoinositol-3-kinase (PI3-kinase), and protein kinase B (PKB/Akt) signaling pathways, depending upon cell type and cellular context (Le Roith et al., 2001; Wood et al., 2005a). On the other hand, the type-II IGF receptor, identified as the cation-independent mannose-6-phosphate (M6-P) receptor, lacks recognizable catalytic domains suggesting that it does not activate conventional signal transduction pathways and works for clearance of IGF-2 (Braulke, 1999).

The "IGF signaling" is now considered to be an evolutionally conserved, and promotes a variety of physiological processes including cell growth, differentiation and proliferation, energy metabolism growth, survival, and migration. At the whole organism level, it is involved in the organismal growth and development, osmoregulation, reproduction, and aging (Dupont and Holzenberger, 2003; Kenyon, 2001; Le Roith et al., 2001; Wood et al., 2005a). The special importance of IGF signaling in embryonic growth and development was demonstrated by knock-out mice studies. Targeting knock-out of either IGF gene shows intermediate growth retardation, whereas mice lacking both IGFs genes or IGF-1 receptor gene result in only 30-45% of body weight compared with wild type, and die shortly after birth (Baker et al., 1993; DeChiara et al., 1990; Dupont and Holzenberger, 2003; Liu et al., 1993). This observation was further confirmed by the reports in human patients where natural mutations such as the deletion of exon 4 and 5 of IGF-1 gene or two point mutations or stop codon in exon 2 of the IGF-1 receptor gene caused severe dwarfism (Abuzzahab et al., 2003; Woods et al., 1996).

The activation of IGF signaling depends on the molecular interaction of IGFs to its receptor,

being modulated by six distinct forms of IGFBPs, referred to as IGFBP-1 through IGFBP-6. These IGFBPs are secreted proteins with molecular mass of 24-25 kDa, sharing highly conserved N-terminal and C-terminal domains and a variable linker (L)-domain. They have equal or greater affinities to IGFs than IGF receptors, and form complexes with IGFs in the extracellular environment. When IGFs form complexes with any of IGFBPs, they are not able to bind to its receptors, therefore, considered as "non-active" forms. Each IGFBP has multi-functions as carrier protein, regulating IGF localization, turnover, and inhibiting or potentiating IGF actions by regulating the interaction between IGFs and IGF-R. For example, the majority of IGFs in the circulation forms a ternary complex with IGFBP-3 and its associated protein, acid-labile subunit (ALS). The ternary complex works as a "reservoir pool" of IGF supply, and also prolong the half-life of IGFs by inhibiting proteolytic degradation. On the other hand, IGFBP-5 is much less in the circulation, but abundantly produced in the vascular smooth muscle cells, and potentiates the biological actions of IGFs (Duan, 2002). Furthermore, IGFBPs help to prevent potential hypoglycemia caused by cross-activation of insulin receptor. Importantly, these IGFBPs provide another level of flexibility (spacious and temporal) for controlling the amounts of free/active IGFs available to the receptor in the target tissues, therefore, more precise and delicate regulation is possible (Clemmons, 2001; Duan, 2002; Duan and Xu, 2005; Firth and Baxter, 2002).

These two lines of issues addressed above (i.e., physiological responses to hypoxia and IGF system during embryogenesis) are indeed interrelated. Several clinical and basic studies have reported the significantly higher levels of IGFBP-1 in the serum of hypoxia-induced IUGR fetus (Giudice et al., 1995; Unterman et al., 1990; Unterman et al., 1993). In addition, maternal serum contains higher levels of IGFBP-1 at higher altitude (Krampl et al., 2002). *In vitro* studies using cultured human cells and *in vivo* studies using rodent models suggest that IGFBP-1 gene expression is elevated in hypoxic condition (McLellan et al., 1992; Popovici et al., 2001; Tazuke et al., 1998). Since IGFBP-1 binds IGFs with high affinity and inhibits IGF activities on cell growth *in vivo* and *in vitro*, it was postulated that the elevated IGFBP-1 might play some role in hypoxia-caused IUGR (Giudice, 2002; Popovici et al., 2001; Tazuke et al., 1998). This appealing model, however, has not been directly tested *in vivo*, and a causation relationships as well as its molecular basis of the elevated

IGFBP-1 levels in hypoxia-induced IUGR fetus have not been established.

The limitation of our knowledge is partly attributed to the nature of mammalian model. First, mammalian fetuses are enclosed in the uterus and affected by a variety of maternal factors via the placental circulation (Crossey et al., 2002; Giudice, 2002). Second, in contrast to a number of *in vitro* studies showing the functions of each IGFBP, the efforts on *in vivo* studies have been hampered by the redundancy in expression patterns and function of IGFBPs. Since IGF system is built on a precise balance, possible phenotypes caused by gain/loss-of function approach could be masked by functional compensation (Duan, 2002; Wood et al., 2005a).

On the other hand, the zebrafish has emerged as an alternative and informative vertebrate model organism for early development (Kimmel et al., 1995; Wood et al., 2005a). This genetically tractable vertebrate develops externally, eliminating the complication of maternal compensation. Fast developing and transparent zebrafish embryos make it possible to manipulate environmental factors and observe the phenotypic changes in organ formation in real time. More importantly, recent advances in genome project as well as genetic/biochemical studies further confirmed that major components of the zebrafish IGF signaling pathway, including IGF ligands, receptors, IGFBPs, and intracellular signal transduction network, are highly conserved (Bauchat et al., 2001; Duan et al., 1999; Li et al., 2005; Maures et al., 2002; Maures and Duan, 2002; Wood et al., 2005a; Wood et al., 2005b). Therefore, if hypoxia affects the zebrafish embryogenesis in the similar way as mammals, information gained from this unique animal model will not only provide new insight into zebrafish developmental biology, but also deepen our understanding of growth and developmental regulation in vertebrates in general.

In this study, therefore, I chose zebrafish as a vertebrate model of embryogenesis, and asked two questions: 1) what is the molecular basis of embryonic growth regulation under hypoxic environment? : 2) how does "hypoxia" cause transcriptional changes during embryonic stages? These questions are addressed in the following chapters. In chapter 1, I have shown that IGFBP-1 mediates hypoxia-induced embryonic growth retardation and developmental delay. Hypoxia up-regulates IGFBP-1 expression and reduces embryonic growth and developmental rates in zebrafish embryos. IGFBP-1 knockdown partially abrogates these hypoxic effects, whereas IGFBP-1

overexpression decreases embryonic growth and development under normoxia. Re-introduction of IGFBP-1 to knocked-down embryos restores the hypoxic effects. The mode of IGFBP-1 actions is further tested in embryonic cells derived from zebrafish, showing that IGFBP-1 suppresses mitogenic action of IGFs. These results suggest that IGFBP-1 plays a major role in coordinating embryonic growth and development in response to hypoxia. In chapter 2, I further explored the molecular mechanisms how hypoxia induces gene expression during embryogenesis, using IGFBP-1 as an example of hypoxia-inducible genes. I have shown that the hypoxia-inducible factor (HIF)-1, a transcription factor, pathway is established in early embryogenesis and mediates hypoxia-induced IGFBP-1 expression. Hypoxia increased the HIF-1 activity, and HIF-1α overexpression was sufficient to induce endogenous IGFBP-1 expression in cultured cell and zebrafish embryo. Although the zebrafish IGFBP-1 promoter contains 13 consensus hypoxia response elements (HREs), deletion and mutational analysis revealed that only one HRE and its ancillary sequence are required for the hypoxia and HIF-1-induction. These results suggest that HIF-1 mediates hypoxia-induced IGFBP-1 gene expression in early development by selectively interacting with a HRE. I believe that these studies provide experimental and direct evidence linking between "physiological response to hypoxia" and "embryonic growth and development" in molecular, cellular, and organism levels.

Chapter 1: IGFBP-1 Mediates Hypoxia-Induced Embryonic Growth and Developmental Retardation.

Introduction

Hypoxia profoundly affects embryonic growth and development, representing a leading cause of IUGR. Recent evidence suggests that hypoxia may influence fetal growth through its connection to the IGF system. Several groups have reported that circulating level of IGFBP-1, a secreted protein that binds to IGF in extracellular environments, is elevated in IUGR fetuses (Giudice et al., 1995; Unterman et al., 1990; Unterman et al., 1993) and that there is a striking inverse correlation between IGFBP-1 levels and the fetal size (Fant et al., 1993). In addition, higher maternal serum IGFBP-1 levels are found at higher altitude (Krampl et al., 2002). *In vitro* studies using cultured human cells and *in vivo* studies using rodent models suggest that IGFBP-1 gene expression is elevated in hypoxic condition (McLellan et al., 1992; Popovici et al., 2001; Tazuke et al., 1998). Since IGFBP-1 binds IGFs with high affinity and inhibits IGF activities on cell growth *in vitro* (Clemmons, 2001; Duan, 2002; Duan and Xu, 2005; Firth and Baxter, 2002), and because IGFBP-1 overexpressing transgenic mice had reduced birth weight (Crossey et al., 2002; Gay et al., 1997; Rajkumar et al., 1995), it was postulated that the elevated IGFBP-1 plays a major role in hypoxia-caused IUGR by binding fetal IGF and inhibiting their growth-promoting activities (Giudice, 2002; Popovici et al., 2001; Tazuke et al., 1998). However, there is no *in vivo* experimental evidence and a causative relationship between the elevated IGFBP-1 expression and IUGR has not been established. Moreover, the impact of hypoxia on early development such as morphogenesis is poorly understood, and the role of IGFBP-1, if any, in mediating the hypoxic effects on embryonic development is unknown.

In this chapter, I first determined the effects of hypoxia on embryonic growth and development, and test the hypothesis that IGFBP-1 mediates these hypoxic effects, using zebrafish embryos as a model of early development in vertebrates.

Materials and Methods

Materials - All chemicals and reagents were purchased from Fisher Scientific (Pittsburgh, PA) unless noted otherwise. Human IGF-I was purchased from GroPep (Adelaide, Australia). RNA polymerase and RNase-free DNase were purchased from Promega (Madison, WI). Restriction endonucleases were purchased from New England BioLabs (Beverly, MA). Superscript II reverse transcriptase (RT) and oligonucleotide primers were purchased from Invitrogen Life Technologies, Inc. (Carlsbad, CA). Morpholino-modified oligonucleotides were purchased from Gene Tools, LLC (Corvalis, OR).

Experimental animals and procedures - Wild-type zebrafish (*Danio rerio*) were maintained on a 14 h light/10 h dark cycle at 28 ˚C and fed twice daily in the fish facility at the University of Michigan. Embryos were obtained by natural cross. Fertilized eggs were raised at 28.5 ˚C and staged according to Kimmel et al (Kimmel et al., 1995). To inhibit embryo pigment formation, embryo medium was supplemented with 0.003% (w/v) 2-phenylthiourea. The dissolved oxygen levels of the system water under ambient condition (normoxia) were approximately 6.5 $\pm$ 0.5 mg/liter. For the hypoxia treatment, oxygen level was reduced to 0.6 $\pm$ 0.1 mg/liter (hypoxia) by bubbling nitrogen gas into water. After embryos of various stages were transferred, the container was sealed and the dissolved oxygen levels were monitored by a dissolved oxygen meter (YSI Model 58, Fisher Scientific, Pittsburgh, PA). After 18-24 h hypoxia treatment, the embryos were fixed in 4% paraformaldehyde in 1 x PBS and stored at –20 ˚C in 100% methanol or frozen in liquid nitrogen and stored at –80 ˚C for further analysis.

Whole-mount in situ hybridization and RT-PCR analysis- Whole-mount *in situ* hybridization using digoxigenin (DIG)-labeled RNA riboprobe was performed as reported elsewhere (Maures et al., 2002). For RT-PCR analysis, total RNA was extracted using Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH). After DNase treatment (Invitrogen), the RNA (2.5 μg) was reverse transcribed with random hexamer primers and SuperScript II reverse transcriptase (Invitrogen) in a total volume of 20 ul. Sequences of primers used for RT-PCR are as shown in Tabble 1-1. PCR cycles and amount of templates were optimized for each primer set in pilot experiments (25 or 500 times dilution of original cDNA samples). PCR cycles are as follows; 94 ˚C for 2 min, 32 cycles (IGFBP-5 and IGF-2) or 35 cycles (IGFBP-1, -2, -3, IGF-1, and IGF-1Ra) of 94 ˚C for 30 sec,

57 ˚C for 30 sec, and 72 ˚C for 1 min. PCR products were analyzed by electrophoresis followed with ethidium bromide staining. The levels of ornithinedecarboxylase (odc) mRNA was measured and used as an internal control (Draper et al., 2001).

Determining the IGFBP-1 gene structure - The zebrafish IGFBP-1 genomic structure was first determined by searching zebrafish genome database (http://www.ensembl.org/Multi/blastview?species=Danio_rerio), and confirmed by genomic PCR methods. Genomic DNA, isolated from adult zebrafish (AB-line), was used for genomic PCR analysis. The amplified PCR product was subcloned to PCR2.1-TOPO vector (Invitrogen) and sequenced.

Morpholino knockdown- Two morpholino oligonucleotides (MO) were designed to target the IGFBP-1 exon1/intron1 boundary (MO1: 5'-ATA AAC CTG TAC CTG GTG CTG TCT C-3') or the exon3/intron3 boundary (MO2: 5'-GAC GAC TCT TCT CAC CTG TTT GAT T-3'). The MOs were dissolved in 1x Danieau Solution (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)$, 5 mM HEPES, ph 7.6) and injected into 1-2 cell stage embryos using a PV830 pneumatic pico pump and glass pipettes (OD=1.2 mm, World Precision Instruments, Sarasota, FL). A standard control MO (5'-CCT CTT ACC TCA GTT ACA ATT TAT A-3') was used in all experiments. To determine the efficiency and specificity of MO knockdown, total RNA was extracted from the injected embryos at various time points and RT-PCR was conducted as described above. The IGFBP-1 PCR products were subcloned into PCR2.1-TOPO vector (Invitrogen) and sequenced.

Overexpression of IGFBP-1- A DNA fragment containing the entire open reading frame of zebrafish IGFBP-1 was generated by PCR using a primer set (5'-GGC GGATCC GCC GCC ACC ATG AAC AGA CTG CTT CTG-3'/5'-CCC GGA TCC GTG GTT GAG TTC CTC GGG-3'). This DNA fragment was subcloned into the pCS 2+ vector to generate IGFBP-1:EGFP fusion protein. The construct was verified by DNA sequencing. To express IGFBP-1:EGFP, linearized plasmid (75 pg) was injected to embryos with or without 2 ng of IGFBP-1 MO1.

Western blotting - Embryos were homogenized in homogenization buffer (100 mM Tris, pH 6.8, 0.1% Tween 20, 10% glycerol, 1 μg/ml aprotinin, 0.3 mM phenylmethylsulfonyl fluoride). Protein levels of each sample were quantified using a commercial protein assay kit (Pierce Biotechnology, Inc., Rockford, IL). Equal amounts of protein (30 μg) were subjected to SDS-PAGE

(12.5%) in reduced condition, and transferred to Immoblin-P membrane (Millipore Corp.). Western immunoblot analysis was performed using a GFP antibody at a 1:1000 dilution (Torrey Pines Biolabs, Inc., Houston, TX). Western ligand blotting, was carried out as described elsewhere (Duan et al., 1999; Kajimura et al., 2003; Shimizu et al., 2000). Briefly, embryo lysates were separated in SDS-PAGE in non-reduced condition, and transferred to nitrocellulose membrane. The membranes were incubated overnight with DIG-labeled human IGF-I at 4 °C in blocking buffer (SuperBlock Blocking Buffer; Pierce Biotechnology). The membranes were washed three times with TBS and incubated with anti-DIG-POD (Roche). The signal was visualized using enhanced chemiluminescence reagent exposing to Hyperfilm ECL (Amersham Pharmacia Biothech, Piscataway, NJ).

Cell culture and* 5'-Bromo-2'-deoxyuridine (*BrdU) incorporation assay- Zebrafish embryonic (ZF4) cells, obtained fromAmerican Type Culture Collection, were grown in a 1:1 mixture of Ham's F12 medium and DMEM with penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% FBS at 28.5 °C. A BrdU incorporation assay was performed as reported as follows. ZF-4 cells were plated onto coverslips in six-well plates and grown to confluence. After overnight serum starvation, the cultures were exposed to 20 μM BrdU and the desired concentrations of IGF-I (Gropep) and/or IGFBP-1 (Bauchat et al. 2001). Each treatment was added to duplicate cultures. After approximately 22 h, cells were washed twice with ice-cold PBS, fixed with 4 % formaldehyde in PBS, and permeablized with acetone/methanol (1:1). The cells were rehydrated in PBS and denatured for 1 h in 2.0 M HCl. Next, the cells were incubated in sodium tetraborate and equilibrated in PBS. The coverslips were then blocked for 2 h in 20% normal goat serum and 0.5% Triton-X followed by overnight incubation with an anti-BrdU antibody. After washing, the coverslips were incubated with a TRITC-linked secondary anti-mouse antibody for 2 h at room temperature. The coverslips were washed, and exposed for 0.5 h in darkness to 4,6-diamidino-2-phenylindole (DAPI) at 0.25 mg/ml. The coverslips were washed three times in PBS and mounted onto slides. Cells were counted under at Nikon E600 fluorescence microscope facilitated with the Optronics Camera System (Melville, NY, USA). For each coverslip, at least ten view fields were randomly selected and a total of approximately 1000 cells were counted. The total cell number was determined by counting DAPI-stained nuclei. The results are expressed as percent of the total BrdU-

positive cells.

Statistics- Values are means ± S.E. Differences among groups were analyzed by one or two-way ANOVA followed by Fisher's protected least significant difference (PLSD) using Stat View software (SAS Institute, Cary, NC). Significance was accepted at $P < 0.05$.

Results

Hypoxia causes growth retardation and developmental delay in zebrafish embryos.

First, the effects of hypoxia on zebrafish embryogenesis were determined and characterized by morphological analysis. As shown in Fig. 1-1 A and B, 24 hours of hypoxia treatment significantly decreased the body length (48 hpf). Hypoxia treatment also caused a significant reduction in the development rate, as indicated by the reducing head-trunk angles (HTA) (Fig. 1-1C), which is a quantitative parameter for determining the developmental stage during 24-48 hpf (i.e., during the pharyngula period) (Kimmel et al., 1995). Based on the HTA data, 48-hpf embryos in the hypoxia group were developmentally equivalent to control embryos at 32 hpf. Similar experiments were carried out by using 6-hpf embryos. Hypoxia treatment for 18 h significantly reduced the somite number from 30.2 ± 1.0 (n = 38) of the control group to 21.4 ± 0.7 (n = 42, $P < 0.001$) of the hypoxia group. The somite number is used for staging embryos 10-24 hpf (i.e., during the segmentation period) (Kimmel et al., 1995). Embryos at 24 hpf in the hypoxia group were therefore developmentally equivalent to control embryos at 19.5 hpf.

Next, I investigated the effect of hypoxia on heart morphogenesis by examining the mRNA expression pattern of *nkx 2.5*, a cardiac marker (Lee et al., 1996). In zebrafish, the shape of the heart changes from a tube-like structure to a looped structure between 24 and 48 hpf under normoxia (Fig. 1-1D, upper panels). Under hypoxia, this looping process was significantly delayed. As shown in Fig. 1-1D, embryos exposed to hypoxia have yet to complete the cardiac looping, and their hearts remained a tube-like structure at 48 hpf, resembling that of the control embryos at 32 hpf. The appearance of pharyngeal arches is another well timed morphogenesis event in the pharyngula period and can be monitored by the mRNA expression patterns of *dlx* 2, a homeobox transcription factor

(Kimmel et al., 1995). Under normoxia, *dlx 2* expression was detected in the primordial region of the budding mandibular (Fig. 1-1D, lower panels, indicated by arrow heads) and hyroid arches (Fig. 1-1D, lower panels, indicated by arrows) at 32 hpf. At 48 hpf, embryos developed distinct branchial arches. In comparison, the mandibular arch and hyroid of embryos on the hypoxia group were located far posterior to the forebrain at 48 hpf, similar to embryos of the normoxia group at 32 hpf. These results suggest that hypoxia not only results in growth retardation but also causes significant delay in embryonic development and the timing of heart and head skeleton morphogenesis.

Hypoxia significantly increases IGFBP-1 mRNA and protein levels.

The IGFBP-1 mRNA expression levels were low under normoxia in all examined stages (Fig. 1-2A). Hypoxia treatment increased IGFBP-1 expression in different spatial domains at different developmental stages. In early embryos (24 and 48 hpf), hypoxia stimulated ubiquitous expression of IGFBP-1 (Fig. 1-2A, lower panels). In advanced embryos (72 and 96 hpf), the hypoxia-induced and basal IGFBP-1 expression became liver-specific (Fig. 1-2A, lower panels, indicated by arrow heads). Semi-quantitative RT-PCR analysis showed that hypoxia significantly increased IGFBP-1 mRNA levels at all of the time points examined (Fig. 1-2B and C). In contrast, no significant changes in the expression levels of IGFBP-2, IGFBP-3, IGFBP-5, IGF-1, IGF-2, and IGF-1 receptor-a were detected (Fig. 1-2B). Ligand blotting analysis revealed that the increase in IGFBP-1 mRNA levels was accompanied by increased functional IGFBP-1 protein levels (Fig. 1-3 A and B). These data suggest that hypoxia treatment significantly increased IGFBP-1 mRNA and protein levels but not those of other components of the IGF-signaling system.

Knockdown of IGFBP-1 abrogates hypoxia-induced growth retardation and developmental delay.

Splice-site-targeting MOs have been proven to be an efficient and specific way to "knock down" targeted genes in zebrafish embryos by altering premRNA splicing (Draper et al., 2001). This approach has the advantage of being verifiable by RT-PCR and thus eliminates the need for specific antibodies. To design specific MOs, I first determined the zebrafish IGFBP-1 gene structure by searching the zebrafish genome database followed by genomic PCR and sequencing analysis. The zebrafish IGFBP-1 gene spans 5 kb in the genome (Fig. 1-4A) and is composed of four exons (exon 1, 465 bp; exon 2, 158 bp; exon 3, 129 bp, and exon 4, 254 bp) and three introns (intron 1, 621 bp; intron

2, 3199 bp; and intron 3, 212 bp). Two MOs were designed to target the IGFBP-1 exon 1/intron 1 and exon 3/intron 3 boundaries, respectively. Each MO was injected into embryos under hypoxia at various doses (0.5, 1, 2, 4, 8, and 16 ng) and the successful knockdown was confirmed by RT-PCR. The results showed that injection of IGFBP-1 MO1 (the exon 1/intron 1 MO) at doses higher than1 ng resulted in the reduction of endogenous IGFBP-1 (387 bp) and an aberrant splice form (1008 bp) (Fig. 1-4B). The knockdown effect lasted until at least 96 hpf (Fig. 1-4C). The exon 3/intron 3 MO2 also efficiently altered splicing, although a higher dose was required (data not shown). Zebrafish IGFBP-1 consists of 262 amino acids, including a 25-aa signal peptide and a 237-aa mature protein that can be divided into a N-terminal domain, middle linker domain, and C-terminal domain (Maures and Duan, 2002). Sequence analysis of the altered splicing product revealed an insertion of 621 bp into intron 1, which caused a reading-frame shift and introduced a premature stop codon (Fig. 1-4D). As a result, the encoded protein is 125 aa and therefore lacks part of the middle linker domain and all of the C-terminal domain. This truncated IGFBP-1 is predicted to be nonfunctional, because deletion of the C-terminal 20 aa or residue changes in this region completely abolish IGF binding (Brinkman et al., 1991; Vajdos et al., 2001). This MO knockdown of IGFBP-1 is highly specific given that RT-PCR analysis revealed that there were no aberrant splice forms nor significant changes in the abundance of other IGFBPs, including IGFBP-2, IGFBP-3, and IGFBP-5 (Fig.1-5).

To test whether the elevated IGFBP-1 plays any role in mediating hypoxia-induced growth and developmental retardation, the IGFBP-1 MO1 or control MO was injected into embryos at the 1- to 2-cell stage. As shown in Fig. 1-6A, the hypoxia treatment caused severe growth retardation and developmental delay indicated by the smaller body size and reduced HTA. Knockdown of IGFBP-1 partially, but significantly alleviated these hypoxic effects by 43-54% and 48-65%, based on the body length and HTA data (Fig. 1-6 B and C). Embryos in the IGFBP-1 knockdown group under normoxia were morphologically indistinguishable from those of the control group under normoxia (Fig. 1-6 B and C), suggesting that knockdown of IGFBP-1 had little effect in growth and development under normoxia. This observation also indicates that the IGFBP-1 MO at the concentration used had little, if any, side effect.

Overexpression of IGFBP-1 inhibits embryonic growth and development under normoxia and restores the hypoxic effects in the knockdown background.

To further test the hypothesis through a gain-of-function approach, an IGFBP-1:EGFP expression construct was generated. The transcript resulting from this construct is intronless and therefore resistant to the IGFBP-1 MOs. Western immunoblotting analysis with a GFP antibody detected a major band at 55 kDa, corresponding to the IGFBP-1:EGFP fusion protein in these embryos (Fig. 1-7, left). This protein was not detected in wild-type or vector-injected embryos. Western ligand blotting analysis indicated that IGFBP-1:EGFP is a functional IGFBP (Fig. 1-7, right). This binding is specific because the band was completely displaced by the addition of an excess amount of unlabeled IGF-1 (data not shown).

To determine whether elevated IGFBP-1 mediates the hypoxia effect, a series of gain-of-function studies were performed. As shown in Fig. 1-8A, overexpression of IGFBP-1 reduced embryo size to 83% of the control under normoxia (Fig. 1-8A). Overexpression of IGFBP-1 also slowed down the developmental rate, as indicated by the significantly reduced HTA (Fig. 1-8B). The body length in the IGFBP-1-plus-MO group was indistinguishable from that of the control group under hypoxia and significantly smaller than the MO group in hypoxia. Likewise, IGFBP-1 overexpression reversed the effects of MO in development as indicated by the reduction in HTA (Fig. 1-8B).

The effects of loss and gain of IGFBP-1 on heart and head skeleton development were also examined. As shown in Fig. 1-8C, the looping of the developing heart and the positions of the mandibular and hyroid arches in the MO group under hypoxia were similar to those of the control group under normoxia. Overexpression of IGFBP-1:EGFP restored these delays (Fig. 1-8C, upper panels). The positions of the mandibular and hyroid arches and the heart structure of the IGFBP-1-plus-MO group were similar to those of the control group in hypoxia (Fig. 1-8C, lower panels).

IGFBP-1 inhibits IGF action in cultured embryonic cells.

The mode of action of IGFBP-1 was examined *in vitro* by using cultured zebrafish embryonic cells. As shown in Fig. 1-9, human IGF-1 or IGF-2 (100 ng/ml, Gropep) resulted in a 6.0 ± 0.5- and 4.9 ± 0.8-fold increases, respectively, in the number of the divided cells over the control ($P < 0.05$).

Addition of purified fish IGFBP-1 (Bauchat et al. 2001) at an equimolar concentration (400 ng/ml) inhibited IGF-1- or IGF-2-stimulated cell proliferation by 60% and 58%, respectively. These inhibitory effects were statistically significant ($P < 0.05$). IGFBP-1 itself had no effect on cell proliferation, suggesting that IGFBP-1 does not directly regulate zebrafish cell proliferation. It is postulated that IGFBP-1 might act by binding to and sequestering the actions of IGFs. To test this idea, IGF and IGFBP-1 were added to the cells at a 2:1 and 4:1 molar ratio. As expected, the inhibitory effect of IGFBP-1 on cell proliferation was diminished when IGF-1 or IGF-2 was added to the cells at a 4:1 molar ratio, presumably due to the saturation of IGFBP-1 with excess amounts of IGFs.

Discussion

This study shows that oxygen availability has a profound impact on embryonic growth. Hypoxia exposure for 18-24 h significantly reduced body size, as it does to human and other mammalian fetuses. It is further revealed that hypoxia also causes significant delays in embryonic development. This conclusion is supported by the reduced somite number and HTA and by the delayed heart and craniofacial skeleton morphorgenesis. Whole-mount *in situ* hybridization suggests that hypoxia causes proportional delays in the morphogenesis of these organs but does not cause patterning abnormality since the expression patterns and intensities of the marker genes are not affected by hypoxia treatment. In fact, the hypoxia-caused growth and developmental retardation is a reversible process. After transferring back to normal oxygen environment, the embryos can "catch up" and become nearly indistinguishable from the control group (Chang et al., unpublished data). This catch-up growth pattern is also observed in IUGR infants and rodent models, and the catch-up growth is often associated with higher serum IGF-1 levels (Albertsson-Wikland et al., 1998; Thieriot-Prevost et al., 1988). In contrast, zebrafish embryos enter suspended animation, and the developmental processes and cell division are stopped under anoxia (Padilla and Roth, 2001).

In developing zebrafish embryos, IGFBP-1 mRNA is expressed at very low levels in many tissues in earlier stages but become liver-specific in advanced embryos after the liver primordium is

formed (Maures and Duan, 2002). The induction of IGFBP-1 expression by hypoxia is operative at both stages: first in multiple embryonic tissues and then only in the liver. IGFBP-1 gene in mammals contains consensus sequence sof hepatocyte nucleotic factor-1 and -3, key transcription factors to regulate liver- specific gene expression (O'Brien et al., 1995; Suh and Rechler, 1997). Zebrafish IGFBP-1 gene also has putative HNF-1 and HNF-3 consensus sequences in 5'- flanking region, which presumably are involved in the observed liver-specific expression.

The induction appears to be specific to IGFBP-1 because all of the other IGF-signaling system components examined, including IGF-1, IGF-2, IGFBP-2, IGFBP-3, IGFBP-5, and IGF-1 receptor, show little change under hypoxia. These findings are consistent with previous studies performed in mammalian and teleost species (Gracey et al., 2001; Maures and Duan, 2002; McLellan et al., 1992; Popovici et al., 2001; Tazuke et al., 1998). No significant changes were found in serum levels of IGF-1 and IGF-2 under hypoxia in ovine fetus (McLellan et al., 1992). Although immunoreactive IGF-1 and IGF-2 were detected in teleost embryonic tissues by immunohistochemistry (Perrot et al., 1999; Radaelli et al., 2003), it is unclear whether hypoxia changes the levels of IGF peptides. The following chapter will examine and discuss the detailed molecular mechanism how IGFBP-1 gene expression is induced by hypoxia.

The gain- and loss-of-function analysis results provide strong evidence supporting the theory that IGFBP-1 plays an important role in mediating hypoxia-caused growth and developmental retardation. Overexpression of a functional IGFBP-1 fusion protein reduced the embryo size to 83% of the control size under normoxia. This result is similar to the reduced birth size in IGFBP-1 overexpressing mice (Gay et al., 1997; Rajkumar et al., 1995). Overexpression of IGFBP-1 also slowed down the developmental rate to a degree similar to that of the hypoxia treatment. Therefore, IGFBP-1 is sufficient to induce growth retardation and developmental delay. Furthermore, targeted knockdown of IGFBP-1 significantly alleviated the hypoxia-induced growth retardation and developmental delay. To my knowledge, this study is the first to provide *in vivo* evidence for a requirement of IGFBP-1 in mediating hypoxia-caused growth and developmental retardation in a vertebrate embryo. However, knockdown of IGFBP-1 only partially (43-65%) alleviated the hypoxia-caused growth retardation and developmental delay. This observation indicates that IGFBP-1 is probably one of many genes

involved in hypoxia-induced embryonic growth and developmental retardation. In fact, a recent microarray analysis study reports a number of genes besides IGF system or its down stream are regulated by hypoxia in zebrafish embryos, including the genes known to be important in cell cycle (cyclin G1 and histone 3), protein synthesis (ribosomal proteins), and metabolism regulation (ATP synthase) (Ton et al., 2003).

Numerous *in vitro* studies have shown that IGFBP-1 inhibits IGF actions in cultured human cells and in transgenic mice (Clemmons, 2001; Duan, 2002; Duan and Xu, 2005; Firth and Baxter, 2002). In this study, it is clear that fish IGFBP-1 inhibited IGF-stimulated zebrafish embryonic cell proliferation, although fish IGFBP-1 itself had no mitogenic activity. This inhibitory effect was abolished when IGF-1 or IGF-2 was added in molar excess. Because the IGF-1 receptor mediates the biological actions of IGFs and because IGFBP-1 bind to IGFs with high affinity, the inhibitory effects of IGFBP-1 can be rationally attributed to its competition for the ligand with the IGF-1 receptor. The lack of ligand-independent activity in fish IGFBP-1 is consistent with the fact that fish IGFBP-1 does not contain an RGD motif (Funkenstein et al., 2002; Maures and Duan, 2002). In contrast, human IGFBP-1 contains a functional RGD motif, which can interact with integrin and is responsible for its IGF-independent activity on cell migration contributing IGF-independent activity (Jones et al., 1993).

It is of interest to note that knock down of IGFBP-1 did not alter the growth and development rate under normoxia. This observation is consistent with recent reports showing that IGFBP-1 knockout mice exhibited normal growth and development under a normal oxygen environment (Leu et al., 2003a; Leu et al., 2003b). This lack of effect is in agreement with the observation that IGFBP-1 mRNA and protein levels are low under normal oxygen conditions. Therefore, the growth and developmental inhibition by IGFBP-1 may be in an "off" mode when there is ample oxygen, thus favoring fast growth and development. In contrast, under hypoxic environment, an unsuitable condition for growth and development, IGFBP-1 becomes in "on" mode to restrict IGF signaling by binding to free IGFs. The biological significance of the up-regulated IGFBP-1 will be discussed in the general discussion.

Chapter 2: Molecular Mechanism of Hypoxia-Induced Gene Expressions in Early Development: In vitro and in vivo Analysis of HIF-1-Regulated IGFBP-1 Gene Expression.

Introduction

Hypoxia triggers the transcriptional changes in a number of genes that promote O_2 delivery and anaerobic respiration, suppress major energy-requiring processes, and inhibit growth and development in animals ranging from invertebrates to mammals (Guillemin and Krasnow, 1997; Huang et al., 2004; Seta and Millhorn, 2004). The many of these transcriptional responses to hypoxia are mediated by the hypoxia-inducible factor-1 (HIF-1) complex. HIF-1 is a heterodimeric complex composed of HIF-1α and HIF-1β, identified as a transcription factor that is induced by hypoxia and binds to 3'-UTR of erythropoietin gene (Semenza and Wang, 1992; Wang et al., 1995; Wang and Semenza, 1995). HIF-1β, also known as aryl hydrocarbon receptor nuclear translocator, is constitutively expressed and insensitive to O_2 availability. When oxygen levels are high, HIF-1α is hydroxylated on its proline residues by prolyl hydroxylase (PDH), which allows the von Hippel-Lindau tumor suppressor (pVHL) to bind in the oxygen-dependent degradation domain. The interaction results in ubiquitination and proteosomal degradation of HIF-1α (Maxwell et al., 1999; Ohh et al., 2000). Under hypoxic environment, the hydroxylation is inhibited HIF-1α is stabilized, and eventually accumulate in the cell (Ivan et al., 2001; Jaakkola et al., 2001; Masson et al., 2001). HIF-1α is then translocated to the nucleus, dimerizes with HIF-1β, binds to DNA, and activates target gene expression (Bruick, 2003; Semenza, 1999; Wenger, 2002). Oxygen tension also regulates the interaction of HIF-1α with the transcriptional coactivators, CREB binding protein (CBP)/p300 though hydroxylation asparagines residue by the enzyme, factor inhibiting HIF-1 (FIH-1) (Mahon et al., 2001). Once HIF-1 binds to the *cis*-regulatory DNA sequences, hypoxia-response element (HRE) that is required for transcriptional induction, HIF-1 drives its target genes cooperative with CBP/p300.

In chapter 1, I have shown that IGFBP-1 is a hypoxia-inducible gene, as is consistent with earlier studies (Gracey et al., 2001; Maures and Duan, 2002; McLellan et al., 1992; Popovici et al., 2001; Tazuke et al., 1998). The loss-/gain-of function studies provided strong evidence arguing that

up-regulation of IGFBP-1 by hypoxia plays a key role in coordinating embryonic growth rate and developmental timing in response to environmental oxygen availability. Although there is *in vitro* evidence that overexpression of HIF-1α in cultured human hepatoma (HepG2) cells increases human IGFBP-1 promoter activity (Tazuke et al., 1998), whether and how hypoxia triggers IGFBP-1 gene expression *in vivo* is not clear, and the *cis*-regulatory elements responsible for hypoxia-induced IGFBP-1 transcription *in vivo* are not well defined.

The objectives of this study are, therefore; 1) to determine when the HIF-1 pathway becomes operational in early development and whether it plays a role in mediating hypoxia-induced IGFBP-1 gene expression in zebrafish embryos, and 2) to identify the key *cis*-regulatory element(s) responsible for hypoxia-induced IGFBP-1 transcription *in vivo*.

Materials and Methods

Materials- All chemicals and reagents were purchased from Fisher Scientific (Pittsburgh, PA) unless noted otherwise. DNA polymerase was purchased from Promega (Madison, WI). Restriction endonucleases were purchased from New England BioLabs (Beverly, MA). Superscript II reverse transcriptase (RT) and oligonucleotide primers were purchased from Invitrogen Life Technologies, Inc. (Carlsbad, CA).

Experimental animals and procedures- Wild-type zebrafish (*Danio rerio*) were maintained on a 14-h light/10-h dark cycle at 28 ˚C and fed twice daily as described in Chapter 1. Hypoxia treatment was conducted following the methods described in Chapter 1. Cobalt chloride ($CoCl_2$, Sigma, St Lewis, WI), a chemical HIF-1 inducer, was dissolved in embryo medium to concentrations ranging from 1×10^{-6} M to 1×10^{-2} M. Embryos at 12 hpf were transferred to and kept in these $CoCl_2$ solutions for 24 hours. The samples were collected and immediately frozen in liquid nitrogen and stored at -80 ˚C.

Molecular cloning- Genomic DNA was isolated from adult zebrafish (AB-line) and used as a template for genomic PCR to amplify the promoter region of the zebrafish IGFBP-1 gene. A primer pair (5'-TAA GGT ACC ATT AAT GTA GAT GTG AAG CAT TTT CCT ATA CGT TTG GAC -3'/5'-AAG AAG CTT ACC GGT CCC AAA GCA CGG CTC AGA ATA AAT AGA TAA-

3') was designed based on Ensembl zebrafish genome data resources (http://www.ensembl.org/Danio_rerio/). The amplified PCR product was subcloned to pCR2.1-TOPO vector (Invitrogen) and sequenced. Sequences were analyzed using TESS: http://www.cbil.upenn.edu/tess (Schug and Overton, 1997). To clone the full open reading frame (ORF) of zebrafish HIF-1α cDNA, a primer set (5'-TT GCC GCC ACC ATG G AT ACT GGA GTT GTC ACT G-3' /5'-TCA GTT GAC TTG GTC CAG AG-3') was designed based on the available sequence information (GenBank/EMBL/DDBJ accession number AY326951). The N-terminus of zebrafish HIF-1α was fused with HA-tag by PCR based amplification using a primer set (5'-ACT AAG CTT GCC GCC ACC ATG TAC CCA TAC GAT GTT CCA GAT TAC GCT GAT ACT GGA GTT GTC ACT GA-3'/5'-GAT GCT GTG ACC GGT CAG AT-3'). The amplified PCR fragment was subcloned into the pcDNA 3.1 vector (Invitrogen) and sequenced.

Plasmid construction- The isolated IGFBP-1 promoter region from -2025 to +89 (relative to the cap site) was digested by KpnI and HindIII and subcloned into a promoter-less luciferase vector (pGL-basic2, Promega) in the sense orientation to generate p2025Luc. The p2025Luc construct was digested with Pme I, Nru I, Xho I, or BstX I, end-filled with Klenow polymerase, and ligated to generate p1430Luc, p1225Luc, p1128Luc, and p282Luc, respectively. To create p148Luc and p50Luc, PCR was carried out using genome DNA as a template with the following primer sets (p248Luc: 5'- ACT GCT AGC AAA GAA CAA GAA GAC TGG ATC CCC GGC A-3'/5'-AAG AAG CTT ACC GGT CCC AAA GCA CGG CTC AGA ATA AAT AGA TAA-3'); p50Luc (5'-GAG GCT AGC AGC GGG ACC CAG TGT GCG TAT AAA TAC-3'/5'-AAG AAG CTT ACC GGT CCC AAA GCA CGG CTC AGA ATA AAT AGA TAA-3'). The amplified PCR products were digested by NheI and HindIII and ligated into pGL-basic2.

To analyze the functional role of the two HREs positioned at -1090 /-1086 and -1070 /-1066, two sets of mutants were constructed by site-directed mutagenesis using Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA). In the first set of mutants, each HRE (A/G CGTG) was mutated to A/G AAAG using specific primers (5'- GCG CTC TGT GGC ACC GAAAGC ACT CGC CCT GTG G-3' and 5'- CAC TCG CCC TGT GGT AAAAGA CCT CAC TCA GGT C-3') to create p1128^{mut1}Luc and p1128^{mut2}Luc, respectively. Complementary sequences of the shown primers were used as

forward primers. p1128^{mut3}Luc was also constructed by mutating both HREs using HRE^{mut1} in the p1128^{mut2}Luc background. In the second set of mutants, the HRE sequence positioned at -1090 /-1086 (ACGTG) was mutated to GCGTG using HRE^{mut4} (5'-CAC TCG CCC TGT GGT GCG TGA CCT CAC TCA GGT C-3') in the p1128^{mut1}Luc background, creating p1128^{mut4}Luc. In a similar manner, p1128^{mut5}Luc was constructed by mutating the HRE positioned at -1070 /-1066 (GCGTG) to ACGTG in the p1128^{mut2}Luc background using HRE^{mut5} (5'-GCG CTC TGT GGC ACC ACG TGC ACT CGC CCT GTG G-3'). To investigate whether the distance of a HRE from the cap site is important, p1113Luc and p1143Luc were constructed using 5'-GTA ATG AGG TGA GAG ACA CAA CGC TTG CGT GCA CTC GCC CTG TGG TAC GT-3' for p1113Luc and 5'-GTG CAC TCG CCC TGT GGT CAC TCG CCC TGT GGT ACG TGA CCT CAC TCA GG-3' for p1143Luc. To test the possible involvement of a putative c-AMP response element (CRE), p1128^{mut6}Luc was generated using CRE^{mut1} (5'-CCC TGT GGT ACG TGA CAAAAC TCA GGT CAG GAC G-3'). To examine the possible requirement of an HAS in the hypoxic induction, the HAS sequence (CAGGT) located at -1099/-1103 was mutated to TTTTT using HAS^{mut1} (5'-GTA CGT GAC CTC ACT TTTTC AGG ACG GCA CCC C-3') to create p1128^{mut7}Luc. Similarly, p1128^{mut8}Luc and p1128^{mut9}Luc were constructed by mutating the HAS of p1128^{mut1}Luc and p1128^{mut2}Luc using the same primer. In order to test whether HAS is sufficient, a HAS sequence was introduced 8 bp upstream of the non-functional HRE at -1070 /-1066 using HAS^{mut2} (5'-GTG CAC TCG CCCAGGT GTA AAA GAC CTC ACT TTTT TCA GGA CGG CA-3') to create p1128^{mut10}Luc and p1128^{mut11}Luc.

Cell culture and DNA transfection- ZFL cells, a cell line derived from adult zebrafish liver, were obtained from American Type Culture Collection (ATCC, Manassas, VA) and grown in a mixture of 50% Leibovitz's L-15 medium, 35% Dulbecco's modified Eagle's medium with 4.5 g/L glucose, and 15% Ham's F12, supplemented with 15 mM HEPES, 0.15 mg/ml of sodium bicarbonate, 0.01 mg/ml insulin, and 50 ng/ml EGF, penicillin (100 units/ml), streptomycin (100 μg/ml) and 5% FBS at 28.0 ˚C in 20% O_2 and 5 % CO_2. Human hepatoma cell line (HepG2), and kidney cell (HEK293) obtained from ATCC were grown in MEM and DMEM, respectively, supplemented with penicillin, streptomycin and 10% FBS at 37.0 ˚C in 20% O_2 and 5 % CO_2.

Twenty-four hours after plated into 6-well plates, cells were transfected with 2 μg reporter construct using Fugene 6 (Roche, Indianapolis, IN). For co-transfection experiments, 1 μg reporter plasmid was co-transfected with 1 μg zebrafish HIF-1α or the empty pcDNA 3.1 vector. To control for transfection efficiency, 100 ng *Renilla* luciferase reporter plasmid (pRL-SV40) was co-transfected. Twenty-four hours after transfection, cells were subjected to hypoxia (1% O_2) or normal oxygen (20% O_2) in a humidified modular incubation chamber (Billups-Rothenberg Inc., Del Mar, CA). Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) following the manufacture's instruction. A human enolase promoter (p2.1) , a well-known HIF-1 target gene, was used as a positive control. As a negative control, p2.4, a mutated HRE, was used for the HIF-1 response (Semenza et al., 1996). These two constructs were purchased from ATCC (Manassas).

Cellular localization of zebrafish HIF-1α-In order to examine the protein expression of the cloned zebrafish HIF-1α, ORF of HIF-1 α without stop codon was fused in frame with EGFP in pcDNA 3.1 vector to generate HIF-1α:EGFP construct. The HIF-1α:EGFP construct or EGFP vector (2 μg) were transfected in HEK293 cells seeded on coverslips. After 24 hours, the transfected cells were transferred to normoxia (20% O2) or hypoxia (1% O2) as described above. The cells were fixed in with 4% paraformaldehyde in 1x phosphate-buffered saline (PBS) and stained with 0.5 μg/ml DAPI. Images were taken with a Nikon DC50NN camera mounted to a Nikon Eclipse E600 fluorescence microscope (Melville, NY).

In vivo promoter analysis - To analyze IGFBP-1 promoter activity *in vivo*, promoter regions of IGFBP-1 (-2025 to +89 and -1128 to +89) were subcloned into a promoter-less pEGFP-N1 vector to create p2025:GFP (pIGFBP-1:GFP) and p1128:GFP, respectively. In addition, $p1128^{mut2}$: GFP was constructed by mutating the HRE at position -1090 /-1086 as described above. As an independent control, a construct containing EGFP driven by CMV promoter was used. After linearization, each of these plasmids (100 pg) was microinjected into zebrafish embryos at the 1- to 2-cell stage as described in Chapter 1. GFP-positive embryos were selected at 24 hpf and randomly transferred to normoxic or hypoxic water. After 24 hours, visible GFP positive cells were counted under a fluorescent microscope. GFP protein levels were measured by Western blotting using a GFP

antibody (Torrey Pines Biolabs). As a control, Western blotting was performed using an anti-tubulin (Sigma) antibody.

Whole-mount in situ hybridization, Northern blotting and RT-PCR- Whole-mount *in situ* hybridization, Northern blotting, and RT-PCR were carried out as reported previously (Maures et al., 2002). For RT-PCR analysis, total RNA was extracted from wild-type zebrafish embryos at various stages using Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH). After DNase treatment (Invitrogen), RNA (2.5 μg) was reverse transcribed with random hexamer primers and Superscript II reverse transcriptase (Invitrogen). Sequences of primer sets are as follows: HIF-1α, 5'-CTA CAA TGA TGT CAT GCT GCC-3'/5'-ACA CAG AGT GAG TGG CAG AA-3'; HIF-1β, 5'-ATG GCA GAC CAA AGA ATG GA-3'/5'-GAA GAG GAA ACC ATC AGC AG-3'; IGFBP-1, 5'-CTT CTG AAC TTC TTC TGG GTG G-3/5'-CTT CTG AAC TTC TTC TGG GTG G-3'; ornithine decarboxylase (odc), 5'-TCA ATC CCA TCT CTT CCA TTC G -3'/5'-TCC GTT TTG CTG GCA CAG TC-3'. PCR cycles and amounts of templates were optimized for each primer set in pilot experiments (25 or 500 times dilution of original cDNA samples). PCR cycles were as follows: 94 ˚C for 3 min, 30 cycles (HIF-1α, HIF-1β and odc) or 35 cycles (IGFBP-1) of 94 ˚C for 30 sec, 57 ˚C for 30 sec, and 72 ˚C for 1 min. PCR products were analyzed by electrophoresis followed by ethidium bromide staining. Cloned zebrafish HIF-1α, HIF-1β, IGFBP-1, and odc DNA fragments were used as positive controls, and no template as the negative control.

Overexpression of HIF-1α in developing zebrafish embryos- Capped zebrafish HIF-1α mRNA was synthesized with mMESSAGE mMACHINE kit (Ambion Inc., Austin, TX). The capped mRNA was microinjected at 1 μg/μl. Injected embryos were sampled 12 hours later. Total RNA was extracted, and reverse transcribed, and the IGFBP-1 mRNA levels were measured by RT-PCR as described above.

Electrophoretic mobility shift assay (EMSA)- The nuclear extract from cultured HepG2 cells was prepared following the procedure by Semenza and Wang (Semenza and Wang, 1992), except that the dialysis step was omitted. To prepare nuclear extract from zebrafish embryos, 100 deyolked embryos raised in normoxic or hypoxic water were homogenized in 750 μl low-salt buffer containing 10 mM Tris-HCl (pH 7.8), 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), and protein

inhibitors (0.4 mM phenylmethylsulfonyl fluoride, 2 μg/ml each of pepstatin, aprotinin and leupeptin). The lysates were then centrifuged at 1000 g for 5 min at 4 ˚C. The pellets were reconstituted in high-salt buffer containing 20 mM Tris-HCl (pH 7.9), 420 mM KCl, 1.5 mM $MgCl_2$, 20% glycerol, 0.5 mM DTT, and protein inhibitors and incubated on a rotator at 4 ˚C for 2 hours. The samples were frozen quickly in liquid nitrogen and stored at -80 ˚C until use. The protein contents of the nuclear extracts were quantified using the BCA Protein Assay kit (Pierce, Rockford, IL).

For EMSA, oligonucleotides (*WT1*, 5'-CAC TCG CCC TGT GGT ACG TGA CCT CAC TCA GGT C-3'; *WT2*, 5'-CGC CCT GTG GTA CGT GAC CTC ACT CAG GTC AGG ACG G-3'; *HAS*, 5'-ACC TCA CTC AGG TCA GGA CGG C-3') were end-labeled using T4 polynucleotide kinase (Promega) and [^{32}P] ATP (ICN), and purified using a G-25 column (Amersham Bioscience, Piscataway, NJ). The radiolabeled double-stranded probe (5 fmol) was incubated with the nuclear extracts prepared from zebrafish embryo (5 μg) or HepG2 cells (1 μg) in a binding buffer containing 10 mM Tris-HCl (pH 7.5), 50mM NaCl, 1mM $MgCl_2$, 0.5mM EDTA, 4% glycerol, 0.5mM DTT, and 0.5 μg poly (dI-dC)-poly(dI-dC) in a final volume of 20 μl. For competition, 50 or 500 fmol unlabeled double-stranded probe or various competing oligonucleotides were added to the binding reaction. To prove the binding activity is indeed due to HIF-1, a human HIF-1α antibody (Lab vision Co., Fremont, CA) or a HA antibody (Covance research products Inc., Berkeley, CA) was added into the binding reaction prior to the probe. An equal amount of mouse IgG was used as control. After 20 min incubation at room temperature, the DNA-protein complexes were separated by electrophoresis on a 4% PAGE gel in 1x TBE buffer. The gels were dried and exposed to X-ray film.

Statistics- Values are means ± S.E. Differences among groups were analyzed by the Student's t-test or by one- or two-way ANOVA followed by Fisher's protected least significant difference (PLSD) using StatView software (SAS Institute, Cary, NC). Statistical significance was accepted at $P < 0.05$.

Results

The HIF-1 pathway is functional and mediates hypoxia induced-IGFBP-1 gene expression in zebrafish embryos.

To determine whether and when the HIF-1 pathway is operational in early development, I analyzed HIF-1α and -1β mRNA expression during zebrafish embryogenesis. Although zebrafish HIF-1α and -1β cDNA sequences have been deposited in GenBank (AY326951 and NM131577), there is no published information on their spatial or temporal expression patterns, regulation, or biological actions. Zebrafish HIF-1α cDNA encodes777 amino acid and share 51% sequence identity with human HIF-1α. RT-PCR analysis indicated that both HIF-1α and HIF-1β mRNAs were expressed abundantly throughout embryogenesis (Fig. 2-1A). Whole mount *in situ* hybridization analysis revealed an ubiquitous distribution pattern of HIF-1α mRNA in zebrafish embryos at all stages examined under normoxic conditions, although higher levels of HIF-1α mRNA were observed in the anterior portion of the embryos at 24 hpf (Fig. 2-1B). HIF-1β mRNA is also expressed in the similar manner during early embryogenesis (Hsu et al., 2001).

To test the function of zebrafish HIF-1α to bind to a HRE, nuclear extracts, isolated from HepG2 cells transfected with a zebrafish HA: HIF-1α expression plasmid, were subjected to EMSA using an oligonucleotide probe containing a functional HRE (WT1). As shown in Fig. 2-2A, strong DNA binding activity specifically bound to the probe was detected. Addition of a HA antibody, but not mouse IgG, eliminated this binding, suggesting that the HA-tagged zebrafish HIF-1α binds to the probe. Next, I tested the transactivation activity of the cloned zebrafish HIF-1α using a well established HIF-1 target gene construct, the human enolase promoter p2.1(Semenza et al., 1996). Co-expression of zebrafish HIF-1α resulted in a 27.5-fold increase in the human enolase promoter activity (Fig. 2-2B). When the HRE in the human enolase promoter construct was mutated (p2.4), this induction was abolished (Fig. 2-2B); suggesting that zebrafish HIF-1α is a functionally conserved protein that can activate gene transcription in a HRE-dependent manner.

To further test if the zebrafish HIF-1 is controlled by hypoxia, zebrafish HIF-1α was expressed and visualized by fusing with EGFP. GFP itself was not changed by hypoxia (Fig. 2-3A,

upper panels), whereas the expression of HIF-1α:EGFP was induced by hypoxia (Fig. 2-3A, lower panels). As expected, HIF-1α:EGFP was localized in the nucleus , but EGFP only was expressed ubiquitously in the cells. In order to test *in vivo*, EMSA experiments were performed using nuclear extracts prepared from zebrafish embryos kept in normoxic or hypoxic water. As shown in Fig. 2-3B and C, hypoxia treatment caused a significant increase in levels of the HIF-1/DNA complex in zebrafish embryos. Taken together, these results suggest that HIF-1 pathway is fully operative in early embryogenesis and it activates IGFBP-1 gene expression in zebrafish embryos under hypoxic stress.

The zebrafish IGFBP-1gene is under the control of HIF-1 in vitro and in vivo.

To determine the molecular mechanism(s) underlying hypoxia-induced zebrafish IGFBP-1 gene expression, a 2.1 kb of the zebrafish IGFBP-1 gene promoter region was isolated (GenBank/EMBL/accession number AB181657). The IGFBP-1 promoter region was subcloned into a promoter-less luciferase vector and transfected into ZFL cells, human HepG2, and HEK293 cells. As shown in Fig. 2-4A, hypoxia caused a significant increase (2.3 fold, $P < 0.05$) in reporter gene activity in ZFL cells. A 2.8 and 2.4 fold, significant increase ($P < 0.001$) was seen in HepG2 cells and HEK293 cells, respectively (Fig. 2-4A). A similar hypoxic induction was reported for the human IGFBP-1 promoter in HepG2 cells (Tazuke et al., 1998). Time course experiments indicated that 4 hours was needed to obtain a significant increase in zebrafish IGFBP-1 promoter activity, and the maximal response was seen after 24 hours of hypoxia treatment (Fig. 2-4B). Since the hypoxic responsiveness of the IGFBP-1 gene is a conserved mechanism and because HepG2 cells can be cultured without insulin (a known regulator of mammalian IGFBP-1 genes) and are easily transfectable, HepG2 cells were used in subsequent studies.

To further study the hypoxia-induced IGFBP-1 promoter activity *in vivo*, the IGFBP-1 promoter region was subcloned into a promoter-less GFP reporter plasmid. Injection of the linearized pIGFBP-1:GFP plasmid into zebrafish embryos resulted in mosaic GFP expression at low levels (Fig. 2-5A). Hypoxia treatment markedly increased the number of cells with visible GFP signals in these embryos (Fig. 2-5A, also see Fig. 2-8). Not only the visible GFP positive cell number, western blot analysis was also performed to measure the total GFP protein levels. Hypoxia

markedly increased the GFP protein levels in the embryos injected with pIGFBP-1:GFP plasmid (Fig. 2-5B). In contrast, hypoxia treatment resulted in a decrease in the levels of tubulin protein (Fig. 2-5B) and several other cellular proteins (data not shown), indicating a global decrease in protein synthesis under hypoxia. As an independent control, a GFP construct driven by the CMV promoter was injected into zebrafish embryos. While mosaic GFP expression was detected, no marked difference was found in the number of GFP expressing cells or GFP protein levels between the normoxia and hypoxia in these control animals (Fig. 2-5C and D), indicating that the hypoxia-induction of GFP driven by IGFBP-1 promoter is due to the promoter activity, rather than changes in GFP stability. These results suggest that the zebrafish IGFBP-1 promoter region contains the promoter activity and the hypoxia responsive *cis*-regulatory element(s).

Next, in order to determine whether zebrafish HIF-1 activates IGFBP-1 gene expression *in vitro*, zebrafish HIF-1α was overexpressed in HepG2 cells. Co-transfection of HIF-1α resulted in dose-dependent increases in zebrafish IGFBP-1 promoter activity. At the concentration of 100 ng DNA, zebrafish HIF-1α caused an increase comparable to the hypoxia-induced increase. At higher doses, zebrafish HIF-1α caused even greater increases (Fig. 2-6A). This was also tested *in vivo* by two ways: 1) overexpression of HIF-1α and 2) pharmacological approach. First, zebrafish HIF-1α mRNA was synthesized and delivered into zebrafish embryos by microinjection. The overexpression of zebrafish HIF-1α resulted in a significant increase in the levels of endogenous IGFBP-1 mRNA (Fig. 2-6B). Alternatively, zebrafish embryos were treated with various concentrations of $CoCl_2$, a chemical HIF-1 inducer. As shown in Fig. 2-6C, $CoCl_2$ increased endogenous IGFBP-1 mRNA levels in a dose-dependent manner. Therefore, these results suggest that zebrafish IGFBP-1 gene is under the control of HIF-1.

The zebrafish IGFBP-1 promoter contains multiple consensus HREs but only one HRE is used in its response to hypoxia and HIF-1.

Although hypoxia has been shown to affect the IGFBP-1 gene expression in zebrafish and burrow-dwelling goby fish *Gillichthys mirabilis* (Gracey et al., 2001; Maures and Duan, 2002), a functional HRE has not been characterized in any fish gene to date (Nikinmaa and Rees, 2005; Semenza, 1999; Wenger, 2002). In mammals, the minimal HRE is (A/G)CGTG (Bruick, 2003). I

searched the zebrafish IGFBP-1 promoter region and found 13 canonical mammalian HREs, including 7 in the forward orientation and 6 in reverse orientation. These HREs are located at -1502 /-1498, -1461 /-1457, -1436 /-1432, -1435 /-1431, -1412 /-1408, -1368 /-1364, -1222/-1218, -1209 /-1205, -1090 /-1086, -1070 /-1066, -215 /-211, -206 /-202, and -57/-53, relative to the cap site (Fig. 2-7A). To determine whether these and/or other *cis*-elements are responsible for hypoxia and HIF-1-induced IGFBP-1 gene expression, a series of deletion constructs were generated. These reporter constructs, i.e. p2025Luc, p1430Luc, p1225Luc, p1128Luc, p282Luc, and p148Luc, contain 13, 9, 7, 5, 3, and 1 HRE, respectively. p50Luc has no HRE site. As shown in Fig. 2-7B, deletion of the sequence between -2025 and -1430 caused a modest but statistically significant increase ($P < 0.01$) in basal promoter activity, suggesting the presence of negative regulatory element(s) in this region. This deletion, however, had little effect on the hypoxia responsiveness. Further deletion of the sequence -1430 to -1225 and from -1225 to -1128 significantly decreased the basal activity ($P < 0.05$). Again, little effect was seen on the hypoxia-induced increase. Because of the reduced basal activity, the hypoxia-induction appeared even greater. Further deletion of sequence -1128 to -282 did not cause further decrease in the basal promoter activity, but it abolished the hypoxia responsiveness. p148Luc was similar to p282Luc. These results suggest the sequence between -1128 and -282 contains the *cis*-regulatory element(s) responsible for the hypoxia responsiveness.

There are two HREs, both in the reverse orientation, between -1128 and -282. They are positioned at -1090 /-1086 and -1070 /-1066 (Fig. 2-7C). To test whether one or both of them are involved in the hypoxia response, either one or both of the HREs were mutated (Fig. 2-7C). As shown in Fig. 2-7D, hypoxia treatment caused a 4.2-fold increase ($P < 0.01$) in the activity of p1128Luc. Overexpression of zebrafish HIF-1α resulted in a 10.5-fold increase ($P < 0.01$) in the activity of p1128Luc. Hypoxia did not cause further increase in the HIF-1α transfected group, suggesting that HIF-1α overexpression is sufficient to account for hypoxia-induced IGFBP-1 promoter activity. Mutation of the HRE at -1070 /-1066 (p1128^{mut1}Luc) did not affect the responsiveness to hypoxia. Likewise, this mutant showed an increase in response to HIF-1α co-expression similar to wild type p1128Luc. Mutation of the HRE at -1090 /-1086 (p1128^{mut2}Luc), however, completely abolished the hypoxia- and HIF-1α-induced increase. The double mutant

p1128^{mut3}Luc acted like p1128^{mut2}Luc (Fig. 2-7D), indicating that only the HRE at -1090 /-1086 is required for the hypoxic induction.

To investigate whether the HRE at position -1090 /-1086 is required for hypoxia-induced IGFBP-1 expression *in vivo*, an EGFP reporter construct driven by the minimal zebrafish promoter region (p1128:GFP) was generated. Injection of p1128:GFP plasmid into zebrafish embryos resulted in mosaic GFP expression at low levels under normoxic conditions (Fig. 2-8A). Hypoxia treatment caused a highly significant ($P < 0.001$), 4-fold increase in the number of visible GFP positive cells (Fig. 2-8B). Western immunoblot analysis showed that p1128:GFP-injected embryos also had a significant increase in the total GFP protein levels in response to hypoxia (Fig. 2-8C and D). Mutation of the -1090 /-1086 HRE did not alter the basic expression levels of GFP, but it completely abolished the hypoxia response. These data suggest that the HRE at -1090 /-1086 is required for the hypoxia and HIF-1-induced zebrafish IGFBP-1 expression in developing zebrafish embryos *in vivo*.

The selective use of the -1090 /-1086 HRE cannot be attributed to its sequence or location.

The two HREs in p1128Luc are only 15 bp apart. Yet, only the HRE at -1090/-1086 is required for the full response to hypoxia and HIF-1. Since the HRE at -1070/-1066 is GCGTG and the HRE at -1090/-1086 is ACGTG, and because a functional HRE has not been reported in any teleost species (Nikinmaa and Rees, 2005), I wondered whether the single nucleotide difference could account for the observed functional difference. To determine whether both forms of HRE can bind HIF-1, EMSA experiments were performed using the oligonucleotides shown in Fig. 2-9A. The labeled probe (WT1) containing the functional HRE bound to nuclear protein or protein complexes in the nuclear extracts prepared from hypoxia-treated zebrafish embryo (Fig. 2-9B). This binding was specific to the HRE because addition of excess amounts of unlabeled WT1 displaced the binding, while Mut A1, the same oligonucleotide with the HRE mutated, did not. MuB1, which has a single nucleotide mutation in the HRE from ACGTG to GCGTG, was equally effective as WT1 in replacing the binding, suggesting that zebrafish HIF-1 can bind to both forms of HRE.

To determine whether the single nucleotide difference causes any functional difference, the nucleotide A at position -1090 was changed into G in the p1128^{mut1} background, resulting in p1128^{mut4}Luc (Fig. 2-9C). If the sequence "ACGTG" is critical, then changing it to GCGTG should

abolish the hypoxia and HIF-1 responsiveness. Conversely, the nucleotide G at position -1066 was changed into A in the p1128^{mut2} background, resulting in the creation of p1128^{mut5}Luc (Fig. 2-9C). If the sequence "ACGTG" is critical, then this mutation should restore the hypoxia and HIF-1 responsiveness. When the activities of these mutants were examined, however, p1128^{mut4}Luc had similar responses to hypoxia and HIF-1α expression to wild type p1128Luc (Fig. 2-9D). p1128^{mut5}Luc, like p1128^{mut2}, did not show any increase in response to hypoxia/HIF-1α overexpression (Fig. 2-9D), suggesting that both HREs (ACGTG and GCGTG) are functional in a fish gene when positioned at the right location.

These data led me to hypothesize alternatively that the precise location (or distance from the TATA box) of a HRE may be critical. To test this idea, two constructs, p1113Luc and p1143Luc, were engineered. In p1113Luc, the -1090/-1086 HRE is changed into -1075/-1071 by deleting 15 nt (Fig. 2-10A). In p1143Luc, an additional 15 nt is added between the two HREs, thus moving the functional HRE to position -1105/-1101 without altering the location of -1070/-1066 HRE. When the activities of these two mutants were tested, they acted like the wild-type p1128Luc (Fig. 2-10B). These data suggest that moving the functional HRE by15 nt in either direction does not affect the responsiveness of the IGFBP-1 promoter to hypoxia or HIF-1α.

A HIF-1 ancillary sequences (HAS) adjacent to the functional HRE is critical for the hypoxic induction.

To determine whether the sequence(s) surrounding the -1090/-1086 HRE is critical, its neighboring sequence for potential DNA binding sites was analyzed. A putative c-AMP response element (CRE) contiguous to the functional HRE was identified (Fig. 2-11A). CRE-binding protein (CREB) and HIF-1 are known to use a common co-activator, CBP/p300, to initiate the transcription of target genes (Arany et al., 1996; Dames et al., 2002; Kallio et al., 1998). It was also reported that an increase in c-AMP levels potentiates the hypoxic induction of the mouse lactate dehydogenase A (LDHA) promoter, and that mutation of the canonical CRE site reduces the hypoxia responsiveness (Firth et al., 1995). I therefore investigated the possible involvement of the putative CRE. As shown in Fig. 2-11B, mutation of the CRE in the zebrafish IGFBP-1 promoter (p1128^{mut6}Luc) did not change its responses to hypoxia or HIF-1α overexpression, suggesting that this CRE is not involved in

hypoxic induction in IGFBP-1 promoter activity.

In addition to the putative CRE site, a putative HAS is positioned adjacent to the functional HRE (Fig. 2-12A). HAS was initially identified in the VEGF promoter, and was shown to be involved in the HIF-1-induced VEGF expression (Kimura et al., 2000; Kimura et al., 2001; Liu et al., 1995). In that case, the HAS may form an imperfect inverted repeat with a HRE with a spacer of 8 nt (Kimura et al., 2001). In the zebrafish IGFBP-1 promoter region, a putative HAS is positioned at -1099/-1103, precisely 8 nt upstream of the functional HRE. No HAS-like sequence was found in the proximity of the other 12 HREs in the zebrafish IGFBP-1 promoter. I asked whether the putative HAS is functional and plays any role in the selective use of the functional HRE. p1128^{mut7}Luc and p1128^{mut8}Luc, two mutants generated by altering the HAS sequence in wild type p1128Luc and p1128^{mut1}Luc backgrounds (Fig. 2-12A), were used to address this question. Mutation of the HAS in either the wild type p1128Luc and p1128^{mut1}Luc backgrounds greatly reduced, but did not abrogate the hypoxic response to hypoxia and/or HIF-1α overexpression (Fig. 2-12B). When the activity of p1128^{mut9}, a HRE and HAS double mutant was tested, it did not respond to either hypoxia or HIF-1α overexpression (Fig. 2-12B). These results suggest that the HAS is important for the magnitude of hypoxia responsiveness of the zebrafish IGFBP-1 promoter. Furthermore, in order to determine whether the HAS core sequence is sufficient to confer the function of a HRE, I generated another mutant construct (p1128^{mut10}Luc), in which the -1090/-1086 HRE and its adjacent HAS were mutated and the minimal HAS sequence (CAGGT) was introduced 8 nt upstream of the -1070/-1066, non-functional HRE (Fig. 2-12A). If the HAS core sequence is solely responsible for the function of the -1090/-1085 HRE, p1128^{mut10}Luc should restore the hypoxia/HIF-1-induced promoter activity. As shown in Fig. 2-12B, however, this mutant did not show improved hypoxia/HIF-1 responsiveness. Since the HRE at -1070 /-1066 is GCGTG and the HRE at -1090 /-1086 is ACGTG, I changed the nucleotide G at position -1066 into A in the p1128^{mut10}Luc background, resulting in the creation of p1128^{mut11}Luc. p1128^{mut11}Luc also failed to show improved hypoxia/HIF-1-induction (Fig. 2-12B), suggesting that the -1103/-1099 HAS is requiredfor the functional involvement of the -1090/-1086 HRE in the hypoxia/HIF-1-regulation. However, ectopic introduction of HAS next to non functional HRE did not restore the responsiveness. Therefore, HAS is not the sufficient factor.

Since mutating the HAS greatly reduces the magnitude of hypoxia responsiveness of the IGFBP-1 promoter, I wondered whether the HAS can directly interact with the HIF-1 complex or whether it can influence the HIF-1 and HRE binding. To test this idea, EMSA was performed using an oligonucleotide probe that contains the functional HRE, HAS, and their surrounding sequence (WT2) (Fig. 2-13A). As shown in Fig. 2-13B, two major bands were detected in the nuclear extracts isolated from hypoxia-treated HepG2 cells, but not in those kept under normoxic condition. These binding activities were displaced by adding excess amounts of unlabeled probe (WT2), but not by Mut A2, the same oligonucleotide with a mutated HRE (Fig. 2-13B). Furthermore, addition of a human HIF-1α antibody prior to the probe eliminated these hypoxia-induced bands, while mouse IgG had no such effect (Fig. 2-13B), indicating that these hypoxia-induced HRE binding activities indeed contained HIF-1α. When MutB2, an oligonucleotide with a mutated HAS, was added in excess, it was as effective as WT2 in displacing the HIF-1/DNA complexes (Fig. 2-13B). MutC2, in which both the HRE and HAS were mutated, did not alter the HIF-1 and HRE binding. These results suggest that having an adjacent HAS does not significantly influence the binding of an HRE to HIF-1.

During the course of these experiments, a strong DNA binding activity was consistently observed in the nuclear extract isolated from normoxic cells (Fig. 2-13B and D). This activity was specific because it was replaced by adding excess amount of unlabeled WT2. This activity was predominantly detected under normoxic condition (Fig. 2-13B and D). To determine whether there is a nuclear protein specifically bound to the HAS site under normoxia, HAS, an oligonucleotide probe only containing the HAS and its surrounding sequence, was used for further EMSA experiments (Fig. 2-13C). This probe was found to interact with the same protein complex (Fig. 2-13D). Addition of excess amounts of unlabeled HAS displaced this binding activity, whereas Mut HAS, which carries a 4 bp mutation in the HAS, was much less efficient in competing for binding (Fig. 2-13 C and D). Importantly, an oligonucleotide containing the functional HRE was unable to compete for binding (Fig. 2-13 C and D), even though 100 times excess amounts of all the competitors displaced the labeled band. These results indicate the presence of a nuclear protein that can specifically interact with the HAS under the normoxic condition.

Discussion

The ability of the IGFBP-1 gene to respond to hypoxia has been documented in a variety of vertebrate species, ranging from fish to humans(Gracey et al., 2001; Maures and Duan, 2002; McLellan et al., 1992; Popovici et al., 2001; Tazuke et al., 1998). A previous *in vitro* study has shown that co-expression of a luciferase reporter construct driven by the human IGFBP-1 promoter and a constitutively active form of human HIF-1α in HepG2 cells results in a 4-fold induction in reporter activity (Tazuke et al., 1998). In this study, I have extended this *in vitro* observation and shown that the HIF-1pathway mediates hypoxia-induced zebrafish IGFBP-1 gene expression in developing zebrafish embryos *in vivo*. Several lines of experimental evidence support this conclusion. First, the HIF-1 pathway is operational in zebrafish embryos. The major components of the HIF-1 pathway are abundantly expressed in most embryonic tissues and hypoxia increases the levels of functional HIF-1. These results suggest that the regulation of HIF-1α in response to environmental oxygen levels is established very early in zebrafish embryogenesis. Knock-out studies in the mouse model have shown that HIF-1 is essential for normal mammalian fetal development in addition to its key role in oxygen homeostasis in adult stages (Adelman et al., 1999; Iyer et al., 1998; Maltepe et al., 1997). Second, the zebrafish HIF-1α is a functionally conserved protein and can bind to a HRE and activate HRE-dependent gene transcription when tested *in vitro*. Third, co-transfection of the zebrafish IGFBP-1 promoter construct with a zebrafish HIF-1α expression in HepG2 cells leads to a 10-fold increase in the IGFBP-1 promoter activity. More importantly, overexpression of zebrafish HIF-1α in developing zebrafish significantly increases endogenous IGFBP-1 mRNA levels. Likewise, $CoCl_2$, a chemical HIF-1α inducer, causes a concentration-dependent increase in IGFBP-1 mRNA levels in zebrafish embryos.

Tazuke et al. (1998) reported that a HRE in the first intron of the human IGFBP-1 gene is required for the hypoxia response in cultured HepG2 cells. In the case of the zebrafish IGFBP-1 gene, however, there is no consensus HRE in the first intron. Instead, I have identified 13 canonical HREs in the promoter region. This region possesses basal promoter activity and contains the *cis*-regulatory element(s) responsible for the hypoxia responsiveness when tested *in vitro* and *in vivo*.

Although the zebrafish IGFBP-1 promoter contains 13 canonical HREs, disruption of the HRE positioned at -1090/-1086, but not the other 12 HREs, abolishes the hypoxia/HIF-1-induced transcriptional response of the IGFBP-1 promoter *in vitro*. The functional importance of the -1090 /-1086 HRE in hypoxia-induced IGFBP-1 expression was further confirmed by *in vivo* promoter analysis. While hypoxia induced a 4-fold increase in the reporter activity *in vivo*, mutation of the -1090 /-1086 HRE completely abolished the hypoxia response. To my knowledge, this is the first *in vivo* study demonstrating the critical role of a HRE in the hypoxia-induced gene expression.

The finding that only one of the 13 canonical HREs is used for the hypoxic induction of the IGFBP-1 gene transcription is both interesting and puzzling. I was particularly intrigued by the fact that there is a canonical HRE located at -1070 /-1066, 15 nt away from the functional HRE. I considered three possible explanations underlying this high degree of selectivity; 1) the HRE at -1070 /-1066 has a single nucleotide difference (GCGTG vs. ACGTG) and this may account for the observed functional difference; 2) the precise location (or distance to the TATA box) of a HRE is critical; and 3) the neighboring sequence surrounding a HRE is critical for its function. The first hypothesis was rejected because EMSA and functional analysis revealed that HIF-1 can bind to both forms of HRE and GCGTG can act as a functional HRE when introduced at the right location (e.g. -1090/-1086). Thus, both GCGTG and ACGTG can be functional HREs in a fish gene. The second possibility was also rejected because p1113Luc and p1143Luc, two reporter constructs in which the location of the functional HRE is moved by 15 nt in either direction, are fully responsive to hypoxia/HIF-1. The conclusion that the location of an HRE is not critical for its function is also in line with the finding that the functional HRE in the human IGFBP-1 gene is located in the first intron (Tazuke et al., 1998). To test the third possibility, I examined a consensus CRE and HAS located in the proximity of the functional HRE. Although mutation of an adjacent CRE in the mouse LDHA promoter was previously reported to reduce its hypoxia responsiveness (Firth et al., 1995), mutation of the CRE in the zebrafish IGFBP-1 promoter has no effect on its hypoxia responsiveness. When the HAS is altered, however, the magnitude of hypoxia responsiveness of the zebrafish IGFBP-1 promoter is greatly reduced. Mutation of both the functional HRE and HAS abolishes its responsiveness to either hypoxia or HIF-1α overexpression, suggesting that cooperative interactions

between the functional HRE and its adjacent HAS may be critical to confer hypoxia responsiveness. The importance of an adjacent HAS in determining a functional HRE is also supported by the sequence analysis results. Among the 13 HREs found in the zebrafish IGFBP-1 promoter region, a HAS motif is present only in the proximity of the functional HRE. In addition, a HAS motif is found next to the functional HRE located in the first intron of the human IGFBP-1 gene (Table 2-1). Likewise, a consensus HAS is found adjacent to a HRE in the mouse and rat IGFBP-1 genes, although they are separated by 9 or 10 nt (Table 2-1) rather than 8 nt found in the zebrafish IGFBP-1 promoter. Whether a HAS is involved in HRE function in a mammalian IGFBP-1 gene needs to be investigated in the future. While the adjacent HAS is clearly required for the hypoxia and HIF-1-induced zebrafish IGFBP-1 promoter activity, introducing the minimal HAS sequence (CAGGT) 8 nt upstream of the -1070 /-1066 HRE was insufficient to restore the hypoxia and HIF-1 regulation, suggesting that other sequence(s) or factors may be also involved in the selective use of a HRE.

It remains an open question how the HAS acts to modulate HRE function. The EMSA results indicate that the HAS does not directly interact with the HIF-1 protein, since no HIF-1/DNA complexes were detected by EMSA when the HAS probe was used. Likewise, in the EMSA experiments using the WT2 probe (containing the HRE and its adjacent HAS), MutB2, an oligonucleotide with a mutated HAS, is as effective as the wild type probe in displacing the HIF-1/DNA complexes. Therefore, it is suggested that an adjacent HAS does not significantly influence the binding of HIF-1 to the HRE. A previous study has shown that a HAS identified in the human VEGF promoter also binds to a constitutive, non-HIF-1 protein in A172 and other cells (Kimura et al., 2001). Of particular interest, I find that this HAS binding activity is reduced or inhibited by hypoxia in HepG2 cells. Since the HAS binding activity is predominantly seen under normoxic condition, I suspect that it may facilitate the selective binding of HIF-1 to its neighboring HRE, perhaps by providing a platform or docking site for the HIF-1 complex. At present, the molecular identity of this HAS binding factor(s) is unknown. HIF-1 has been reported to interact with ATF-1/CREB-1 in the mouse LDHA gene (Firth et al., 1995), with AP-1 in the VEGF gene and the tyrosine hydroxylase gene (Galson et al., 1995; Kvietikova et al., 1995), and with the orphan receptor hepatic nuclear factor-4 (HNF-4) in the erythropoietin gene (Norris and Millhorn, 1995). These transcription factors are

unlikely to be the HAS binding factor because they are either constitutively bound to a HRE or are induced by hypoxia, while the HAS binding activity is inhibited or reduced under hypoxia. In addition, mutation of the CRE in the zebrafish IGFBP-1 gene has no effect on the hypoxic induction. Clearly, more studies are needed to identity this HAS binding factor and determine whether and how it modulates the HRE function. Identifying the HAS binding factor, protein "X", and elucidate its role in regulating IGFBP-1 gene expression should deepen our understanding of hypoxia-regulated gene expression in early development.

GENERAL DISCUSSION

Homeostasis is a consequence of genetic programs that individual cell possesses to be able to sense and respond to the changes in a given environment. When exposed to a different environment, animals maintains homeostasis through "adaptation", a trait that 1) enhance the fitness of an organism, and 2) whose current beneficial characteristics reflect the selective advantage of the trait at its time of origin (Hochachka and Somero 2002). The main focus of this dissertation is to understand how the zebrafish embryo "adapts" to hypoxic environment and coordinates embryonic growth through modifying IGF system. In chapter 1, I examined the biological role of the up-regulated IGFBP-1 on the zebrafish embryo under hypoxia. Chronic hypoxia treatment results in significant embryonic growth retardation and developmental delay in zebrafish, concomitant with a significant increase in IGFBP-1 mRNA and protein levels. Overexpression of IGFBP-1 reduced the growth and developmental rate under normoxia condition, and targeted knockdown of IGFBP-1 partially abrogated the hypoxia-caused growth retardation and developmental delay. Furthermore, re-introduction of a MO-resistant IGFBP-1 to the IGFBP-1 knocked down embryos restored the hypoxia effects. The effects are, at least in part, through the inhibitory effects of IGFBP-1 on IGF-dependent cell proliferation through reduced "free/active" forms of IGFs and the consequent suppression of IGF signaling (Fig. 3-1). These findings demonstrate a clear causative relationship between the elevated IGFBP-1 expression and hypoxia-induced embryonic growth and developmental retardation. This work also have provided unequivocal evidence supporting the hypothesis that IGFBP-1 plays a key role in mediating the hypoxic effects on embryonic growth and development.

This induction of IGFBP-1 expression by hypoxia may be an evolutionally conserved physiological mechanism to restrict the IGF-stimulated growth and developmental process under stressful conditions. In addition to hypoxia, the IGFBP-1 gene is highly responsive to food deprivation, malnutrition, stress, and chronic diseases in a wide variety of vertebrate species ranging from teleosts to human (Clemmons, 2001; Duan, 2002; Firth and Baxter, 2002; Kajimura et al., 2003;

Kelley et al., 2002; Takenaka et al., 1993; Takenaka et al., 2000; Wood et al., 2005a). Moreover, the involvement of IGF-signaling under hypoxic environment has been indicated even in invertebrates. It has been demonstrated in *C. elegans* that wild type does not survive under hypoxic environment, whereas *daf-2* mutant (insulin receptor mutation) is highly tolerant to hypoxia, mediated through an Akt-1/PDK-1/forkhead transcription factor pathway overlapping with but distinct from signaling pathways regulating life-span (Scott et al., 2002). Because these stressful and catabolic conditions lead to adaptive changes in metabolic reorganization, such as the activation of the anaerobic ATP-generating pathway (glycolysis), the biological significance of IGFBP-1 may be to serve as a "molecular switch" by controlling the availability of IGFs to its receptor, and to divert the limited energy resources away from growth and development toward those metabolic processes essential for survival.

In chapter 2, a molecular mechanism is proposed how IGFBP-1 gene expression is triggered in response to hypoxia. I have shown that the HIF-1 pathway is established and operational in the earliest stages of vertebrate development and that it plays a major role in mediating hypoxia-induced IGFBP-1 gene expression in zebrafish embryos at the cellular and organism levels. Intriguingly, although the zebrafish IGFBP-1 promoter contains 13 consensus HREs, only one of these HREs is required for the hypoxia/HIF-1 regulation of IGFBP-1 gene expression. Further analysis shows that a HAS adjacent to the functional HRE is required for its hypoxia/HIF-1 responsiveness. Therefore, the selective interaction of HIF-1 with the HRE (-1090/-1086) through its adjacent HAS may be the underlying mechanism of hypoxia-inducible IGFBP-1 gene expression (Fig. 3-2). So far, the data support that HAS is a new *cis*-element to which an unidentified nuclear protein binds. Future study will focus to identify the *cis*-acting nuclear protein (X) and its functions. To date in mammals, more than 40 genes have been characterized as HIF-1 targets including VEGF (Levy et al., 1995; Liu et al., 1995), erythropoietin (EPO) (Semenza and Wang, 1992), LDHA (Firth et al., 1994; Firth et al., 1995), and elonase1 (ENO1) (Semenza et al., 1996; Semenza et al., 1994). Since the presence of HAS is also found close to the authentic HRE site in other hypoxia-responsive genes including VEGF, EPO, LDHA, this working model may be applicable not only to IGFBP-1 gene, but also to many, if not all, of the hypoxia-inducible genes. Another emerging and intriguing question is to clarify the

"sufficient "factors for the function of HRE to induce IGFBP-1 promoter activity in response to hypoxia. As data shown, ectopic introduction of HAS itself next to non-functional HRE did not restore the function of HRE. Recent observation implies that there may be alternative putative *cis*-elements between HRE and HAS in the IGFBP-1 promote r sequence, which will be a future direction of this study.

Recently, intensive efforts have been devoted to address what molecule is "oxygen sensor". PDH, that regulates the interaction between HIF-1α and pVHL, is proposed to be a oxygen sensor, due to its property (Semenza, 2001). The activities of PDHs are sensitive to oxygen availability and decreases from ambient air O_2 concentration through anoxia (Km= 20.9 %) (Hirsila et al., 2003). Besides the regulations by PDHs, reactive oxygen species (ROS) through mitochondria electron transport (complex III) and subsequent ROS signaling including p38 MAPK is required for the stability of HIF-1α under hypoxia (Brunelle et al., 2005; Emerling et al., 2005; Guzy et al., 2005; Mansfield et al., 2005). In addition, mammalian target of rapamycin (mTOR) pathways, induced by ROS-induced PI3-K/Akt pathways seem to be involved in stabilization of HIF-1α (Gao et al., 2002). However, it still remains unclear how these multiple pathways are integrated and regulate HIF-1 activity in response to the decreased oxygen levels.

I believe that the significance of this study are: 1) to incorporate genetic and developmental perspectives into the research area of endocrinology and physiology, and 2) to use one of the best models, zebrafish embryo, for testing the particular hypothesis and clarify relevant physiological phenomena in vertebrates in general. For example, loss-of-function studies using mouse model have gain a limited success in dissecting functions of each IGFBPs, partially because of redundancy in function and expression patterns. IGFBP-1 knockout mice have no apparent phynotype under normal conditions, even though liver regeneration is impaired after hepatoectomy (Leu et al., 2003a; Leu et al., 2003b). IGFBP-2 knockout mice also have minor phenotype besides smaller spleen and larger liver (Wood et al., 2000). Knockout mice for IGFBP-3, -5, and –6 have been generated, but these animals appeared normal in size and shape (Pinter et al.2001). In contrast, recent studies using zebrafish embryo have been very successful to address the function of IGFBP-1 (this study), IGFBP-2, and IGFBP-3. Target knockdown of IGFBP-2 resulted in delayed development, reduced body

growth and abnormal cardiovascular development (Wood et al., 2005b). When IGFBP-2 was knockdown ed, the pharyngeal skeleton and cartilage development were disrupted (Li et al., 2005). The different outcomes between mouse model and zebrafish may be explained by three morphological and physiological characteristics in fish model. First, there exist no maternal compensation in gene knockout progeny via placental circulation, in contrast to mammalian model. Second, the spacious expressions of each IGFBPs are much less redundant in zebrafish embryo, compared with those in mammals (Duan et al., 1999; Li et al., 2005; Maures et al., 2002; Maures and Duan, 2002; Wood et al., 2005a; Wood et al., 2005b). Third, transient konockdown of IGFBP-1 (this study) and IGFBP-2 did not alter the expression of other known IGF system, whereas disruption of any genes in IGF system often influence other IGF members in mouse model. For instance, serum levels of IGFBP-1-3 and -4 were increased in IGFBP-2 knockout mice (Wood et al., 2000), which may mask the potential phenotypes caused by loss/gain of function approaches. As the Krogh principle proposed (Krogh, 1929; Krebs, 1975), the approach, choosing the best animal for a question in the comparative context , will be more powerful and effective for the study of the relevant nature of animal physiology. In the same time, it needs caution against the differences in mature (or "diversity") between fish and mammalian model, which may cause discrepancies depending on the questions to ask.

Since teleosts are the most diverse and species-rich group of vertebrates with more than 25,000 species, and evolved independently from the tetrapod lineage of vertebrates 300-400 million years ago with repeated genome duplication (Venkatesh, 2003), they display a range of anatomic, behavioral, and physiological strategies for hypoxia-adaptation. In addition, under an aquatic environment, 1/30th of the oxygen contents and 1/10,000 times slower rate of oxygen diffusion compared with air in the same volume, teleosts have acquired unique properties of molecular pathways for oxygen consumption (Nikinmaa and Rees, 2005). Understanding the special properties based on their habitual environment and life history in molecular, cellar, and organism levels will further clarify the relevant physiological mechanisms not known otherwise in mammalian models.

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