HYPOXIA-INDUCIBLE FACTOR 2-ALPHA PARTICIPATES IN THE REGULATION OF INTERSEGMENTAL VESSELS ANGIOGENESIS AND NEUROMAST DEPOSITION

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Recommended Citation
Chou, Chi-Fu; Lin, Han-Jia; Tsai, Chih-Yu; Lin, Tzung-Yi; Chung, Hsin-Yu; Chiang, Chia-Yin; Li, Chung-Hao; Pai, Tun-Wen; Hu, Chin-Hwa; and Tzou, Wen-Shyong (2016) "HYPOXIA-INDUCIBLE FACTOR 2-ALPHA PARTICIPATES IN THE REGULATION OF INTERSEGMENTAL VESSELS ANGIOGENESIS AND NEUROMAST DEPOSITION," Journal of Marine Science and Technology. Vol. 24 : Iss. 2 , Article 29.  
DOI: 10.6119/JMST-015-0629-1
Available at: https://jmstt.ntou.edu.tw/journal/vol24/iss2/29

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Acknowledgements
We thank Taiwan Zebrafish Core Facility at Academia Sinica (TZCAS) for providing Tg(fli1:EGFP) transgenic fish. TZCAS is supported by the grant of NSC 103-2321-B-001- 050 from the Ministry of Science and Technology. This work was supported by the Ministry of Science and Technology, Taiwan, R.O.C. (Grant No. MOST 104-2321-B-019-005-MY3) and the Center of Excellence for the Oceans, National Taiwan Ocean University.

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HYPOXIA-INDUCIBLE FACTOR 2-ALPHA PARTICIPATES IN THE REGULATION OF INTERSEGMENTAL VESSELS ANGIOGENESIS AND NEUROMAST DEPOSITION

Chi-Fu Chou¹, Han-Jia Lin¹, Chih-Yu Tsai¹, Tzung-Yi Lin¹, Hsin-Yu Chung¹, Chia-Yin Chiang¹, Chung-Hao Li¹, Tun-Wen Pai², Chin-Hwa Hu¹, and Wen-Shyong Tzou¹

Key words: hypoxia-inducible factor 2-alpha, lymphoid enhancer factor 1, spleen tyrosine kinase, intersegmental vessels, lateral line primordium.

ABSTRACT

Subtle morphogenesis begins during trunk development after the segmentation period in zebrafish. Sprouting angiogenesis forms the trunk vascular system after the extension of the dorsal aorta and cardinal veins. The posterior lateral line primordium migrates from head to tail, forming the sensory organs on the skin. It is still unclear whether this morphogenesis requires several master regulators to control the ongoing process. Hypoxia-inducible factor 2-alpha (Hif2-alpha) plays an essential role in the maturation of both central neural system and digestive system during embryogenesis. We hypothesized that Hif2-alpha also regulates trunk development. Through loss-of-function experiments, we found that Hif2-alpha, but not Hif1-alpha, is necessary for angiogenesis and the formation of neuromasts in the trunk. Hif2-alpha regulates the expression of spleen tyrosine kinase (Syk), maintaining sprouting in the trunk. Furthermore, Hif2-alpha also regulates lymphoid enhancer factor 1 (Lef1), moderating the deposition of neuromasts. The regulation of Syk and Lef1 expression by Hif2-alpha occurs through the direct binding of Hif2-alpha to the hypoxia-responsive elements (HRE) of syk and lef1. This study provides the first evidence that Hif2-alpha plays a pivotal role in the vertebrate embryonic development of intersegmental vessels and the mechanosensory system in the zebrafish trunk.

I. INTRODUCTION

Zebrafish somitogenesis occurs from 10.5 hours post fertilization (hpf) until 24 hpf. Zebrafish undergo dynamic morphogenetic modifications during embryonic development (Stickney et al., 2000). After segmentation, the body lengthens, and the posterior lateral line (PLL) primordium moves steadily from trunk to tail (Kimmel et al., 1995). Once the dorsal aorta and cardinal vein form, the intersegmental vessels (ISV) sprout from them to constitute the trunk vascular system (Isogai et al., 2001). This subtle morphogenesis requires a vigorous regulation of cell migration and proliferation.

Vessel development comprises multiple processes, including the migration, proliferation, and differentiation of vascular endothelial cells, and can be divided into vasculogenesis and angiogenesis (Semenza, 2007). Angiogenesis is the process of endothelial cell proliferation and migration from existing blood vessels, forming new capillaries (Eilken and Adams, 2010). Sprouting angiogenesis requires the cooperation of two specific types of endothelial cells. Tip cells migrate toward vascular endothelial growth factor A (VEGFA) and Notch ligand. Subsequently, the stalk cells form the lumen (Gerhardt et al., 2003; De Smet et al., 2009). Sprouting angiogenesis requires the cooperation of two specific types of endothelial cells. Tip cells migrate toward vascular endothelial growth factor A (VEGFA) and Notch ligand. Subsequently, the stalk cells form the lumen (Gerhardt et al., 2003; De Smet et al., 2009). Known angiogenic factors such as VEGFA, basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF) function as the direct stimulation. Tumor necrosis factor alpha (TNF-alpha), transforming growth factor beta (TGF-beta) and interleukin-1 (IL-1) function as the indirect stimulation (Brogi et al., 1994).

The lateral line is a unique mechanosensory system in fish and amphibians that senses water movement. It is comprised of a series of hair cell organs (neuromasts). Those deposited...
throughout the skin on the head, jaw, operculum and the sensory neurons from rostral to the ear are called the anterior lateral line (ALL). The primordium extends from the ear to the tail by only approximately 100 cells to form the PLL (Gompel et al., 2001; Ghysen and Dambly-Chaudiere, 2004). Appropriate spatial expression patterns of the chemokine receptors Cxcr4b and Cxcr7b, which are regulated by Wnt and Fgf signaling, direct the collective migration of the PLL primordium (Haas and Gilmour, 2006; Dambly-Chaudiere et al., 2007). The cell fate determination of the supporting cells in maturing neuromasts also requires Notch signaling (Matsuda and Chitnis, 2010).

Hypoxia-inducible factors (HIFs) regulate many important physiological mechanisms, including angiogenesis, metabolism, erythropoiesis, cell proliferation, and apoptosis (Tian et al., 1998; Schofield and Ratcliffe, 2004). HIFs are transcriptional activators that regulate oxygen homeostasis (Semenza, 2014). Hypoxia regulates sprouting angiogenesis through multiple pathways, including both pro-angiogenic and anti-angiogenic genes (Krock et al., 2011). In mammals, Hif1-alpha participates in the VEGF signaling pathway to regulate blood vessel development (Hoeben et al., 2004). In tumor cells, VEGF is also stimulated by Hif1-alpha-regulated angiogenesis (Kuwahara et al., 2002). Hif2-alpha is essential for the development of organs, including retina, heart, liver, bone marrow, and central nervous system (Scortegagna et al., 2003; Ko et al., 2011; Lin et al., 2014). Although Hif2-alpha reduces the growth rate of both neuroblastosoma in mice and breast cancer cells, it promotes and enhances the efficiency of tumor angiogenesis (Leek et al., 2002; Giatromanolaki et al., 2006; Favier et al., 2007). Zebrafish incubated in a hypoxic environment showed enhanced ISV angiogenic development (Rizvi et al., 2013).

Spleen tyrosine kinase (Syk) is a member of the non-receptor type Tyr protein kinase family. It is broadly expressed in hematopoietic stem cells and binds to the immunoreceptor tyrosine-based activation motif (ITAM), regulating many downstream cell signaling reactions including cell proliferation, differentiation and phagocytosis (Turner et al., 1995; Parsa et al., 2008; Christie et al., 2010). Syk is also important for maintaining vascular completeness, the separation of the lymphatic and vascular systems, and the development of B-cells and T-cells (Cheng et al., 1995; Abtahian et al., 2003; Sebzda et al., 2006). In mammalian cell culture experiments, Syk participates in endothelial cell proliferation and migration (Inatome et al., 2001). The lymphatic defect in Syk null mice results from the loss of endothelial precursor cell signaling necessary for lymphatic vascular development (Sebzda et al., 2006).

Lymphoid enhancer factor 1 (Lef1) is a member of the Lef/Tcf family of transcription factors that activates downstream genes by association with beta-catenin in the Wnt/beta-catenin pathway (Eastman and Grosschedl, 1999). Canonical Wnt signaling is important in embryonic patterning, cell-fate determination, cell proliferation, and cell differentiation during development (Logan and Nusse, 2004; Husken and Carl, 2013). Wnt signals from the paraxial mesoderm drive the specification of posterior neuronal differentiation (Nordstrom et al., 2002). Wnt modulates posterior hypothalamic neurogenesis through Lef1 and regulates neural progenitor differentiation (Lee et al., 2006; Wang et al., 2009). The zebrafish Lef1 protein sequence is highly conserved with the human, mouse, and African clawed frog. In zebrafish, Lef1 is expressed in the neural crest, tailbud, and developing mesoderm during embryogenesis (Dorsky et al., 1999). Lef1 is also required for neuromast formation and spacing during lateral line development in zebrafish (McGraw et al., 2011; Valdivia et al., 2011; Matsuda et al., 2013).

Zebrafish are well characterized developmental model animals, with easy breeding, external fertilization, transparent embryos, and a short generation interval. They are suitable for genetic manipulation, and broad selection of mutants is available (Alstrom et al., 2006). The in vivo imaging of the neuromasts on the surface of the lateral line makes zebrafish an excellent system for studying collective cell migration during organogenesis (Dambly-Chaudiere et al., 2003). The detailed anatomy of the developing zebrafish vascular system has been described, and shares high structural similarity with other vertebrates (Isogai et al., 2001). Transgenic zebrafishes, exhibiting vascular-specific fluorescence, provide an opportunity to detect individual cells or cellular compartments during vessel formation (Lawson and Weinstein, 2002; Schuermann et al., 2014).

Hif2-alpha plays an important role in the development of the zebrafish central nervous system and liver (Ko et al., 2011; Lin et al., 2014). Hifs also influence cell proliferation and migration (Schofield and Ratcliffe, 2004), both of which are active in trunk development (Kimmel et al., 1995). We hypothesized that Hif2-alpha also participated in the regulation of trunk and tail development. In this study, we examined a potential role for Hif2-alpha in the trunk by loss-of-function experiments. Zebrafish embryos with morpholino (MO) knockdown of Hif2-alpha showed aberrant angiogenesis and shortened neuromast formation. The defects in Hif2-alpha morphant embryos were rescued by the ectopic injection of syk or lef1 cRNA. We also performed chromatin immunoprecipitation sequencing (ChiP-seq) and discovered several hypoxia-responsive elements (HRE) that were bound by Hif2-alpha protein.

II. MATERIAL AND METHODS

1. Zebrafish Maintenance

Adult zebrafish and Tg(βl1:EGFP) transgenic fish (Lawson and Weinstein, 2002) were maintained under standard laboratory conditions. The developmental stages of embryos were determined based on standard protocols (Kimmel et al., 1995). All animal procedures were approved by the Animal Ethics Committee of the National Taiwan Ocean University (Affidavit of Approval of Animal Use Protocol code 101045).
2. Morpholino and cRNA Injection

Morpholino (Gene Tools LLC, Oregon, USA) sequences and dosages were as follows: **lefl**, 5’-CTCCTCCACCTGTGACACTGCCGCACT-3’ (3 ng); **hif1-alpha**, 5’-CAGTGGACAACTCAGTATTCATTCC-3’ (9 ng); and **hif2-alpha**, 5’-CGCTGTTCCTCGTAATTCGCCGAC-3’ (6 ng) (Ishitani et al., 2005, Ko et al., 2011). For capped-RNA synthesis, the full-length **hif2-alpha** and **syk** cDNAs were cloned into the pT7TS vector, and **lefl** was cloned into the pGM-T vector. After linearization, the plasmids were transcribed in vitro with mMESSAGE mMACHINE T7 ULTRA transcription kit from Ambion (Austin, TX, USA, cat. no. AM1345). For the rescue assay, 75 pg of purified **hif2-alpha** cRNA, 50 pg of purified **syk** cRNA or 100 pg of **lefl** cRNA were used.

3. Chromatin Immunoprecipitation

ChIP was performed using a Magna ChIP™ G Chromatin Immunoprecipitation Kit (Millipore, Billerica, MA, USA, cat. no. 17-611) as described (Lin et al., 2014). Approximately 500 embryos at 24 hpf were washed with 1X PBS and cross-linked with 1% formaldehyde for 15 minutes. Unreacted formaldehyde was quenched with 125 mM glycine. The cross-linked samples were washed with 1X PBS and homogenized by tissue grinder in Cell Lysis Buffer. The cell pellets were suspended in Nuclear Lysis Buffer after centrifugation. The cell lysates were sonicated on wet ice with twenty 30-s pulses (90-s pause between pulses) by a sonicator (MISONIX, Farmingdale, NY, USA, cat. no. S-4000), and the DNA was sheared to ~200-500 base pairs in length. Recombinant protein G magnetic beads were incubated with a polyclonal antibody against anti-Hif2-alpha (GeneTex, Hsinchu, Taiwan, cat. no. GTX103707) or normal rabbit IgG (Millipore, Billerica, MA, USA, cat. no. 12-370) as the negative control (Roche Applied Science, Indianapolis, IN, USA, cat. no. 11 096 176 001). The samples were incubated with Anti-Digoxigenin-AP Fab fragments (1:10,000 dilution, Roche Applied Science, Indianapolis, IN, USA, cat. no. 11 093 274 910) overnight at 4°C. The samples were colored with nitroblue tetrazolium chloride (NBT) (Roche Applied Science, Indianapolis, IN, USA, cat. no. 11 383 213 001) and 5-Bromo-4-chloro-3-indolyl-phosphate, 4-toluidine salt (BCIP, 4-toluidine salt) (Roche Applied Science, Indianapolis, IN, USA, cat. no. 11 383 221 001) in the dark after 1X PBT wash. Finally, the color reaction was blocked by 4% PFA incubation for 20 minutes. Cryosections were cut at a thickness of 10 μm for embryos (LEICA CM3050 S, Leica, Wetzlar, Germany). Color staining images were acquired using a microscope (OLYMPUS BX 51, Olympus, Tokyo, Japan). Double fluorescent *in situ* hybridization was performed as described (Ko et al., 2011). hif2-alpha probe was labelled with fluorescein (Roche Applied Science, Indianapolis, IN, USA, cat. no. 11 685 619 910) and detected using HRP-labeled anti-FITC antibody (Roche Applied Science, Indianapolis, IN, USA, cat. no. 11 426 346 910). Dig-labeled syk probe was detected using HRP-labeled anti-DIG antibody (Roche Applied Science, Indianapolis, IN, USA, cat. no. 11 207 733 910). After washing, Antibodies were visualized with TSA Plus Cy3 solution or TSA Plus Fluorescein Solution (Perkin-Elmer, Waltham, MA, USA, cat. no. NEL741001KT and NEL744001KT). Fluorescent images were acquired with confocal microscope (Nikon C2+ Confoical Microscope System, Nikon, Tokyo, Japan).

5. Whole-Mount Immunostaining and TUNEL Assay

Whole-mount immunostaining with an EGFP antibody was performed as described (Lin et al., 2014). Tg(fli1:EGFP) transgenic fish embryos were fixed with 4% PFA overnight at 4°C. The fixed embryos were incubated with 100% methanol overnight at -20°C and were washed thrice with 1X PBT for five minutes. Washed samples were incubated with 3% bovine serum albumin for one hour at room temperature. Samples were incubated with mouse anti-GFP primary antibodies (1:50 dilution, Abcam, Cambridge, UK, cat. no. ab290)
overnight at 4°C. After washing with PBT for 20 minutes, samples were further incubated with Alexa Fluor 488 conjugated anti-rabbit IgG secondary antibodies (1:600 dilution, Invitrogen, Carlsbad, CA, USA, cat. no. A11070) for 2 hours at room temperature in the dark. Finally, samples were washed thrice with 1X PBT for 30 minutes and preserved at 4°C.

TUNEL assays were performed with an in situ cell death detection kit using TMR red (Roche, cat. no.12 156 792 910). Briefly fixed 36 dpf Tg(fli1:EGFP) transgenic fish embryos were incubated in permeabilization solution for 15 minutes on ice. The samples were labeled with the TUNEL reaction mixture for 2 hours at 37°C in the dark. Finally, the labeled samples were washed thrice with 1X PBT for 30 minutes and preserved at 4°C. All images were acquired using a fluorescence microscope (OLYMPUS BX 51, Olympus, Tokyo, Japan).

6. Neuromast Staining

The neuromasts in live zebrafish embryos were stained with the fluorescent dye 4-(4-Diethylaminostryryl)-1-methylpyridinium iodide (4-Di-2-ASP) (Sigma-Aldrich, St. Louis, MO, USA, cat. no. D3418) at 4 dpf as described (Schuster and Ghysen, 2013). Images were acquired using a fluorescence microscope (OLYMPUS BX 51, Olympus, Tokyo, Japan).

III. RESULTS

1. Gene Expression Pattern of Hif-alpha mRNA

Hif2-alpha is necessary for neuronal and liver development (Ko et al., 2011; Lin et al., 2014). We analyzed the hif1-alpha and hif2-alpha gene expression patterns by whole-mount in situ hybridization (WISH) at different stages during development. Expression of hif2-alpha was widespread in the head, somites and dorsal aorta at 24 hpf (Fig. 1A, B). The hif2-alpha expression level decreased in the somites and dorsal aorta at 30 hpf (Fig. 1C, D), except in the head region. At 48 hpf, hif2-alpha expression in the notochord emerged (Fig. 1E, F) and was enhanced at 72 hpf (Fig. 1G, H). Intestinal hif2-alpha expression was also observed at 72 hpf. The hif1-alpha (Fig. S1) and hif2-alpha expression patterns were similar during the indicated developmental stages.
2. Hif2-alpha, but Not Hif1-alpha, Regulates ISV Angiogenesis

To determine the function of Hif1-alpha and Hif2-alpha during vascular development, we microinjected transgenic fluorescent zebrafish Tg(fli1:EGFP) with hif1-alpha and hif2-alpha MOs. Compared with wild type and mismatch-injected transgenic zebrafish (Fig. 2A, B, I, J), ISV sprouting was delayed in hif2-alpha morphants at 30 hpf (Fig. 2C). At 48 hpf, most hif2-alpha morphants revealed a fused dorsal longitudinal anastomotic vessel (DLAV), with ectopic branching or vanishing ISV. Severe phenotypes displayed a failure in DLAV formation, sprouting, and branching (Fig. 2D). These ISV growth defects were rescued by co-injection of hif2-alpha MO with hif2-alpha cRNA (Fig. 2E, F). No obvious phenotype was observed in hif1-alpha MO-injected Tg(fli1:EGFP) zebrafish at 30 and 48 hpf (Fig. 2K, L). These results suggest that Hif2-alpha is required for embryonic ISV development in zebrafish.

3. Syk Functions Downstream of Hif2-alpha to Control ISV Angiogenesis

Syk promotes angiogenesis and cell migration (Turner et al., 1995; Christie et al., 2010). To understand whether Hif2-alpha participates in angiogenesis by regulating Syk, we analyzed the syk expression pattern. At 24 hpf, syk was highly expressed in the head and ventral trunk, and at a low level throughout the whole body (Fig. 3A). This pattern was maintained until 48 hpf, when expression decreased in the ventral trunk (Fig. 3D). In hif2-alpha morphants, the head expression was slightly reduced and the ventral trunk expression was strongly decreased at 24 hpf (Fig. 3B). Gene expression in the head also significantly decreased at 48 hpf (Fig. 3E). We co-injected the hif2-alpha MO with hif2-alpha cRNA, and syk expression was restored (Fig. 3C, F). The expressions of hif2-alpha and syk are co-localized in the ventral trunk including dorsal aorta and axial vein at 30 hpf (Fig. 3G-I). These results indicate that Syk functions downstream of Hif2-alpha.

To investigate whether Hif2-alpha regulates ISV angiogenesis through Syk, we co-injected the hif2-alpha MO with syk cRNA into Tg(fli1:EGFP) zebrafish embryos. At 30 and 48 hpf, the growth delay and defects caused by Hif2-alpha knockdown were rescued (Fig. 2G, H). Therefore, we concluded that Hif2-alpha regulates ISV angiogenesis by regulating Syk expression.

4. Knockdown of Hypoxia Inducible Factor 2-alpha Causes Apoptosis and Atypical Morphogenesis in Somites

Hif2-alpha protects CNS neural progenitors from cell death (Ko et al., 2011). We investigated whether the whole body expression of Hif2-alpha prevented apoptosis. Whole-mount TUNEL assay and immunostaining against EGFP in Tg(fli1:EGFP) embryos at 36 hpf revealed evidence of apoptosis in hif2-alpha morphants. Compared with hif2-alpha morphants, the wild type had far fewer apoptotic cells (Fig. 4A). The hif2-alpha morphants exhibited apoptosis mainly in the brain (data not shown), spinal cord, and caudal veins (Fig. 4B). Apoptosis was also observed around the delayed sprouting tip cells in ISV (Fig. 4F). These results suggested that the loss of Hif2-alpha led to apoptosis and, subsequently, aberrant ISV angiogenesis.

To examine the morphology of somite, we analyzed myod1 expression pattern. In Fig. 5A, the somite formed a chevron-shaped pattern but with the injection of hif2-alpha MO (Fig. 5B) resulted in a more obtuse angle of the somite and abnormal narrow intersomitic boundaries. It suggested that the spatial environment (somites) for ISV spouting was only perturbed.
5. Hypoxia-Inducible Factor 2-alpha Regulates Neuromast Deposition in Trunk

The zebrafish lateral line provides a convenient model to study cell migration. We hypothesized that Hif2-alpha may play a role in lateral line development. We injected zebrafish embryos with hif2-alpha morpholinos with and without hif2-alpha cRNA, and we counted the numbers of neuromasts from one side on the trunk at 4 dpf. The neuromasts were visualized by 4-Di-2-ASP live staining. Approximately 87% of the wild type lateral lines contained 7-10 neuromasts (Fig. 6A). These numbers were significantly reduced to less than 37% in Hif2-alpha morphants (Fig. 6B). Co-injection of
hif2-alpha MO with hif2-alpha cRNA rescued the numbers of 7-10 neuromasts to 71.7% (Fig. 6C).

On the other hand, Hif1-alpha knockdown revealed no significant effect on neuromast deposition (Fig. 6G) or primordium migration. These results suggest that Hif2-alpha is required for the neuromast deposition in the trunk of embryonic zebrafish.

6. Lef1 Regulates Neuromast Deposition Downstream of Hif2-alpha

Lef1 is required for neuromast formation (McGraw et al., 2011; Valdivia et al., 2011; Matsuda et al., 2013). To further understand the potential regulatory relationship between Hif2-alpha and Lef1, we analyzed the lef1 expression pattern. At 48 hpf, lef1 was weakly expressed at the posterior lateral line primordium, but strongly expressed in the optic tectum (ot) and posterior hypothalamus (Fig. 7A). Expression of lef1 was abolished in hif2-alpha morphants (Fig. 7B) and rescued by co-injection with hif2-alpha cRNA (Fig. 7C). Moreover, lef1 expression did not change in hif1-alpha morphants (Fig. 7D). This result indicates that Lef1 functions downstream of Hif2-alpha.

To investigate whether Hif2-alpha regulates neuromast deposition through Lef1, we co-injected hif2-alpha MO with lef1 cRNA. We discovered that 64.4% of embryos retained 7-10 neuromasts after co-injection with hif2-alpha MO and lef1 cRNA (Fig. 6D). Moreover, co-injection of lef1 MO with lef1 cRNA rescued the number of 7-10 neuromasts from...
Wild type | hif2α MO
---|---
A | B

hif2α MO + hif2α cRNA | hif1α MO

Fig. 7. Lef1 expression patterns in hif2-alpha morphants. The expression patterns of lef1 were examined by WISH at 48 hpf. Images of WT (A), hif2-alpha morphant (B), hif2-alpha morphant co-injected with hif2-alpha cRNA (C), and hif1-alpha morphant (D). ot, optic tectum. hy, hypothalamus. ha, habenula.

39.5% to 75.8% (Fig. 6E, F; these results are summarized in Fig. 6H). Therefore, we concluded that Hif2-alpha regulates neuromast formation by regulating lef1 expression.

7. Hif2-alpha Regulates syk and lef1 Expression by Binding to the HREs in the cis-regulatory Elements Located in the 3’ Flanking Region of syk and the Intron of lef1

We performed ChIP-seq to determine the putative Hif2-alpha binding targets and identified signals within lef1 and syk. To confirm the ChIP-seq results, we examined whether Hif2-alpha directly binds to these HREs. Zebrafish embryos at 24 hpf were assayed by ChIP with antibodies against Hif2-alpha or rabbit IgG as a negative control. After the immunoprecipitation, DNA extracts were assessed by PCR. The expected regions were amplified in the Hif2-alpha pull-down extract but not in the IgG extract. This result indicates that Hif2-alpha indeed binds to the predicted HREs (Fig. 8A). The binding regions were located in the third intron of lef1 and 3’ flanking region of syk, respectively (Fig. 8B). These results suggest that Hif2-alpha directly binds to the HREs and regulates syk and lef1 expression.

IV. DISCUSSION

In the present study, we examined Hif2-alpha participation in the subtle morphogenesis after somitogenesis. First, we showed that Hif2-alpha is required in the vascular system during trunk development and regulates syk expression. Second, Hif2-alpha regulates neuromast formation by regulating lef1 expression. We also analyzed the ChIP-seq results, and verified that HIF2-alpha directly binds the HREs of lef1 and syk.

1. Hif2-alpha Regulates syk and lef1 Expression by Binding to the HREs in the cis-regulatory Elements Located in the 3’ Flanking Region of syk and the Intron of lef1

Hif2-alpha was cloned from endothelial cells (EC) and reported as endothelial PAS domain protein-1 (EPAS1), (Tian et al., 1997). In mammals, HIF1-alpha is ubiquitously expressed, whereas HIF2-alpha is found in a subset of tissues (Semenza, 2003; Wiesener et al., 2003). In zebrafish, hif1-alpha and hif2-alpha shared very similar widespread expression patterns in the head and somites during the pharyngula and hatching periods (24-72 hpf) (Fig. 1, S1). The only difference is that hif2-alpha, but not hif1-alpha or hif3-alpha, was expressed in the pharyngeal arch when entering the larval period (Lin et al., 2014). Murine Hif1-alpha modulates endothelial cell proliferation, migration, and vessel sprouting. However, Hif2-alpha regulates vascular morphogenesis, integrity, and assembly (Fraisl et al., 2009). Knockout mouse embryos or embryonic stem cells lacking HIF1-alpha showed severe vascular defects, absence of large vessel formation, and impaired vascular functions (Carmeliet et al., 1998; Iyer et al., 1998). Loss of Hif2-alpha caused improper vascular development in mice, and this phenotype was rescued by endothelium-specific expression of Hif2-alpha cRNA (Duan et al., 2005). Here, we demonstrated that loss of Hif2-alpha, but not Hif1-alpha, caused aberrant ISV development in zebrafish.

2. Hif2-alpha Regulates Angiogenesis at Multiple Levels

Zebrafish Hif2-alpha protects neural progenitor cells and neural differentiation by upregulating survivin orthologues
during embryogenesis (Ko et al., 2011). Our results show severe apoptosis in hif2-alpha morphant somites (Fig. 4), a phenomenon similar to the survivin morphant. Depletion of survivin causes apoptosis in the axial vessel region, caudal vein plexus, and neural structures. The aberrant sprouting cell migration of the ISV was also observed in survivin morphants (Ma et al., 2007; Delvaeye et al., 2009). Moreover, Lef1 cooperates with c-Myc to activate Bcl-2 and survivin expression in leukemia cells (Zhou et al., 2011). Loss of Lef1 in Xenopus tropicalis resulted in the abnormal formation of posterior cardinal veins and loss of angiogenic sprouting of endothelial cells (Roel et al., 2009). Hif2-alpha therefore likely participates in ISV angiogenesis through multiple hierarchical regulations.

In zebrafish, Syk and Zap-70 promote vascular cell migration and ISV formation. Zap-70 is a cytosolic tyrosine kinase, regulating hematopoiesis activation and differentiation (Arpaia et al., 1994). Knockdown of either gene resulted in the angiogenic delay of ISV and dorsal ISV patterning defects. Replenishment of syk mRNA rescued the angiogenic migration and ISV patterning defects in syk or vegfa morphants, suggesting that Syk might participate in the VEGF signaling pathway (Christie et al., 2010). However, the zap-70 expression level was not significantly different between wild type and hif2-alpha morphants (data not shown). In mouse subcutaneous xenografted tumors, hypoxia and reoxygenation regulate Syk/Lck-mediated gene expression, thereby controlling angiogenesis and tumor growth (Chakraborty et al., 2006). Hif1-alpha activates many factors involved in the regulation of angiogenesis, such as VEGF, PLGF, Ang1, Ang2 and PDGF (Kelly et al., 2003). Therefore, although VEGF is necessary for angiogenesis, we speculate that Syk is also important in the regulatory networks underlying ISV angiogenesis.

3. Participation of Notch Signaling and Its Relation with Hifs

VEGFA stimulation and Notch/Dll4 signaling together regulate tip cell formation (Phng and Gerhardt, 2009). Hifs regulate Notch signaling activity by stabilizing the Notch intracellular domain and, therefore, the expression of Notch target genes (Gustafsson et al., 2005; Bertout et al., 2009). Notch-regulated ankyrin repeat protein (Nrarpr), which is directly regulated by Notch signaling, inhibits the Notch signaling pathway in some mouse cells and in hematopoietic cells (Krebs et al., 2001; Yun and Bevan, 2003; Pirot et al., 2004). It is suggested that Nrarp coordinates endothelial Notch and Wnt signaling to stabilize nascent blood vessels during retinal angiogenesis and ISV formation in zebrafish (Phng et al., 2009). Nrarp can also stabilize Lef1 by blocking its ubiquitination, promoting neural crest cell development in a Notch independent manner (Ishitani et al., 2005). It is interesting to note that DeltaD/Notch signaling is required for proneuromast determination (Matsuda and Chitnis, 2010). Therefore, in primordium cells, the interaction between Hif and Notch signaling is also potentially important for cell fate determination.

4. Hif2-alpha Regulates Neuromast Formation

The development of the zebrafish PLL system occurs from head to tail. The migration path followed by the primordium is guided by the expression of the chemokine stromal-derived factor 1 (SDF1), which is recognized through its receptor Cxcr4b in the leading zone (Li et al., 2004). In the primordium trailing zone, Cxcr7b inhibits Cxcr4b and is required for the directionality of migration by the antagonistic interactions of Wnt and FGF signaling (Dambly-Chaudiere et al., 2007; Dalle Nogare et al., 2014). Interestingly, Hif1-alpha controls neural crest chemotaxis through the SDF1/Cxcr4b axis in zebrafish and Xenopus (Barriga et al., 2013). In our study, primordium migration results at 96 hpf were not influenced in hif1-alpha, hif2-alpha or left1 morphants. We also observed the somite morphogenesis was perturbed in hif2-alpha morphant. In zebrafish, Sulf1 regulates the formation of myoseptum and deposition of neuromasts (Meyers et al., 2013). Sulf1 is also involved in the formation of Wnt signaling complex in Xenopus (Fellgett et al., 2015). However, Sulf1 is deregulated and its promoter is directly bound by both Hif1-alpha and Hif2-alpha in human breast cancer cell (Kuwahara et al., 2002). The relationship between Sulf1 and Hif in zebrafish remains undetermined.

Rosette formation in the migrating primordium is critical for neuromast deposition. Progenitor cells in the leading zone form new rosettes, which are subsequently deposited from the trailing zone (McGraw et al., 2011). Cell proliferation in the PLL primordium is a key determinant of periodic neuromast deposition (Aman et al., 2011). Moreover, Lef1 is required for progenitor cells to maintain cell proliferation in the primordium leading zone, and regulates the FGF signaling inhibitor Dusp6 to influence neuromast formation (Matsuda et al., 2013). Hif1-alpha regulates Wnt/beta-catenin signaling in embryonic stem cells or tumor cells by enhancing beta-catenin activation and the expression of the downstream effectors Lef1 and Tcf1 (Mazumdar et al., 2010; Jeong and Park, 2013; Rampazzo et al., 2013). The Wnt inhibitor Dkk1, modulated by the Nogo-C2/Nogo receptor complex, regulates the morphogenesis of zebrafish PLL primordium (Han et al., 2014). In this study, compared with Hif1-alpha, Hif2-alpha plays the major role in PLL morphogenesis. The evidence shown here could result from the direct regulation of Lef1 by Hif2-alpha and/or the complicated and less-defined relationship between Hif2-alpha and Wnt pathway.

5. HRE Binding Sites in Zebrafish

In our results, we identified the direct binding of HIF2-alpha in the third intron of left1 and the 3’ flanking sequence of syk, respectively. This is the first evidence that Hif2-alpha binds the HRE in syk and regulates its expression. Hif1-alpha directly binds the left1 5’ UTR to regulate Wnt/beta-catenin signaling in ES cells (Mazumdar et al., 2010). Interestingly, we did not find the HRE cluster in the 5’ UTR of zebrafish left1 but the intronic binding site. We speculated that Hif2alpha regulation of left1 through binding to the HRE in the intron is...
an adaptation in teleost also seen in legl (Lin et al., 2014). Further investigation is required to determine proximal and even possibly the distal regulatory elements to illustrate the whole picture of gene regulation.

V. CONCLUSION

We demonstrated that Hif2-alpha plays an essential role in the embryonic development of ISV and PLL in zebrafish through directly controlling syk and left expression, respectively. The role Hif2-alpha plays in embryonic development after somitogenesis in the zebrafish trunk awaits further investigation.

ACKNOWLEDGMENTS

We thank Taiwan Zebrafish Core Facility at Academia Sinica (TZCAS) for providing Tg(fli1:EGFP) transgenic fish. TZCAS is supported by the grant of NSC 103-2321-B-001-050 from the Ministry of Science and Technology. This work was supported by the Ministry of Science and Technology, Taiwan, R.O.C. (Grant No. MOST 104-2321-B-019-005-MY3) and the Center of Excellence for the Oceans, National Taiwan Ocean University.

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