Characterization of Zebrafish Pax1b and Pax9 in Fin Bud Development

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Both Pax1 and Pax9 belong to the important paired box gene family (PAX), which mainly participates in animal development and sclerotome differentiation. To date, the precise molecular mechanism and related signaling pathway of Pax1 remain unclear. In our study, microinjection of morpholino- (MO-) modified antisense oligonucleotides against pax1b induced pectoral fin bud defects. Furthermore, we demonstrate that the phenotypes caused by the knockdown of Pax1b in zebrafish could not be phenocopied by pax9 MO and could not be rescued by either Pax1a or Pax9 overexpression. We further find that Pax1b affects the expression of col2a1, Uncx4.1, Noggin3, and aggrecan, confirming the role of Pax1b in chondrocyte differentiation and bone maturation. Moreover, we identify an interaction between PAX1 and FOXO1 and find that the interaction was enhanced under hypoxia stress. Together, this evidence for cell death caused by pax1b knockdown provides new insight into the role of the Pax protein family in cell fate determination and tissue specification.

1. Introduction

The Pax protein family, consisting of numerous transcription factors with a paired box domain containing 128 amino acids, plays a central role in embryonic patterning and organ differentiation [1]. In vertebrates, Pax genes are divided into four subfamilies according to their structures. The Pax1/9 subfamily participates in the formation of skeletal muscle and sclerotome differentiation [2, 3].

In most vertebrates, Pax1 and Pax9 have similar expression patterns and functions. For example, the expression of both chicken PAX1 and PAX9 genes was the strongest in undifferentiated cells of precartilage condensations or at the margins of differentiated cartilages and was absent from cartilage itself [4]. Both induce chondrogenic differentiation in the sclerotome via targeting Nkx3.2 [5]. Murine PAX1 and PAX9 have overlapping expression profiles and respond to fibroblast growth factor (FGF) and hedgehog (HH) signaling during the progression of limb bud formation [6]. More interestingly, there are four kinds of spontaneous Pax1 mutant mice (Pax1im, Pax1im-ex, Pax1im-i, and Pax1im-s) which show different phenotypes [7]. It has been reported that PAX1 is a candidate gene in vertebral malformations and congenital scoliosis from the study of clinical genetics and the mouse mutant undulated [8, 9]. Using the teleost medaka, a closely related species to zebrafish, Japanese scientists determined the similarity of pax1 and pax9 expression patterns in the sclerotome and pharyngeal pouch. MO knockdown of either Pax1 or Pax9 causes defects in the neural arch and scoliosis and double knockdown revealed that Pax1 and Pax9 function synergistically in sclerotome development [10].
However, the expression patterns of pax1b and pax9 in zebrafish are quite different. pax1b is a maternally expressed gene and is zygotically expressed in the pharyngeal pouches, fin bud, and notochord and weakly expressed in the dorsal aorta and axial vein at 48 hpf [11], while pax9 is expressed after segmentation, primarily in part of the somites and branchial arches and not in the fin bud (ZFIn). These differences in their expression patterns suggest divergent functions in transcriptional activity and cell differentiation between Pax1b and Pax9 in zebrafish. To address whether the functions of Pax1b and Pax9 have distinct roles in zebrafish embryonic development, we designed two morpholinos (MOs) against pax1b and pax9 to study their mechanism of action.

FOXO1, a member of the Forkhead family proteins of the O subclass, is not only one of the most critical regulators of cell death [12], but also an early molecular regulator during mesenchymal cell differentiation into osteoblasts. In mouse embryos, the expression of FoxO1 is higher in skeletal tissues, and FoxO1 silencing has a drastic impact on skeletogenesis and craniofacial development [13]. Gene fusions involving PAX3/7 and FOXO1 in alveolar rhabdomyosarcoma have been reported [14]; however, the interaction between PAX1 and the FOXO family has not yet been described. In this research, we studied the relationship between PAX1 and FOXO1 to determine whether FOXO1 participates in the developmental processes regulated by Pax1.

2. Materials and Methods

2.1. Fish Maintenance and Embryo Collection. Zebrafish (Danio rerio), AB strain, were kept at 28.5°C under a light and dark cycle of 14 and 10 hours, respectively. Embryos were collected and staged as described [15].

2.2. Plasmid Construction. The pax1b cDNA sequence was deposited in GenBank with an accession number of XM_695785. The full coding sequence of pax1b was amplified from cDNAs derived from 24 hpf embryos with a forward primer (zpF: 5'-atgagactctcctcctggattg-3') and a reverse primer (zpR: 5'-ttatgaggctcattcctggattg-3') and subcloned into pX7 and pBlueScript to generate vectors for synthesizing mRNA and antisense RNA probes in vitro, respectively. Zebrafish pax9 and amphioxus pax1/9 were subcloned using the same strategy as pax1b and the primers were as follows: zp9F (5'-atggagccagctttgtgg-3'), zp9R (5'-ttatgagctcctcctggattg-3'), aplF (5'-ttatgagctcctcctggattg-3'), and aplR (5'-ttatgagctcctcctggattg-3'). Expression plasmids were all subcloned into pCMV5 vector with various tags. Template for PCR was cDNA from different species including human, mouse, and zebrafish.

2.3. Reverse Transcription-PCR. To quantify nk3.2, col2a1, and aggrecan transcripts in embryos, injected embryos were digested at 24 hpf or 48 hpf. First strand cDNAs synthesized from total RNA (Trizol from Takara) were used as templates with the SuperScript Kit (Invitrogen). Specific primers with the sequences listed in Supplemental Table 1 (see Supplementary Material available online at http://dx.doi.org/10.1155/2014/309385) were used to amplify markers [16, 17]. TE buffer was used as negative control. For qPCR assays, fold change for each group of embryos was determined using the delta-delta Ct method. Data were normalized to the control embryos. Quantified mRNA levels were normalized to β-actin and are presented relative to control embryos.

2.4. RNA Synthesis, Whole-Mount In Situ Hybridization. Capped mRNAs were synthesized using T7 Cap Scribe (Roche) according to the manufacturer’s instructions. For preparation of digoxigenin-labeled antisense probe, plasmid containing pax1b cDNA was linearized with KpnI. In situ hybridizations were performed as previously described [18].

2.5. Morpholinos and Microinjection. Four morpholino oligonucleotides were synthesized by Gene Tools (paxlb-MO1: 5'-CATTTGCACTGTATTTCCCCAT-3', positioned from 176 to 200 in the ENSDART00000132835 sequence; paxlb-MO2: 5'-CCCGTGTCCTCCGCTAAAGACTGCCC-3', positioned from 84 to 108 in ENSDART00000132835; zebrafish pax9-MO1: 5'-CAAGGCTGGCTCCATGCTTATAG-3', positioned from 136 to 160 in the U40931.1 sequence; and zebrafish pax9-MO2: 5'-CCTTGATTAATTATGCACCGAGCGG-3', positioned from 47 to 71 in the U40931.1 sequence). The sequence of control MO is 5'-CTCTTACCTCACGTATCATTATA-3'. All MOs were dissolved in nuclease-free water to make a 20 μg/μL stock. Western blots and RT-PCR assays were used to check MO efficiency. All morphants were injected using a 1:1 mixture of the two MOs. mRNAs and morpholino oligonucleotides were injected into the yolk of fertilized eggs at the single-cell stage [16].

2.6. Cell Culture and Cell Death Assay. Mammalian cells were grown in DMEM (GIBCO) supplemented with 10% fetal calf serum (Hyclone). In the DNA damage induced cell death assay, U2OS cells were exposed to lethal treatments (80 J/m² UV or 2.5 μM doxorubicin) and were kept in culture medium for 8 h before Hoechst staining. The positive cell numbers in 10 random sweeps were summed, and an error bar was calculated from 3 independent replications in each panel [19]. pax1b DNA induction was mediated by the IRES-TOMATO lentivirus system.

2.7. Western Blot, Immunoprecipitation, and Immunofluorescence Staining. 36 hpf embryos and 293FT cells were lysed with lysis buffer [18]. The total lysis was mixed with an equal volume of 2× SDS sample buffer and was analyzed by Western blotting. Antibodies used are the following: rabbit polyclonal antibody to Pax1 (83312 from Abcam), Uncx4.1 (ARP47548 from Aviva Systems Biology), Noggin3 (16054 from Abcam), and FOXO1 (sc-11350 from Santa Cruz) and mouse monoclonal antibody to Flag (F1804 from Sigma) or Myc (M4439 from Sigma). For immunoprecipitation, anti-Flag M2 affinity gel was purchased from Sigma. For immunofluorescence staining, 24–36 h after transfection with or without hypoxia stimulation, HeLa cells grown on coverslips were fixed with
4% formaldehyde for 20 min at room temperature, followed by 0.5% Triton X-100 treatment for 5 min and 3% BSA blocking. The cells were then incubated with corresponding primary and secondary antibodies along with DAPI staining for visualization of nuclei. Fluorescence images were acquired with a Nikon microscope. Fluorescent secondary antibodies, Alexa Fluor 546 (A10040), and FITC-Goat anti-mouse antibody (62-65II) were purchased from Invitrogen.

2.8. Hypoxia Treatment. 293FT and HeLa cells were treated with CoCl₂, a well-known hypoxia mimetic agent [20] at different concentrations (2, 20, 200, and 400 μmol/L) for 14 h.

2.9. Statistical Analysis. Data are presented as means ± SE. Differences between treatment groups were analyzed using ANOVA. Differences were considered significant at the P < 0.05 level.

2.10. Ethics Statement. Our experiments were conducted with the permission of the ethics committee of Chongqing Medical University.

3. Results

3.1. Pax1b Is Required for Zebrafish Morphogenesis and Embryonic Development. In the context of bone mineralization and sclerotome differentiation, few studies to date have examined Pax1b function in zebrafish. We designed two MOs against pax1b to block its translation (Figure S1). Western blot assays and RT-PCR showed that the bands in the 2nd and 3rd lanes had reduced signals with respect to the control lane (Figure S2A), confirming the efficiency of pax1b MOs on protein and RNA levels, respectively. Zebrafish embryos injected with 2 ng pax1b MO showed small eyes as well as a curved axis and tail, while 5 ng pax1b MO caused more severe phenotypes: head atrophy and a shorter body axis, indicating that the pax1b MO functions in a dose-dependent manner (Figures 1(b) and 1(c)).

To test the specificity of pax1b MOs, we carried out coinjection of pax1b mRNA and pax1b MO and found that 200 ng of pax1b mRNA could rescue pax1b morphants to normal axial length, while GFP mRNA could not rescue the axis defects at any concentration (Figures 1(d)–1(g)). Interestingly, paxla, the closest homologue of pax1b, could not rescue pax1b morphants in our experiments, neither could amphioxus pax1/9 nor could zebrafish pax9, the other member of the pax1/9 subfamily (Figure 1(h)). These results indicated that, compared with paxla and zebrafish pax9, pax1b plays different roles in early embryonic development and teleost Pax family members have more diverse and complex functions than previously shown.

3.2. Pax9 Inhibition Causes a Tail Defect. Two MOs against zebrafish pax9 were designed (Figure S1). Due to the lack of a Pax9 antibody, we verified zebrafish pax9 MO efficiency using its target nkx3.2 (Figure S2B). As expected, the knockdown of zebrafish pax9 downregulated nkx3.2 transcription, confirming the efficiency of pax9 MOs. Zebrafish pax9 morphants have different phenotypes than pax1b morphants. pax1b morphants showed short body axis and a fin bud defect; however, zebrafish pax9 morphants only showed a kinked tail. Coinjection of pax1b and zebrafish pax9 MO showed all of the defects mentioned above (Figures 2(a)–2(d)). The tail defect in zebrafish pax9 morphants could be rescued only by zebrafish pax9 mRNA, while the aberrant phenotype could not be rescued by mRNA of members of the same subfamily, paxla and pax1b. These results further confirm that pax1 and pax9 have unique functions in zebrafish embryo development.

3.3. Loss of Function of Zebrafish pax1b Causes Fin Bud Defects. Compared with the control group, single-cell stage injection of 2 ng pax1b MO caused moderate defects, with smaller and asymmetric pectoral fin in 55% of embryos, and severe defects including the almost complete lack of fin buds in 27% of embryos. Embryos injected with 5 ng pax1b MO had more serious phenotypes: fin buds were nearly abolished in about 52% pax1b morphants (Figures 3(a)–3(c) and 3(e)). Coinjection of pax1b MO and pax9 mRNA rescued the aberrant phenotypes, confirming the specificity of the pax1b MO (Figure 3(d)). In order to further characterize the observed phenotypes, we evaluated the expression of the pectoral fin markers erm and pea3, as means to assess defects in fin bud development. In pax1b morphants at 28 hpf, the expression of erm and pea3 was dramatically reduced. Coinjection of pax1b MO and pax9 mRNA rescued the defects (Figures 3(f)–3(m)). These data confirm that pax1b plays a vital role in zebrafish fin bud development.

3.4. Pax1b Controls Bone Maturation. Further investigation at the molecular level found that collagen type II (col2a1), a chondrocyte differentiation marker, was downregulated in pax1b morphants (Figure 4(a)), suggesting that chondrocytes differentiation was affected in pax1b morphants. Using polyclonal antibodies against Noggin3 and Uncx4.1, we found that the protein level of Uncx4.1 was downregulated, while Noggin3 was upregulated in pax1b morphants (Figure 4(b)). Due to a lack of available antibody against Aggrecan, we detected its transcript and found a significant reduction in pax1b morphants (Figure 4(c)). These results suggested that pax1b correlates with the progression of bone maturation.

3.5. Forced Expression of Pax1b Decreases Cell Death Potential on Physiological Stress. The obvious fin bud defects in pax1b morphants led us to investigate whether Pax1b affected cell death in an overexpression system. A range of biological stressors or DNA damage can induce cell death. In control cell culture, UV treatment with 80 J m⁻² dose induced 72.0% cell death in the U2OS cell line, but transfection of 0.5 μg or 1.5 μg Pax1b DNA reduced this rate to 49.2% and 42.9%, respectively (Figure 5(a)). Consistently, 2.5 μM doxorubicin caused 76.4% U2OS cell death; transfection of 0.5 μg or 1.5 μg Pax1b DNA decreased this ratio to 55.9% and 48%, respectively (Figure 5(b)). Thus, Pax1b serves as a cell death inhibitory molecule, and its knockdown might increase apoptosis or other types of cell death.
3.6. PAX1 Interacts with FOXO1. We studied the relationship between PAX1 and FOXO1 using immunoprecipitation assays. Results showed that PAX1 interacts with FOXO1 in HEK293FT cells and that the interaction is conserved in different species including human, mouse, and zebrafish (Figures 6(a) and 6(b)). However, there was no interaction between PAX9 and FOXO1 (Figure 6(c)).

We next tested the subcellular localization of PAX1 and FOXO1. Immunofluorescence assays revealed that PAX1 was only located at the nucleus while FOXO1 was distributed in both the cytoplasm and nucleus. Moreover, the colocalization of PAX1 and FOXO1 increased when stimulated with CoCl₂ (Figure 7(a)). Further communoprecipitation analysis showed that hypoxia stress enhanced the interaction between PAX1 and FOXO1 in a dose-dependent manner (Figures 7(b) and 7(c)). Taken together, these data provide evidence that Pax1 might participate in fin bud development together with FOXO1.
Figure 2: Fin bud and tail defects caused by injection of pax1b MO and/or zebrafish pax9 MO. (a) Fin bud and axis defects caused by injection of 5 ng pax1b MO. (b) Tail defect caused by injection of 5 ng zebrafish pax9 MO. (c) Fin bud and tail defects caused by coinjection of 2.5 ng pax1b MO and 2.5 ng zebrafish pax9 MO. (d) Statistics of phenotype ratios. White columns indicate the ratio of fin bud defect; shaded columns indicate the ratio of kinky tail. (e) Statistical analysis of phenotypes caused by coinjection of different mRNAs with 5 ng zebrafish pax9 MO. The amount of mRNA injected for every embryo is as follows: 200 ng gfp mRNA, 200 ng pax1b mRNA, 200 ng pax1a mRNA, and 200 ng zebrafish pax9 mRNA. All embryos were observed at 36 hpf.
Figure 3: Fin bud defects caused by pax1b knockdown. ((a)–(d)) Dorsal view of the region near the fin bud with orientation of head towards the top. (a) Control embryos. ((b)–(c)) pax1b morphants. (d) Rescue embryos: coinjection with 5 ng pax1b MO and 200 ng pax1b mRNA. (e) Statistical analysis of fin bud defects caused by injection of control MO or pax1b MO. Total numbers of injected embryos are labeled. ((f)–(m)) Expression pattern of erm and pea3 in control embryos, pax1b morphants, and co-injected embryos. Numbers of defective embryos and total numbers of stained embryos are labeled in the bottoms. The embryonic stage is 72 hpf in panels (a)–(e) and 32 hpf in panels (f)–(m). All the embryos are viewed from the dorsal side with head towards the top.
**Figure 4:** Molecular mechanism of sclerotome development mediated by Pax1b. (a) The relative expression level of zebrafish collagen type II (col2a1) monitored by qPCR. (b) Sclerotome differentiation analyzed by molecular markers Uncx4.1 and Noggin3 by Western blot. (c) The relative expression level of zebrafish *aggrecan* monitored by qPCR. The fold change for each group was determined using the delta-delta Ct method. Quantified mRNA levels were normalized to *β*-actin and are presented relative to control embryos. 50 embryos at the 48 hpf stage were used in each group, performed in triplicate.

**Figure 5:** Forced expression of Pax1 decreases cell death potential on physiological stress. U2OS cells were exposed to 80 J/m² UV (a) or 2.5 μM doxorubicin (b) for 8 h. Hoechst-positive cells were counted and subjected to statistical analysis using Student’s *t*-tests. All *pax1b* DNA induction was mediated by the IRES-TOMATO lentivirus system. Data are presented as means ± SE from three independent experiments. *P < 0.05*, compared with vector.
Figure 6: The interaction between PAXI and FOXO1. (a) FOXO1 is a new PAXI-interacting protein. HEK293FT cells were transfected with Myc-tagged FOXO1 and Flag-tagged PAXI cloned from human cDNA. Cells were harvested for immunoprecipitation with anti-Flag affinity resin and immunoblotted with the indicated antibodies. (b) The interaction of Pax1 and FOXO1 was conserved. Myc-tagged FOXO1 was immunoprecipitated by Flag-tagged Pax1 or mouse Pax1. (c) PAX9 did not interact with FOXO1 in HEK293FT cells. zPax1b: zebrafish Pax1b; mPax1: mouse Pax1. TCL: total cell lysate. IP: immunoprecipitation.

4. Discussion

The PAX protein family was first identified almost thirty years ago [21]. We used MEGA4.0.2 software to do a phylogenetic analysis and found that there are high identities among *Homo sapiens* Pax1, *Mus musculus* Pax1, *Xenopus* Pax1, *Danio rerio* Pax1, *Danio rerio* Pax9, and *Branchiostoma* Pax1/9 (Figure S3). The amino acid alignment performed by DNAssist software indicated that the amino acid sequence is highly conserved among *Branchiostoma* Pax1/9, *Danio rerio* Pax1a, and *Danio rerio* Pax1b as well as *Danio rerio* Pax9 (Figure S4). The knockout of Pax1 in mouse produced malformed sternum and scapula [7]. In this report, we used zebrafish as an animal model to investigate the biological functions of Pax1b and Pax9, demonstrating that *pax1b* morphants display serious defects in fin buds and the axis which is different than *pax9* morphants. Pax1b overexpression rescued the morphants to a moderate phenotype, whereas Pax1a or zebrafish Pax9 could not rescue *pax1b* morphants. On the other hand, several reports have revealed differences between undulated mutations of Pax1 and its knockout model, proving the haploinsufficiency of Pax1 and redundancy of Pax9 [7, 22]. Moreover, it has been reported that the loss of Pax9 function in the vertebral column in Pax9lacZ mutant mice might be rescued by Pax1 and another report showed that Pax9 might partially substitute for Pax1 [7, 23]. Our results suggest
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morphants might have a close relationship with cell death. Previous studies have illustrated that Pax3 and Pax7 are associated with cell survival in numerous cancer cell lines and silencing of pax2 promotes renal carcinoma apoptosis [29, 30]. Our original study shows that knockdown of paxlb induced cell death in the specific tissue of zebrafish embryos and Paxlb overexpression decreased stress-induced apoptosis in the U2OS cell line. FOXOs not only promote mammalian cell survival by inducing cell cycle arrest and quiescence in response to oxidative stress, but also regulate longevity in model organisms [31]. FOXO1 can be phosphorylated by JNK or Mst1 proteins, which phosphorylate FOXO1 under conditions of oxidative stress. This phosphorylation causes the translocation of FOXO1 from the cytoplasm to the nucleus [12]. It has been reported that the transcription of FoxO3, another member in the FoxO subfamily, is induced by hypoxia and the increased expression of FoxO3 results in enhanced cellular survival by attenuating HIF-induced apoptosis [32]. We supposed that FOXO1 might play a role in resistance to hypoxic stress during development together with PAX1. In this study, we demonstrate for the first time that PAX1 interacts with FOXO1 and that this interaction is strengthened under hypoxia stress. We postulate that fin bud malformation in paxlb morphants is caused by cell death via FOXO1 signaling. The downstream events remain unclear, and much more work is needed in the future to address the exact mechanism.

5. Conclusions

In summary, we have discovered that paxlb plays a pivotal role in zebrafish fin bud development. Overexpression of paxlb can relieve cell death induced by stress. Furthermore, we found an interaction between PAX1 and FOXO1 for the first time, an interaction enhanced under hypoxia stress. Together, the evidence for cell death caused by paxlb knockdown provides new insights into the role of the Pax protein family in cell fat determination and tissue specification.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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