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Damage in brain development by morpholino knockdown of zebrafish *dax1*

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***DAX1* is an orphan nuclear receptor and involved in development of steroidogenic organs, which activates transcription of genes involved in steroidogenesis. In this study, we analyzed the function of the zebrafish *dax1* during early development of central nervous systems to appear unidentified aspects of *DAX1* and decrease confusions concerned with functions of *DAX1* in early development of vertebrates. By whole-mount *in situ* hybridization of embryo at the 32 h post fertilization (hpf), expression of zebrafish *dax1* was detected around the forebrain, midbrain, hindbrain, and the extending tail tip. Embryos injected with zebrafish *dax1* morpholino antisense nucleotide (MO) exhibited delayed development. When the developmental stage of wild type embryos was at Prim-15 (32 hpf), zebrafish *dax1*MO injected embryos were at Prim-5 (24 hpf). Concurrently with developmental delay, the MO injected embryos showed high mortality. At 48 hpf, the MO injected embryos exhibited abnormal development in the central nervous systems. The enlarged tectum and the protruded rhombomeres were observed. Moreover, development of central nervous systems, especially midbrain–hindbrain boundary, became narrower. At 5 day post fertilization, the MO injected embryos formed edemas around head, pericardial sac and abdomen. Collectively, our results indicated that the zebrafish *dax1* is important for brain development.**

[**Key words:** Dax1; Zebrafish; Brain development; Sexual differentiation; Morpholino]

Nuclear receptors act as transcriptional factors that regulate the expression of genes involved in development, growth, sexual maturation, reproduction, and metabolic homeostasis (1). More than 70 members of the nuclear receptor superfamily have been discovered in humans. Many nuclear receptors have one DNA-binding domain (DBD) consist of two zinc finger modules and a ligand binding domain (LBD) located at the C-terminal region (2,3).The ligand binding pocket at C-terminus interacts with ligands and changes conformation of the nuclear receptor (4).

Various lipophilic ligands, such as steroids and thyroid hormones, bile acids, fatty acids, and retinoids, interact with LBDs. Binding of lipophilic ligands induces allosteric changes in the conformation of the receptors (2,5). This conformational change converts an apo-type receptor to an active holo-type receptor (2,6,7). In particular, the configuration changes in the AF-2 core at the LBD C-terminus lead to transcriptional activation or repression of associated genes (2,4,8–10).

Several transcriptional co-factors, such as steroid receptor co-activators (*P160* families), interact with holo-type nuclear receptors (11). They have helical leucine-rich lxxll motifs (l is leucine and x is any other amino acid). Co-factor lxxll motifs interact with LBDs in holo-type receptors (10,12). These co-activators interacting with holo-type nuclear receptors recruit histone acetyltransferases and methyltransferases to specific enhancer/promotor regions, which facilitates chromatin remodeling, assembly of general transcription factors, and transcription of target genes (13).

DAX1 (dosage-sensitive sex reversal, adrenal hypoplasia congenita (AHC) critical region, on the chromosome X, gene 1) is a member of the NR0B family of orphan nuclear receptors. DAX1 has atypical DBD including the lxxll motifs instead of zinc fingers at its N-terminus and LBD including the AF-2 motif at its C-terminus. *DAX1* is considered to act as an lxxll motif-containing co-repressor (2,14). *DAX1* mutations cause AHC with hypogonadotropic hypogonadism. Similarly, over-expression of the *DAX1* gene in mice leads to phenotypic sex reversal in males (14). *DAX1* is expressed in the hypothalamic–pituitary–adrenal–gonadal (HPAG) axis and reflects phenotypic features of *DAX1* deficiency, specifically AHC and hypogonadal hypogonadism (2,15).

It has been demonstrated that *DAX1* represses transcriptional activities of nuclear receptors such as steroidogenic factor 1 (*SF1*) (14). Moreover, *DAX1* regulates the transcriptional activity of steroidogenic enzyme genes, and some of them are also regulated by *SF1* (16). *DAX1* and *SF1* coordinate to regulate transcriptional repression and activation of these genes in HPAG- axis (17).

Several researchers reported possible functions of *DAX1* except for the HPAG axis. In previous studies using zebrafish as a vertebrate model, expressions of *dax1* were detected in liver and bilateral structure cephalad, and medial to the pectoral fins in the region of the 5th branchial arch (15,18). Transcripts of zebrafish *dax1* around the 5th branchial arch were co-localized with those of even-skipped-like (*eve1*) gene which is a candidate marker of early tooth initiation in zebrafish. Furthermore, it is reported that down regulation of *eve1* was caused by knockdown of zebrafish *dax1* (18). In previous study of other model organism, mouse *Dax1* was expressed in embryonic stem

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(ES) cell derived from the inner cell mass of blastocysts and showed as a negative regulator of *Oct3/4* which is an indispensable transcriptional factor in the self renewal of ES cells (19). However, *in vivo* functions of mouse *Dax1* has not been clearly understood yet (20).

Zebrafish is a small cyprinoid teleost fish that is used as a model organism for studying physiology and genetics of vertebrates (21–23). It has been used as a model organism for examining toxicity of chemical compounds during early development, reproduction, and sexual differentiation (24,25). Several researchers have studied zebrafish *dax1* (Gene ID: 100001692). Zebrafish Dax1 has one lxxll motif on DBD and one AF-2 motif on LBD. Comparing with mammalian DAX1, zebrafish Dax1 has fewer lxxll motifs; mammalian DAX1 has three lxxll motifs (15,17). Previous studies have shown that zebrafish *dax1* signals were detected at anterior region to the pituitary (15). However, other reports have reported that expression of zebrafish *dax1* was detected in a wider area including the whole brain (26). Although morpholino antisense nucleotide (MO) knockdown of zebrafish *dax1* was performed, there are only two previous reports about MO knockdown during early development. In one report, zebrafish *dax1* MO injected embryos showed edemas in their body cavities, predominantly in the abdomen, after 4 day post fertilization (dpf) (15). In other report, injection of MO targeting zebrafish *dax1* down-regulated *eve1* expression (18). The functions of zebrafish *dax1* remain unknown in/outside the HPAG-axis (20). Moreover, genetic information on zebrafish *dax1* in the NCBI database is also a little confusing/unclear. Previously, there was a computationally predicted gene, LOC798707 similar to DAX [*Danio rerio*] (Gene ID: 798707) in NCBI database supported by ESTs. In this study, we represent the gene as "*dax1* similar gene". As a result of bioinformatics analysis, we concluded that zebrafish *dax1* and *dax1* similar gene are splice variants. At present, *dax1* similar gene was removed as a result of standard genome annotation processing by NCBI. In this study, we performed whole-mount *in situ* hybridization (WISH) studies and MO knockdown experiments of *dax1* in order to understand the important role of zebrafish *dax1* during early development.

MATERIALS AND METHODS

Gene In this study, we focused on a zebrafish *dax1*, which was identified from the NCBI database. Zebrafish *dax1* mRNA (GenBank accession number: NM_001082947.1) has putative coding regions of a DBD and LBD that contains one lxxll motif and one AF-2 motif as described in previous studies and was reported to be involved in/out of HPAG axis (15,18) (Figs. 1A and B).

Extraction of total RNA Total RNA was extracted from hatching-period embryos at 72 h post fertilization (hpf). Hand-dechorionated embryos (approximately 15) were rinsed in 1× phosphate buffered saline (PBS; 2.9 mM NaH2PO4, 9.0 mM Na2HPO4, 138 mM NaCl; pH 7.4) before extraction of total RNA. Extraction was performed using the SV (spin vacum) total RNA isolation system (Promega, Heidelberg, Germany) according to a standard protocol recommended in the instruction of the kit.

cDNA synthesis The target cDNA was synthesized from total RNA using a SMART RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA). Approximately 1 μg of total RNA was used to synthesize template cDNA. Reactions were carried out according to the standard protocol recommended in the instruction of the kit. Finally, the cDNA products were dissolved in 100 μl Tricine-EDTA buffer, pH 8.5 (Clontec).

cDNA amplification The synthesized cDNA was amplified using an Advantage 2 PCR Kit (Clontec). Zebrafish *dax1* gene primers were as follows: forward 5′-TCAAGACCCTGAAGTTCGT-3′ and reverse 5′-TTGACCGCGCCAATGAC-3′. Polymerase chain reaction (PCR) was performed under the following conditions: 95°C for 1 min, 35 cycles at 95°C for 30 s, 56°C for 30 s, 68°C for 3 min, and a final extension at 68°C for 3 min.

cDNA cloning PCR products were purified by agarose gel electrophoresis and used for cDNA cloning. A pTAC-2 vector containing M13F and M13R sites was used. cDNA cloning was performed using a DynaExpress TA PCR Cloning Kit (pTAC-2) with Jet Competent Cells (Biodynamic Laboratory, Tokyo, Japan). Nucleotide sequences were determined on an ABI 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA) using M13 universal primers (forward 5′-GTAAAACGACGGCCAGT-3′ and reverse 5′-GGAAACAGCTATGACCATG-3′). The sequence was analyzed using a GENETYX software (ver. 8.0, GENETYX, Tokyo, Japan) and the NCBI BLASTN programs.

Whole-mount *in situ* hybridization For *in vitro* synthesis of DIG-labeled RNA probes for zebrafish *dax1*, linearized pTAC-2 vectors containing the partial sequences in LBD were used as templates for a digoxygenin (DIG) RNA Labeling Kit (SP6/T7; Roche, Mannheim, Germany). Whole-mount *in situ* hybridization (WISH) experiments were performed as described previously (27), with minor modifications. Zebrafish *dax1* signals were detected using Fluorescent Antibody Enhancer Set for DIG Detection (Roche).

Signals in embryos were detected using fluorescence microscopy (Olympus CKX41 equipped with an Olympus U-RFLT50 lamp; Olympus, Tokyo, Japan). Photographs were taken with an ART CAM-130MI (ARTRAY, Tokyo, Japan). Acquired photo data were analyzed and transformed to pseudo colored photo data by using Image J software (ver. 1.45 s, NIH, ND, USA). Different colors correspond to fluorescence signal intensity.

Morpholino antisense nucleotide knockdown of zebrafish *dax1* Morpholino antisense nucleotides (MOs) (Gene Tools, Philomath, OR, USA) were used for gene knockdown. The MO1 targeted intron–exon junction of zebrafish *dax1* pre mRNA to inhibit mRNA splicing (28) (Fig. 1A). Standard control MO (st. control MO) had no complementary sequences to zebrafish genome. The MO1 sequence was 5′-CAGCAACATCTGAGAACAAGAGATT-3′ and st. control MO sequence was 5′-TATTAATAGATCGTGAGTTATTCGT-3′. Approximately 10 nL of MOs (50 μM) were injected into zebrafish 1-cell stage embryos. Concentration and injected volume of morpholino solutions were followed by former studies (29,30). If more than 60% of MO injected embryos—except for the dead individuals—showed same characteristics (the number of MO injected embryo was approximately 60–200 individuals), we recognized them as phenotypes caused by MO injection.

RESULTS

Whole-mount *in situ* hybridization The RNA probes were synthesized from a 535 bp template cDNA which contained the partial sequences in LBD (Fig. 1). Zebrafish *dax1* mRNA signals were detected in the central nervous systems including the forebrain, the midbrain

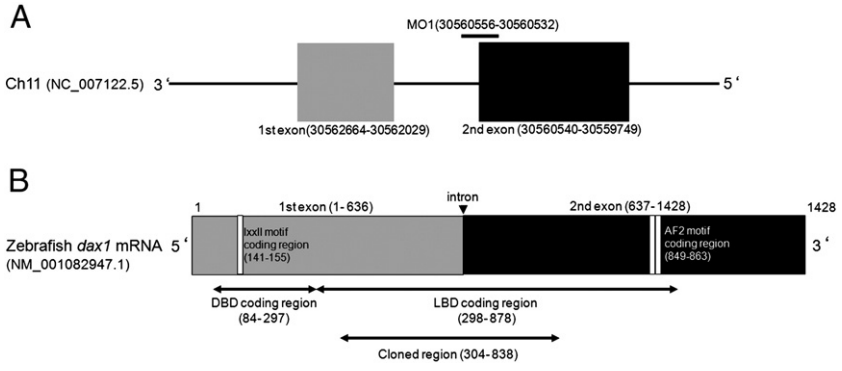

FIG. 1. Overview of the gene structure of zebrafish *dax1*. (A) Schematic representations of the exon and the intron of zebrafish *dax1* gene (Gene ID: 100001692) in the chromosome 11. The gray-colored region represents the 1st exon. The black-colored region represents the 2nd exon. MO1 that target zebrafish *dax1* is shown as short bars. MO1 targets intron–exon junctions. (B) Schematic representation of zebrafish *dax1* mRNA (GenBank accession number: NM_001082947.1). The gray-colored region represents the 1st exon. The black-colored region represents the 2nd exon. The arrowhead shows the position corresponding to an intron. The white vertical bar indicates lxxll motif coding region. The double white vertical bar represents an AF-2 motif coding region.