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Functional characterization of the zebrafish *WHSC1*-related gene, a homolog of human *NSD2*

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ABSTRACT

Methylation of specific lysine residues of histone H3 and H4 has been reported to be important in the structuring of chromatin and for the transcription of certain genes. Proteins with SET domains have been shown to methylate specific lysine residues of histone H3 and H4.

We isolated a SET domain-containing gene from the zebrafish (*Danio rerio*). The gene has the highest sequence similarity to human *NSD2*, also known as Wolf–Hirschhorn syndrome candidate 1 or *WHSC1*, and therefore, was named *DrWhsc1*. *DrWhsc1* mRNA is expressed in various tissues with the highest level in testis. Morpholino oligonucleotides for the *DrWhsc1* gene affected early embryogenesis in zebrafish, such as endbrain enlargement, abnormal cartilage, marked reduction of bone, and incomplete motor neuron formation. Such developmental abnormalities are also observed in Wolf–Hirschhorn syndrome patients and *Whsc1*-deficient mice. In addition, suppression of the *DrWhsc1* gene or defect in the SET domain of *DrWhsc1* resulted in impairment of di-methylation of histone H3K36 at early embryogenesis. These results indicate that *DrWhsc1* is a functional homolog of *WHSC1* and that the SET domain of *DrWhsc1* is essential for di-methylation of histone H3K36 in zebrafish.

1. Introduction

Modifications of histone proteins affect cell proliferation through transcriptional regulation. In heterochromatin, histone proteins are deacetylated by histone deacetylases, leading to repression of transcription. In euchromatin, histones are highly acetylated, and many transcription factors are recruited to the transcriptional initiation site to initiate transcription [1–3]. Histone proteins are not only modified by acetylation, but also by methylation, phosphorylation, ubiquitination, and sumoylation [4], and these modifications play important roles in chromosome condensation and DNA replication [5]. Especially, lysine methylation of histone proteins affects the transcription of certain genes [6]. Methylation of histone 3 lysine 4 residue (H3K4), H3K36, and H3K79 has been shown to cause transcriptional activation, whereas that of H3K9, H3K27, and H4K20 cause transcriptional repression [7]. Other lysine or arginine residues are also reported to be methylated by specific methyltransferase [4,8] and specific histone demethylases have also been reported [9–11].

Except for histone H3K79 methyltransferase, other histone H3 or H4 methyltransferases for specific lysine residues have a common SET domain [12]. The SET domain was originally identified in the polycomb family (PcG), thrithorax family (trxG), and Su(var) genes, and alterations of some of the SET domain-containing genes have been implicated in human diseases [13–17]. Previously, we reported that the nuclear receptor-binding SET domain protein 1 (*NSD1*) gene is responsible for Sotos syndrome which is characterized by overgrowth in childhood, specific craniofacial features, and mental retardation [14]. NSD1 protein contains one SET domain and other functional domains including PHD and PWWP domains, both of which are involved in protein–protein interaction. Furthermore, the recombinant protein containing the SET domain of NSD1 was demonstrated to have the ability to methylate H3K36 and H4K20, and embryos of NSD1-deficient mice exhibited marked apoptosis at E7.5-E8.0, indicating that the NSD1 protein is required for gastrulation [18].

NSD1-related genes *NSD2* and *NSD3* were also identified in human, and both contain a SET domain. Hemizygous deletion of *NSD2* is associated with Wolf–Hirschhorn syndrome characterized by severe growth retardation, mental delay, and microcephaly [15]. And mouse lines in which homologs of the Wolf–Hirschhorn syndrome gene (*Nsd2*) were deleted showed symptoms similar to human Wolf–Hirschhorn syndrome [19]. NSD2 (also known as MMSET

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or WHSC1) was demonstrated to possess histone H3K4 and H4K20 methyltransferase activity and function as a transcriptional corepressor in multiple myeloma [20]. Recently, murine Nsd2 (Whsc1) was reported to exhibit histone H3K36 trimethyltransferase activity in embryonic stem cells [21]. *NSD3* is expressed in human breast cancer cell lines [13]. One NSD3 isoform (also known as WHISTLE from WHSC1-like 1 isoform 9 with methyltransferase activity to lysine) is considered to have histone H3K4 and H3K27 methyltransferase activity [22].

In order to gain more insights into the physiological roles of *NSD*-related genes, we isolated one *NSD*-related gene from zebrafish. The cloned gene shows a high sequence similarity to human *NSD2*, or Wolf–Hirschhorn syndrome candidate 1 (*WHSC1*), and, because it is a *Danio rerio* (zebrafish) *WHSC1*-related gene, was designated *DrWhsc1*. To better understand the physiological roles of this gene, we examined the methylated lysine residues of histones and the eliminated the function of *DrWhsc1* in knockdown experiments.

2. Materials and methods

2.1. Isolation of a WHSC1-related gene from zebrafish

The sequence of the SET domain in the human *NSD1* gene was used to search the zebrafish EST database (UCSC Genome Bioinformatics; http://genome.ucsc.edu/), and fc12c04.x1 and fk47b04.y1 were identified as the EST cDNA clones with significant sequence similarity to human *NSD1* cDNA. Because the 5′ region of the open reading frame was missing in fc12c04.x1 and fk47b04.y1, we utilized 5′-RACE. The resulting 5′ region of the gene was ligated with the fc12c04.x1 or fk47b04.y1 to obtain full-length cDNA. The primers used for 5′-RACE were 5′-CATTTCCCTTGGGACCGGCGTCAAT-3′ and 5′-GCCACCTTTCTCTTCTGAGAGCCTTTT-3′. The cloned zebrafish cDNA was a zebrafish *WHSC1*-related gene, and therefore, was designated *DrWhsc1*.

The total RNA isolated from zebrafish embryos were a generous gift from Dr. K. Araki of the National Research Institute of Aquaculture, Fisheries Research Agency. EST clones (fc12c04.x1, and fk47b04.y1) were purchased from RZPD Deutsches Ressourcenzentrum fuer Genomforschung GmbH (Berlin, Germany). The 5′-RACE reaction was performed using a RACE kit from Invitrogen (Carlsbad, CA).

2.2. RT-PCR using total RNA isolated from zebrafish embryos and adult tissues

Fertilized eggs of zebrafish (*D. rerio*) were incubated at 28 °C for 8, 16, 24, 32, or 48 h and collected by brief centrifugation. Brain, testis, kidney, liver, spinal cord, embryo, heart, ovary, fin, regenerated fin, and gill samples were dissected from adult male and female zebrafish under anesthesia. Embryos and tissues were lysed with Trizol (Invitrogen), and total RNAs were extracted with chloroform followed by precipitation with isopropyl alcohol [23]. Single stranded-complementary DNA was synthesized with 0.1 μg of total RNA as the template and an oligo(dT) primer, and the resulting cDNAs were used for PCR. PCR was carried out with 30 cycles of consecutive reactions at 94 °C for denaturation, 60 °C for annealing, and 72 °C for elongation. RT-PCR products were analyzed by 1% agarose gel electrophoresis. The RT-PCR kit was purchased from Takara (Shiga, Japan).

2.3. Injection of morpholino oligonucleotides into zebrafish embryo

One-cell-stage fertilized zebrafish eggs were injected with morpholino oligonucleotides, which target the translation initiation site (ATG-MO) or SET domain region (SET-MO), and incubated for 48 h at 28 °C. The morpholino oligonucleotides and the five mismatched oligonucleotides used as negative controls were purchased from Gene Tools, LLC (Philomath, OR). The sequences of the morpholino oligonucleotides were 5′-GATCCCCCCTTACTGTCT GTCATTG-3′ for the translation initiation site and its negative control 5′-GATGCCGCCTTAGTGTGTGTGATTG-3′ and 5′-TCGGGTCTCT GAAATATAATGTCAC-3′ for the SET domain in the C-terminal region and its negative control 5′-TCCGGTGTCTCAAATATAAAGT GAC-3′.

2.4. Morphological analysis of zebrafish embryos

The zebrafish embryos were fixed with formalin followed by hydrogen peroxide to remove proteins and the cartilage and bone structures were visualized by staining with Alcian Blue following an established protocol [24].

To detect the motor neurons, the zebrafish embryos were treated with znp-1, a mouse monoclonal antibody (ZFIN: The Zebrafish Model Organism Databases, University of Oregon; http://zfin.org/action/antibody), followed by Alexa Fluor 594 conjugated anti-mouse secondary antibody. Transgenic zebrafish in which the promoter/enhancer of the *islet-1* gene expressed in motor neuron was fused to a green fluorescent protein (GFP) was also used to visualize the motor neurons, main axons and the peripheral branches within the muscles [25].

2.5. Preparation of acid extract from zebrafish embryos and western blotting using specifically methylated histone antibody

Zebrafish embryos were collected 1 or 2 days after fertilization, suspended in a 10-fold volume of lysis buffer containing 10 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, and 1.5 mM PMSF and agitated with microtube pestles, followed by the addition of HCl to obtain a final concentration of 0.2 M. After 30 min on ice, the supernatants were recovered by centrifugation at 11,000*g* for 10 min at 4 °C, dialyzed against 200 ml of 0.1 M acetic acid, and then against H_2O and aliquots of the extract were stored at −80 °C until use.

Acid extracts from the zebrafish embryos injected with or without morpholino oligonucleotides were separated by 15% SDS–polyacrylamide gel electrophoresis, electrophoretically transferred to a PVDF membrane and incubated with antibodies against mono-, di-, or trimethylated lysine residues of histone H3 or H4 protein, followed by the addition of horseradish peroxidase-conjugated anti-rabbit IgG. Immunoreacted proteins were detected using an ECL-plus reagent (GE Healthcare Bio-Sciences, NJ).

Upstate™ antibodies specific for histone H3 and mono-, di-, or trimethylated H3K4, H3K27, H3K36, H4K20 were purchased from Millipore (Billerica, MA).

3. Results and discussion

3.1. Characterization of the zebrafish gene which is homologous to the human Wolf–Hirschhorn syndrome candidate 1 (WHSC1) gene

The full-length cDNA which we isolated has one SET domain, two PWWP domains, and three PHD domains but contains no nuclear receptor binding domain (NID) (Supplementary Fig. 1A). A comparison of the amino acid sequences of the human NSD proteins showed that *DrWhsc1* had the highest sequence similarity to the protein encoded by human *NSD2* (or *WHSC1*) gene and that the sequence homology is particularly high in the region containing the SET domain (Supplementary Fig. 1B). RT-PCR showed that *DrWhsc1* mRNA was detected in most tissues of adult zebrafish;