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Transcriptional regulation and O-GlcNAcylation activity of zebrafish OGT during embryogenesis ☆

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Abstract

Zebrafish OGT (zOGT) sequence was identified in zebrafish (*Danio rerio*) genome and six different transcriptional variants of zOGT, designated *var1* to *var6*, were isolated. Here we describe the developmental regulation of zOGT variants at transcriptional level and characterization of their OGT activities of protein O-GlcNAcylation. OGT transcriptional variants in zebrafish were differentially generated by alternative splicing and in particular, *var1* and *var2* were contained by 48 bp intron as a novel exon sequence, demonstrating that this form of OGT was not found in mammals. Transcript analysis revealed that *var1* and *var2* were highly expressed at early phase of development including unfertilized egg until dome stage whereas *var3* and *var4* were begins to be expressed at sphere stage until late phase of development. Our data indicate that *var1* and *var2* are likely to be maternal transcripts. The protein expression assay in *Escherichia coli*–p62 system showed that OGT activities of *var3* and *var4* were found to be only active whereas those of other variants were inactive.

Keywords: z*O*-GlcNAc transferase; Transcriptional variants; *O*-GlcNAc; Developmental regulation

Nucleocytoplasmic protein O-GlcNAcylation is proceeded by *O*-GlcNAc transferase (OGT) and in mammalian cells, a variety of proteins have been known to be modified by single GlcNAc residue through the unique action of OGT [1–4]. Functional role of OGT has been known to be involved either directly or indirectly in many biological processes, such as dynamic interplay with phosphorylation of signaling factors [5–7], cytoskeletal organizations [8], and hexoseamine biosynthetic pathway [9,10]. OGT has been known to be localized both in cytosol and nucleus [11] either alone or as a complexed form with other molecules [7], and also in mitochondria [12].

Recently, it has been reported that OGT-mediated protein O-GlcNAcylation may function in cellular sensing of nutrients [13], toxic stresses [14], and heat [15]. Moreover, several studies showed that O-GlcNAcylation could be linked to oocyte maturation in xenopus [16,17]. However, the role of OGT during embryo development is poorly understood. During past several years, cDNAs encoding OGT have been cloned in mammalian species including human [4], rat [3], and mouse [18], and also reported in other species such as *Drosophila*, xenopus, and plant [19]. However, little information is available for zebrafish OGT (zOGT).

Zebrafish is now well utilized as a model system of vertebrates to identify gene-function during embryogenesis [20,21]. We searched the whole genome of zebrafish to find out a homolog of the mammalian OGT. Here, we show the zOGT sequence in genomic organization and its six different transcriptional variants, and demonstrate, for the first time, that these transcriptional variants of zOGT are differentially regulated during development. Furthermore, we expressed these transcriptional variants and analyzed their OGT activities in *Escherichia coli*–p62 system.

Materials and methods

Bioinformatics. The zebrafish genome search was initially performed using human OGT sequence (NM_181672) and rat OGT

☆ *Abbreviations:* OGT, *O*-GlcNAc transferase; zOGT, zebrafish *O*-GlcNAc transferase; GlcNAc, *N*-acetyl-glucosamine; hpf, hour post fertilization.

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sequence (NM_017107) as query by BLAST search programs at Zebrafish Genome Browser (www.ensemble.org/Danio_rerio). The genomic sequence (contig BX323828.7.1-206011 on chromosome 14) with the closest similarity to mammalian OGT sequence was finally identified and translated protein sequences were further searched by BLAST X.

Zebrafish embryo preparation. Zebrafish embryos were maintained and prepared as previously described in the Zebrafish Book [22]. Briefly, based on morphological criteria of embryos, developmental stages were classified as hour post-fertilization (hpf). After natural spawning, embryos were collected and cultured in fishwater system containing 0.2 mM 1-phenyl-2-thiourea (PTU) to prevent pigmentation and 0.01% methylene blue to prevent fungal growth.

Gene-specific primer sequences. Standard PCR amplifications were performed using the following oligonucleotides:

Primer 1: 5′-AAGGATCCACCATGGCGAGCTCGGTGGG-3′
Primer 2: 5′-AACTCGAGTCAGGTGCTCTCGCTGGTCTCC-3′
Primer 3: 5′-GAGCTCGGTGGGGAACGT-3′
Primer 4: 5′-CCAGTGTGCTGAAGTGAGC-3′
Primer 5: 5′-GAGCTCGGTGGGAAACGC-3′
Primer 6: 5′-GCATGGGGTTCTGCTTGG-3′
Primer 7: 5′-ATGGTCAAGAGGTTGCTGAC-3′
Primer 8: 5′-GTCACCACAATGGTTCTGG-3′
Primer 9: 5′-TGTGATGATCAAGTGGCG-3′
Primer 10: 5′-CGGGTGGTCACCACAATT-3′
Primer 11: 5′-TTGTAGTATCTGGGACTGTAG-3′
Primer 12: 5′-TGGCCACCACACAG…ATTAAC-3′
Primer 13: 5′-TGCCAAGCTTTACTGCAAC-3′
Primer 14: 5′-TGCCAAGCTTTACTGCAATG-3′
Primer 15: 5′-AGATCTGGCTGGCCATCC-3′
Primer 16: 5′-GGGAACACCTCCAGTGC-3′
Primer 17: 5′-GAGGAGCACCCCGTCCTGCTCAC-3′
Primer 18: 5′-GATGGCTGGAACAGGGCCTCTGG-3′
Primer 19: 5′-AAAGGATCCATGGCGTCTTCCGTGGGCAAC-3′
Primer 20: 5′-TTTGTCGACTTATGCTGACTCAGTGACTTC-3′
Primer 21: 5′-TTTGTCGACTCAGGCTGACTCAGTGACTTC-3′
Primer 22: 5′-CCGGATCCATGAGTGGGTTTAACTTTGG-3′
Primer 23: 5′-CCCAAGCTTCTAGTCAAAGGCAATGCGCAG-3′

Primer 1 (forward)/primer 2 (reverse) (primer 1/2) is for zOGT full length amplification, primer 3/4 is for zOGT *var1*, *3* (234 bp) and *var2*, *4* (204 bp) amplification, primer 5/6 is for zOGT *var5* (257 bp) and *var6* (227 bp) amplification, primer 7/8 is for zOGT *var1*, *2* (256 bp) and *var3*, *4* (208 bp) amplification, primer 9/10 is for zOGT *var5*, *6* (218 bp) amplification, primer 11/13 is for zOGT *var1*, *2* (546 bp) amplification, primer 12/13 is for zOGT *var3*, *4* (531 bp) amplification, primer 12/14 is for zOGT *var5*, *6* (531 bp) amplification, primer 15/16 is for zOGT all variants (457 bp) amplification, primer 17/18 is for zebrafish β-actin (500 bp) amplification, primer 19/20 is for human OGT (full length) amplification, primer 19/21 is for rat OGT (full length) amplification, and primer 22/23 is for rat p62 (full length) amplification.

DNA constructions. Total RNA was purified from zebrafish embryo samples which were collected during 17 developmental stages by RNAzol B (Tel-Test) and cDNA library was synthesized using random hexamers (Takara) by M-MLV reverse transcriptase (Promega) according to the manufacturer's instructions. Transcriptional variants of zebrafish OGT (zOGT) were cloned from cDNA library by PCR amplification using 50 pmol forward and reverse gene-specific primers, 200 μM dNTPs and 1.25 U exTaq (Takara-Shuzo) in the presence of 10 mM Tris–Cl (pH 8.3), 50 mM KCl, and 1.5 mM $MgCl_2$. PCR was carried out under the condition of 25 cycles of denaturation for 3 min at 94 °C, annealing for 30 s at 58–62 °C, and elongation for 30–40 s at 72 °C. zOGT *var1*, *2*, *5*, and *6* were cloned from cDNA of unfertilized egg, and zOGT *var3* and *4* were cloned from cDNA of 24 hpf embryo. Each variant of zOGT was cloned in pGEM-T easy vector (Promega) and further constructed in pMAL-c2 (New England Biolabs) for protein expression in *E. coli* system. cDNA sequences of human and rat OGT were cloned by RT-PCR using primer 19 (forward)/20 (reverse) with cDNA from H293 cells, and using primer 19/21 with cDNA from PC12 cells [15], respectively.

Transcript analysis of zOGT variants by RT-PCR. Six transcriptional variants of zOGT in 17 different developmental stages were analyzed using gene-specific primers by RT-PCR and quantitative RT-PCR. For analysis of total expression levels of zOGT, RT-PCR was performed using cDNAs of 17 stage-specific embryos and primer 15 (forward)/16 (reverse) resulting in 457 bp PCR product. Primer 11/13, and primer 12/13 were used for analysis of expression level of zOGT *var1* and *2* (546 bp), and for zOGT *var3* and *4* (531 bp), respectively. Finally, either primer 12/14 or primer 9/10 was used for analysis of expression level of zOGT *var5* and *6* (531 bp). Expression level of zOGT *var1* and *2* (256 bp), and zOGT *var3* and *4* (208 bp) was further analyzed by quantitative RT-PCR using primer 7/8 (Copy-I specific). Expression of exon 2a in Copy-I, and Copy-II genomic sequence was also analyzed by quantitative RT-PCR using primer 3/4, and primer 5/6, respectively (expected size of 234 and 257 bp in case of presence of exon 2a whereas expected size of 204 and 227 bp in case of absence of exon 2a). Primer 9/10 was used for competitive RT-PCR analysis for presence of exon 19 sequence on Copy-II genomic sequence (expected size of 218 bp in case of absence of exon 19 whereas expected size of 266 bp in case of presence of exon 19). For a control, zebrafish β-actin was amplified using primer 17/18 resulting in 500 bp PCR product.

Southern blot analysis of genomic DNA. Genomic DNA was prepared from zebrafish fin and muscle tissues as previously described [22]. Ten micrograms of genomic DNA was digested overnight with 50 U *Bam*HI (Roche) and then, digested DNA was separated on 0.8 % (w/v) agarose gel followed by ethidium bromide staining (0.5 μg/ml). Denatured DNA in gel was transferred to Hybond-N^+ nylon membrane (Amersham Biosciences) using a vacuum apparatus (Appligene). Hybridization probe was prepared by PCR using primer 11/13 and zOGT *var1* as a template. Resulting PCR product was digested with *Pst*I and *Kpn*I, and ^{32}P-labeled using [α-^{32}P]dCTP (3000 Ci/mmol) by Ready-To-Go DNA labeling bead (Amersham biosciences) followed by separation on microspin G-50 column (Amersham biosciences). Hybridization using radioactive probe was performed according to standard protocols as described [23]. Hybridization and washing steps were carried out under high stringency conditions. Radioactive signals were visualized with a BAS2000 Image Analyzer (Fuji Photo Film).

OGT activity assay in E. coli–p62 system. Assay for OGT activity in *E. coli*–p62 system was newly developed as follows (unpublished). Full-length cDNA of p62 in expression vector of pET28a(+) (Novagen) and variant cDNA of zOGT including human and rat OGT in expression vector of pMAL-c2 (New England Biolabs) were co-transformed to BL21 (DE3) codon plus-RIL cells (Stratagene) according to manufacturer's instructions. Bacterial clone containing two plasmids was selected in presence of both 100 μg/ml ampicillin and 50 μg/ml kanamycin. Bacterial cell lysates were subjected to 10% SDS–PAGE and immunoblot analysis for *O*-GlcNAcylated proteins was carried out using RL2 antibody (Alexis, Switzland) as previously described [15]. Expression levels of OGT and p62 were confirmed by immunoblotting using anti-MBP-OGT antibody (rabbit polyclonal antibody prepared in our laboratory) and T7 tag antibody (Novagen). Immuno-signals were developed on Super RX film (Fuji) using SuperSignal West Pico chemiluminescent substrates (Pierce).

Phylogenetic tree. Raw DNA sequences were aligned using CLUSTAL_X [24] and neighbor-joining analysis were carried out using MEGA 3 [25]. Sequences (GenBank Accession No.) available to generate the phylogenetic tree are as follows. *Homo sapiens* transcript variant 1, NM_181672 [26], *H. sapiens* transcript variant 2, NM_181673 [26], *H. sapiens* mitochondrial form, U77413 [27], *Mus musculus*, NM_139144 [28], *Rattus norvegicus*, NM_017107 [3], *Xenopus laevis*, BC082353 [29], *Xenopus tropicalis*, NM_001024576 (direct submission), and *Drosophila melanogaster*, AF217788 (direct submission). The aligned data set are reproducible and significant.