Characterization of N-Myristoyltransferases in Vertebrate Embryos by Using Zebrafish: Appearance of Low Molecular Weight N-Myristoyltransferase 1 in Early Development

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Abstract

For the first step to explore the biological characteristics of NMTs during vertebrate’s embryogenesis, we concentrated on zebrafish as a useful model of higher organisms. By in silico analysis of amino acid sequences, we found that zebrafish NMT1 (zNMT1) and zNMT2 which are translated from nmt1a and nmt2 genes had high homology to human or mouse NMT1 and NMT2, respectively. Expression analysis of nmt1a and nmt2 by RT-PCR and RNA blotting revealed that both genes were expressed during early development, and nmt2 expression was major initial developmental stages. Inhibition of zNMTs by 2-hydroxymyristic acid (2-OHMyr) at early development resulted in embryonic lethal. Morpholino antisense oligo against nmt1a caused development arrest in early epiboly stage. Those results suggest that zNMTs are necessary for development after early epiboly stage. The recombinant zNMT1 was then prepared and its N-myristoyltransferases activity was confirmed. Finally, we analyzed expression of zNMT1 proteins in embryos at several developmental stages, and found that low molecular weight zNMT1 (29 kDa), which included N-terminal part of zNMT1, appeared specifically during embryonic development. Expression of full length zNMT1 fused to myc-His tag at its C-terminal resulted in production of low molecular weight protein (35 kDa). Detection of intact NMTs in protein extract from early embryos showed that there existed low molecular weight NMTs with substrate binding activity. From these findings, we concluded that zNMTs, like mammalian one, are also essential for zebrafish development, and low molecular weight zNMT1 appeared specifically in early developmental stage. Those isoforms might play important role in developmental processes in early embryos.

Keywords: Zebrafish; N-myristoyltransferase; Gene expression development

Abbreviations: NMT: Protein N-myristoyltransferase; Src: Src tyrosine kinase; Lck: Lymphoid specific cytosolic protein tyrosine kinase; MARCKS: Myristoylated alanine-rich C-kinase substrate; ARF: ADP-ribosylation factor; HIV: Human immunodeficiency virus; Amp: Ampicillin; Km: Kanamycin; E. Coli: Escherichia coli; IPTG: Isopropyl β-D-1-thiogalactopyranoside; NUS: NusA protein; GST: Glutathion S transferase; MARCKSL: MARCK-like protein; EF-1α: Elongation factor-1 alpha

Introduction

The covalent lipid modifications on proteins are an important aspect of its functional regulation. The acylation by long fatty acid chain on amino acid residues in proteins alters biochemical characteristic of proteins, that is, conjugation of hydrophobic group heighten its affinity to the lipid membrane structures such as organelles or plasma membrane [1,2]. The lipid modified proteins targeting to plasma membrane appears their functions by interacting to other proteins nearly located the membrane. For example, recruiting of proteins involving specific intracellular signaling on cell membrane is often achieved by lipid modifications, and the effect enables rapid and effective signal transduction depending on the external environment. Due to this function, the cells finally achieve their proper cellular functions [1,2].

Three common lipid modifications (myristoylation of N-terminus glycine residue and palmitoylation or prenylation of serine and cysteine residue) are considered to be important for cellular functions [1]. Among these, myristoylation is recently received considerable attention, because the potential target amino acid sequence for myristoylation has been found in many signaling and regulatory proteins such as pp60csrc, pp56ck [3-8], CAMP-dependent protein kinase [9], MARCKS [10], G-protein α subunit [11], ARF protein [12], calcineurin [13] and HIV-1 matrix protein [14]. Further, the N-myristoylation on several proteins has been shown to be important for the biological events such as appearance of cellular functions [1,2], transformation [8] or production of the virus particles [14,15].

N-myristoyltransferases (NMTs) are group of enzyme that mediates protein amino terminal myristoylation. The NMTs recognize amino terminal consensus amino acid sequences like Gly-X-X-Thr/Ser and catalyze the covalent transfer of myristic acid from myristoyl-CoA to amino group of glycine residue. Orthologues are widely recorded in the database of eukaryote genome, found in the organisms including human [16], mouse [17], rat [18], bovine [19] and yeast [20]. The wide distribution of NMTs in eukaryotes suggests its importance for basic cellular function. There exist at least two genetically distinct forms but structurally similar NMTs, NMT1 and NMT2, in mammalian [16,21]. Although those two NMTs seem to exist in wide variety of vertebrates, its biochemical characteristics or functions are not much revealed. In case of mammalian, there is one report that NMT1 but not NMT2 is important for the development of mouse. However, the detail roles of NMT1 during embryogenesis did not addressed in that study, because the NMT1 knockout mice died between embryonic days 3.5 and 7.5 [22].

For the first step to explore the biological characteristics of NMTs during vertebrate’s embryogenesis, we concentrated on zebrafish as a...
useful model of higher organisms. This model animal has been used commonly in many researches for developmental biology. In this study, we first confirmed the existence of zebrafish NMT's (zNMTs) which has high homology to mammalian's NMTs, and examined their gene expression during embryogenesis. We next analyzed an effect of inhibition of zNMT1 on their embryogenesis. Finally, we analyzed expression of zNMT proteins at several embryonic stages. The results suggest that zNMTs like mammalian NMT are necessary for proper embryonic development of zebrafish. It was also suggested that novel low molecular weight zNMT1 appears specifically during embryogenesis. We think that those findings would be useful information for understanding NMT's function during development of higher organisms.

Materials and Methods

Zebrafish care and collection of eggs

Adult zebrafish were purchase from local provider and maintained in aquarium under artificial condition (28°C, light period for 14 hrs and dark period for 10 hrs). For collection of fertilized eggs, two female and three male fish were mated. Spawning was induced by switching from dark period to light period.

Cloning of zNMTs and plasmid construction for their expression in Escherichia coli

Total RNA from the zebrafish embryo at 6 hpf was reverse-transcribed with oligo-dT primer in a volume of 20 µl. Aliquots of 1 µl were used for amplification of zNMT1 or zNMT2 cDNA. Polymerase chain reaction was carried out with following forward and reverse primers: 5'-CCACCATGCGGATGAG-3' and 5'-TCACCTGCAGAACCAATCC-3' for zNMT1, and 5'-ATGGCGGAGACCGAGTCGCC-3' and 5'-TTACGTAAACAAAGGCAAC-3' for zNMT2, respectively. Amplification was done by 35 cycles of denaturation at 95°C for 30 sec, annealing at 50°C for 30 sec, and extension at 72°C for 90 sec. Amplified DNA fragments were purified by agarose gel electrophoresis and cloned into pT7Blue-T vector (Novagen, San Diego CA). The constructs (pT7blue-zNMT1 and -zNMT2) were analyzed by DNA sequencing and confirmed its identity to the sequences on public databases.

Expression of recombinant zNMTs

E. coli BL21 strain was transformed with pET50b-zNMT1, pGEX-zNMT1 and pGEX-zNMT2 vectors and cultured on the LB plate supplemented with 50 µg/ml of kanamycin (Km) or 50 µg/ml of ampicillin (Amp) at 37°C overnight. Several colonies were obtained, and one of them was transferred to 4 ml of LB broth with 50 µg/ml of Km or Amp. After incubation at 37°C for 16 hrs, aliquots of 20 µl was transferred to 2 ml of 2×YT broth, further cultured approximately for 2 hrs at 37°C until OD600nm reached 0.6. The expression of the recombinant protein (NUS-zNMT1, GST-zNMT1s and GST-zNMT2s) in BL21 was induced by addition of 100 µM of IPTG followed by culture for 16 hrs at 20°C or 37°C. E.coli were then collected from the medium by centrifugation at 5000 xg for 10 minutes, washed once with 1×PBS and bacterial cells were collected again by centrifugation. The target protein was extracted from the cells by ultra-sonication in 50 µl of 1% Triton-X 100 in PBS or 2×NMT reaction buffer [50 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 0.45 mM 2-mercaptoethanol, 1% Triton-X-100] or direct addition of 5×SDS-PAGE loading buffer [0.1 M Tris-HCl (pH 6.8), 5% SDS, 30% glycerol, 0.06% BPB, 5% 2-mercaptoethanol]. The protein extract was then centrifuged at 15000 xg for 15 min at 4°C, and separated into soluble and insoluble fractions. Each fraction was used for further analysis.

Purification of recombinant zNMT1 and preparation of the polyclonal antibodies against zNMT1

Antibodies against zNMT1 were obtained by immunization with purified recombinant NUS-zNMT1 fusion protein. The recombinant NUS-zNMT1 fusion protein was expressed in E. coli BL21 strain as described above, and collected as insoluble fraction. NUS-zNMT1 fusion was extracted from inclusion body with 3 M urea supplemented with 1% 2-mercaptoethanol at 4°C. NUS-zNMT1 protein was purified by Ni affinity chromatography with HisTrap HP column (GE healthcare Amersham Place, UK). Purified NUS-zNMT1 including its degraded peptides were collected and concentrated with AmiconR filter devices 5000 NMWL filter unit (Millipore). The target protein concentration of the sample reached to approximately 1 mg/ml. Immunization of rabbits with purified NUS-NMT1 was requested to Kerry (Wakayama Japan). As for titer check of anti-zNMT antibodies, the recombinant GST-zNMT1s and GST-zNMT2s were used. GST-zNMT1s and GST-zNMT2s were expressed as above and extracted from insoluble fraction with 5×SDS-PAGE sample buffer. The samples were accessed by SDS-PAGE, followed by immunoblotting with anti-zNMT antibodies.

Detection of NMT activity by MS analysis

The total volume of soluble fraction of E. coli protein extracts in 2×NMT reaction buffer was adjusted to 100 µl with water, then myristoyl-CoA Li salt (Sigma) and substrate peptide (GARASVLSK in 2×NMT reaction buffer was adjusted to 100 µl with water, then myristoyl-CoA Li salt (Sigma) and substrate peptide (GARASVLSK-16)-GARASVLSK (Sigma) were added, and the reaction was started by addition of 10 µl of recombinant GST-tagged zNMT1s and GST-zNMT2s. The reaction was incubated at 30°C for 30 min, and the reaction was stopped by addition of 5×SDS-PAGE loading buffer [0.1 M Tris-HCl (pH 6.8), 5% SDS, 30% glycerol, 0.06% BPB, 5% 2-mercaptoethanol]. The protein extract was then centrifuged at 15000 xg for 15 min at 4°C, and separated into soluble and insoluble fractions. Each fraction was used for further analysis.

Construction of the expression plasmid for myc- and His-tagged zebrafish NMT1

The nmt1a gene was amplified from pT7blue-zNMT1 template.

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DNA fragments | sense primer (5' → 3') | antisense primer (5' → 3')
--- | --- | ---
zNMT1 full (for pET50b) | ATGGCGGCACGCGGATGAGAATGAGAC | ATAGGGGCGCCTGACAGAAACAATCC
zNMT1 full | CGAGAATTCACATGGGCGGATGAG | ATAGGGGCGCCTGACAGAAACAATCC
zNMT1 deltaC1 | GCTATGAACTCGGAGAGGGCAGAGG | ATAGGGGCGCCTGACAGAAACAATCC
zNMT1 deltaC2 | GCGAGAATTCGCGGAGGACAGCGAGTC | ATAGGGGCGGCCTGACAGAAACAATCC
zNMT1 deltaN1 | GCTATGAACTCGGAGAGGGCAGAGG | ATAGGGGCGCCTGACAGAAACAATCC
zNMT1 deltaN2 | GCTATGAACTCGGAGAGGGCAGAGG | ATAGGGGCGCCTGACAGAAACAATCC
zNMT2 full | ATGCCAATTCGCGGAGAGGACGGAG | ATAGGGGCGCCTGACAGAAACAATCC
zNMT2 deltaC1 | ATGCCAATTCGCGGAGAGGACGGAG | ATAGGGGCGCCTGACAGAAACAATCC
zNMT2 deltaC2 | ATGCCAATTCGCGGAGAGGACGGAG | ATAGGGGCGCCTGACAGAAACAATCC
zNMT2 deltaN1 | GCTATGAACTCGGAGAGGGCAGAGG | ATAGGGGCGCCTGACAGAAACAATCC
zNMT2 deltaN2 | GCTATGAACTCGGAGAGGGCAGAGG | ATAGGGGCGCCTGACAGAAACAATCC

Table 1: Primer set for construction of recombinant zNMTs.

Expression and Purification of zNMT1-myc-his Protein

The pXI pro-zNMT1-myc-his pEFP and pXI pro-myc-his pEFP constructs were injected into embryos at one cell stage, the embryos were incubated for 6 hours after fertilization. Protein was extracted from 60 embryos, the extract was then mixed with Ni-Sepharose beads (GE healthcare) and incubated for 15 minutes at room temperature with stirring. The beads were then collected by centrifugation at 3000×g for 3 minutes, washed three times with 30 mM imidazole/PBS solution (pH 7.0). Finally, the proteins bound to the beads were eluted with 250 mM imidazole/PBS solution (pH 7.0). The samples were analyzed by SDS-PAGE followed by immunoblotting with anti-myc antibody.

SDS-PAGE, CBB staining and immunoblotting

The protein extracts were boiled with 2.5×SDS-PAGE loading buffer for 5 minutes. The samples were then separated on the 10-12.5% polyacrylamide gel with Tris-glycine SDS buffer (25 mM Tris, 192 mM glycine, 0.1% SDS). For CBB staining, the gels were then stained with Coomassie Brilliant Blue R-250 (CBB R-250 Wako Japan) for several minutes, removed excess CBB with decolorization buffer (25% methanol, 10% Acetic acid in water) until separated proteins were detected.

For immunoblotting, separated proteins by SDS-PAGE were transferred to PVDF membrane. The membranes were reacted with indicated antibodies as described previously. For reaction with primary antibodies, anti-zNMT antibodies (No.1 and No.2) were diluted 1:1000 in blocking solution, respectively and used. Peroxidase conjugated anti-rabbit IgG (GE healthcare) was used as secondary antibody. Bound antibodies were detected with DAB substrate or ECL plus detection reagents (GE healthcare) and chemiluminescence was captured by Light Capture II cooled CCD camera system (ATTTO Tokyo, Japan).

RT-PCR Analysis

Zebrafish embryos (20 embryos) in each developmental stage were homogenized in Qiazol (Qiagen, Valencia CA) and total RNAs were prepared according to the manufacturer’s instructions. Aliquots of total RNA (2 µg) were reverse-transcribed with using oligo dT primer in a volume of 20 µl. After reverse transcription, aliquots of 1 µl were used for PCR. PCR amplification of zebrafish N-myristoyltransferase 1 (NM_001020480: nmt1a), zebrafish N-myristoyltransferase 2 (NM_001020480: nmt2) and zebrafish β-actin 2 (NM_181601.3 : zbac2 ) were done with following primer sets: 5'-ACTCTCGACCTAGGAAAC-3' and 5'-GCCAAGCTCAGGAGGAAC-3' and 5'-GTAAATCTCCACCATCTCTCC-3' for zNMT1, and 5'-GCCAAGCTCAGGAGGAAC-3' and 5'-GCCAAGCTCAGGAGGAAC-3'.
Mammalian occurs in uterus, detail observation and analysis of NMTs until zNMT1 and zNMT2 RNA was detected.

Challenging with 2-hydroxomyristic acid

Approximately 10 fertilized eggs were distributed in 24 well plate. 20 μM 2-hydroxomyristic acid (MP biomidicals, LLC) or myristic acid (Sigma-Aldrich) was added to each well at several time points. The embryos were incubated at 27°C until 24 hpf and living and dead embryos were counted under microscope.

RNA Blotting

The NMT1 specific sense and anti-sense digoxigenin labeled RNA probes were prepared by in vitro transcription kid (DIG RNA labeling kid, Roche Diagnostics, Basel, Switzerland). The pT7blue vectors encoding 410-975 or 1217-1447 nucleotides region of zNMT1 cDNA and 177-443 or 912-1200 nucleotides region of zNMT2 cDNA were used as template for in vitro transcription. Total RNA was prepared from zebrafish embryos at 6 hpf and aliquots of 30 μg were digested in 50% formamide, denatured at 75°C for 10 minutes. After immediate cooling on ice, RNA was separated by electrophoresis on 1% agarose gel containing 2% formaldehyde in MOPS buffer [20 mM MOPS, 5 mM sodium acetate 660 mM formaldehyde (pH 7.0)]. The separated RNA was transferred to nylon membrane (Hybound-N GE healthcare) by capillary blotting with 10×SSC buffer (Sigma) for 19 hrs. The signal of zNMT2 RNA was weaker than that of zNMT1 RNA. RNA blotting with RNA from 6 hpf embryos showed that zNMT1 and zNMT2 RNA was detected as single band, suggested that expression of zNMTs start initial developmental stage. Therefore, zNMTs, like mammalian’s one, are exist in mammalian, its orthologues proteins only exist in fish and invertebrates.

Morpholino Experiments

Antisense morpholino oligo against zNMT1 was purchased from Gene Tools LIC (Philomath, OR). The MO sequences of zNMT1 and control were 5'-tggctgccttcacgctcag-3' and 5'-gtaccgctcttcacttcgct-3', respectively. MOs at a concentration of 50 ng/μl were injected into embryos at the one- to two-cell stage.

Gene expression of zNMTs in the embryos at early developmental stage

To investigate the expression of zNMT1 and zNMT2 gene on zebrafish development, we performed RT-PCR analysis and RNA blotting. In RT-PCR analysis, the expression of zNMT1 and zNMT2 was observed even in the embryos at 4 hours post-fertilization (4 hpf), both expression continued to at least 72 hpf (Figure 3A). More detail analysis of zNMTs’ expression by RT-PCR showed that expression of zNMT1 and zNMT2 was observed even after 2 hpf and the expression was kept during early development. In initial stages (1-3 hpf) nmt2 expression seems to be more evident than that of nmt1a (Supplemental Figure 1). RNA blotting with RNA from 6 hpf embryos showed that zNMT1 and zNMT2 RNA was detected as single band, suggested that both zNMT1 and zNMT2 RNA did not have splicing variants. The signal of zNMT2 RNA was weaker than that of zNMT1 RNA (Figure 3B). These data suggest that expression of zNMTs start initial developmental stage. Therefore, zNMTs, like mammalian’s one, are suggested to play an important role in early stage of embryogenesis.

Results

In silico analysis shows existence of zebrafish N-myristoyltransferases (zNMTs) with high homology to mammalian’s NMTs

Knock out of mouse N-myristoyltransferase 1 (NMT1) gene resulted in embryonic lethal [22], suggesting importance of NMT1 for development of mammalian. However, due to the embryogenesis of mammalian occurs in uterus, detail observation and analysis of NMTs in embryos is difficult. Thus, we have concentrated on a model animal, zebrafish, which can easily observe and analyze their embryonic development.

When we study an organism as a model of mammalian, most important thing, however, is whether the organism has a similar gene to mammalian. To identify the zebrafish N-myristoyltransferase (zNMT) which has homology to mammalian’s one, we first searched NMTs on several databases, and found three N-myristoyltransferases (NP_001018316, NP_001156321 and NP_001186683). NP_001018316 (N-myristoyltransferase 1a) and NP_001186683 (N-myristoyltransferase 2) proteins were predicted to be orthologues of human NMT1 and NMT2, respectively. On the other hand, NP_001156321 (N-myristoyltransferase 1b) does not exist in mammalian, its orthologues proteins only exist in fish and invertebrates.

Inhibition of zNMT1 causes development arrest and decreases viability of embryos in early developmental stages

To examine a function of zNMTs during embryogenesis, we first analyzed the effects of NMT inhibitor, 2-hydroxymyristic acid (2-OHMyr) on zebrafish. The 2-OHMyr or myristic acid (Myr) was arrested at dome or early epiboly...
Figure 1: Comparison of the amino acid sequences of NMT orthologues. Amino acid sequences of zebrafish NMT1 and NMT2 (NP_001018316, NP_001186683), human NMT1 and NMT2 (NP_066565, NP_004799), mouse NMT1 and NMT2 (NP_032733, NP_032734), fruit fly NMT (NP_523969) and yeast NMT (NP_013296) were aligned by using CLUSTAL W online program. Conserved amino acids were highlighted gray and light gray.
stage compared with no-injection or control MO injection. These data suggests that zNMT1a is essential for development of zebrafish; other NMTs (zNMT2 or zNMT1b) could not rescue the function of zNMT1a.

**Recombinant zNMT1 exhibited myristoyltransferase activity**

For the purpose of confirming enzymatic activity of nmt1a gene product (zNMT1), we next carried out production of recombinant zNMT1 in *E. coli*. As shown in Supplemental Figure 2, NUS-zNMT1 fusion protein (about 120 kDa) was induced in *E. coli* with IPTG. The NUS-NMT1 partially existed in soluble fraction when induced at 25°C for 6 hrs. Up to 50% of recombinant zNMT1 were produced as soluble protein when induced at 20°C O/N (data not shown).

We next attempted to detect the NMT1 activity in unpurified bacterial lysate (Figure 6A). Peptide substrate (GARASVLSK-biotin), *E. coli* lysate and myristoyl-CoA were mixed and reacted at 30°C for 1 hour. Reacted peptide was then analyzed by MS spectrum. Mass spectrum of substrate peptide reacted with bacterial lysate containing NUS tag showed one major peak (m/z 1114.6617) derived from substrate peptide (Figure 6A). On the other hand, MS of reacted peptide with the lysate containing NUS-zNMT1 fusion protein contained another extra peak (m/z 1324.8723) corresponding to the MS of myristoyl-GARASVLSK-biotin peptide. Amino acid sequences of these peaks were further analyzed by MS/MS analysis and confirmed they were derived from substrate peptide (data not shown).

Purification of soluble zNMT1 was then attempted. However, degradation of recombinant protein was observed during purification process, small amount of zNMT1 was finally obtained (Figure 6B). With this purified protein, we attempted to detect substrate binding activity of recombinant zNMT1. As shown in Figure 6C, the recombinant protein was binding its substrate peptide without depending on the existence of myristoyl-CoA. In contrast, pre-myristoylated substrate peptide did not bind to the recombinant protein in the presence of myristoyl-CoA. These data suggest that NUS-zNMT1 has enzymatic activity characteristic of NMT, thus nmt1a gene encodes functional protein.

**Low molecular weight zNMTs is produced specifically in early developmental stages**

We next developed the antibody against zNMT1, the zNMT1 protein in zebrafish embryo was then determined by immunoblotting (Figure 7). To confirm the reactivity of the anti-zNMT1 serum, GST-zNMT1 (full length), deltaC1 (1-487 aa), deltaC2 (1-273 aa), deltaN1 (94-491 aa), deltaN2 (240-491 aa) and GST-zNMT2 (full length), deltaC1 (1-491 aa), deltaC2 (1-293 aa), deltaN1 (94-491 aa), deltaN2 (240-491 aa) were expressed in *E. coli* and whole cell lysates from the bacterial cell were used for immunoblotting with the antiserum. As shown in supplemental

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**Table 2:** The number of total and dead embryos when challenged with 2-hydroxymyristic acid.

| Addition time | 2 | 4 | 6 | 8 | 10 | 12 | 14 | 16 hpf |
|---------------|---|---|---|---|----|----|----|-------|
| Experiment 1  | Myr 5(5)  | 6(6)  | 7(6)  | 7(5)  | n/a  | n/a  | n/a  | n/a  |
| Experiment 2  | Myr 10(1) | 10(0) | 9(1)  | 11(2) | 11(1) | n/a  | n/a  | n/a  |
| Experiment 3  | Myr 7(7)  | 9(9)  | 10(5) | 10(2) | 10(1) | n/a  | n/a  | n/a  |
| Experiment 4  | Myr 10(4) | 10(8) | 10(9) | 10(7) | 10(2) | 11(4) | 9(2) | 10(3) |

**Figure 2:** Phylogenic tree of NMT orthologues. Phylogenic tree was drawn by Jalview ver. 2.6.1 soft (http://www.jalview.org/). According to the amino acid identity of zebrafish NMT1 and NMT2 (NP_001018316, NP_001186683), human NMT1 and NMT2 (NP_066565, NP_004799), mouse NMT1 and NMT2 (NP_032733, NP_027341), fruit fly NMT (NP_523699) and yeast NMT (NP_013296). The position of zebrafish NMT1 and NMT2 were underlined. Protein clusters of vertebrate NMT1 and NMT2 were shown by red and green squares. Average distance of each protein was calculated and expressed.

**Figure 3:** Gene expression analysis of endogenous zNMT during embryogenesis. (A) Total RNA was prepared from embryos at 6 hpf and aliquots of 30 μg were separated on agarose gel containing formaldehyde. The RNA were then transferred to nylon membrane by capillary blotting, hybridized with DIG-labeled RNA probes which hybridize specifically to zNMT1 and zNMT2 mRNA. The hybridized probes were detected with AP conjugated anti-DIG antibody and BM purple AP substrate. (B) Total RNA was prepared from the embryos at indicated stages and cDNAs were reverse-transcribed. 30 cycles of PCR for zNMT1, zNMT2 and actin 2 were performed, amplification of those cDNAs were analyzed by agarose gel electrophoresis followed by EB staining.
Figure 3, anti-zNMT1 serum recognized specifically with recombinant full length zNMT1 protein, deltaC1 and deltaC2 zNMT1 mutants, indicates that the epitopes recognized by anti-zNMT1 serum exist near amino-terminal. Then, we prepared embryonic proteins extracted from 2 to 72 hpf embryos and reacted with anti-zNMT1 serum by immunoblotting. The result revealed that the approximately 29 and 65 kDa zNMT was detected specifically during early embryonic stages and approximately 50-67 kDa zNMTs were detected in 48 and 72 hpf (Figure 7A and Supplemental Figure 4). These results suggest that zNMT1 has several isoforms with different molecular weight, and that low molecular weight (29 kDa) zNMT1 isoform appeared specifically in early embryos.

Considering from the reactivity of zNMT1 antiserum, we expected that the 29 kDa zNMT1 detected in early developmental stages included an amino-terminal part of zNMT1 peptide which is probably produced from full length zNMT1 gene. Therefore, we next expressed tagged zNMT1 in early developmental stage (Figure 7B). Full length zNMT1 fused to myc-His tag at its C-terminal was expressed in the embryos at 6 hpf, the protein extracted from the embryos was purified with Ni-Sepharose beads. Immunoblotting of purified zNMT1-myc-His with anti-myc antibody revealed that its molecular weight became smaller (35 kDa) than expected from the full length zNMT1s amino acid sequence (58 kDa). Intact NMT in protein extract from 6 hpf embryos was then detected by substrate binding assay and found that main bands were detected in low molecular weight (30 kDa and 33 kDa). The NMTs in early embryo could be detected by using other potential NMT substrate peptides originated from EF-1α, MARCKS, MARCKS-L, Src (Supplemental Figure 5). These findings suggest that potential NMT substrate peptides originated from EF-1α, MARCKS, MARCKSL, Src (Supplemental Figure 5). These facts implied that the 29 kDa zNMT1 detected in early developmental stages is essential for embryonic development of mouse [22], and inaccessibility of mammalian’s one.

Thus, it is important to study with enzyme which has close relation to NMT of Saccharomyces cerevisiae was used as model for NMT and well examined biochemically, probably because the enzyme in yeast can be prepared and purified easily [6,18,20]. Those studies addressed the protein structure and biochemical characteristics of yeast NMT, provided a good understanding of a NMT enzyme [25-27]. However, the yeast NMT does not have high homology to mammalian’s one (Figure 1 and 2). Some studies described different activity of rat NMT from yeast NMT to same substrate peptide [18,28]. These facts implied distinct characteristics of mammalian’s NMTs from yeast NMT, thus it is important to study with enzyme which has close relation to mammalian’s one.

To elucidate the physiological functions of mammalian’s NMTs, the NMT1 deficient mouse was established previously and their phenotype was analyzed. The report implied that NMT1 but not NMT2 is essential for embryonic development of mouse [22]. The detail roles of NMT1 in development, however, are largely unknown because of lethality of NMT1 KO mice [22] and inaccessibility of mammalian’s embryos.

In our study, we concentrated on zebrafish as a useful model for developmental studies to elucidate the functions of NMT1 during embryogenesis. The usefulness of this model is availability of a large number of embryos at one time, external fertilization, rapid development and easier observation of their embryonic processes. In

**Discussion**

Although N-myristoyltransferases (NMTs) are expected to have important roles in development of mammalian [22], its characteristics during development have not been revealed. In previous studies, the NMT of Saccharomyces cerevisiae was used as model for NMT and well examined biochemically, probably because the enzyme in yeast can be prepared and purified easily [6,18,20]. Those studies addressed the protein structure and biochemical characteristics of yeast NMT, provided a good understanding of a NMT enzyme [25-27]. However, the yeast NMT does not have high homology to mammalian’s one (Figure 1 and 2). Some studies described different activity of rat NMT from yeast NMT to same substrate peptide [18,28]. These facts implied distinct characteristics of mammalian’s NMTs from yeast NMT, thus it is important to study with enzyme which has close relation to mammalian’s one.

To elucidate the physiological functions of mammalian’s NMTs, the NMT1 deficient mouse was established previously and their phenotype was analyzed. The report implied that NMT1 but not NMT2 is essential for embryonic development of mouse [22]. The detail roles of NMT1 in development, however, are largely unknown because of lethality of NMT1 KO mice [22] and inaccessibility of mammalian’s embryos.

In our study, we concentrated on zebrafish as a useful model for developmental studies to elucidate the functions of NMT1 during embryogenesis. The usefulness of this model is availability of a large number of embryos at one time, external fertilization, rapid development and easier observation of their embryonic processes.
addition to these advantages, the information about zebrafish genome is recently available on the web site such as ZFIN or Ensembl. For the first step to investigate NMTs in zebrafish, we searched database and found three transcript of zebrafish NMTs (zNMTs) (nmt1a, NM_001020480, nmt1b, NM_001162849 and nmt2, NM_001199754). As we expected, nmt1a and nmt2 product (NP_001018316 and NP_001186683) had significant high homology to human or mouse NMT1 and NMT2, respectively (Figure 1 and Figure 2), and nmt1a product had N-myristoyltransferase activity (Figure 6). The nmt1a and nmt2 were expressed in embryos at early developmental stages (at least in 2 hpf embryos) and the expression lasted during embryogenesis (Figure 3 and Supplemental Figure 1). These data suggest that zNMTs play pivotal roles in embryonic development of zebrafish like mammalians’ one. Consequently, zebrafish can be a good substitute for mammalian studies for NMT’s roles during development. A NMT inhibitor, 2-hydroxymyristic acid (2-OHMyr) impaired the embryonic development (Figure 4 and Table 2). Inhibition of NMTs revealed that inhibition of NUS tag affinity chromatography. The purified sample was reacted with HRV3C protease to removal of NUS tag. Purification of zNMT1 at each process was confirmed by SDS-PAGE followed by CBB staining. (C) The purified recombinant zNMT1 protein was reacted with substrate peptide labeled with biotin as indicated, after linkage between zNMT1 and its substrate peptide. The zNMT1-substrate complex in each samples were analyzed by SDS-PAGE followed by immunoblotting with anti-biotin antibody.

Figure 6: Enzymatic activity of nmt1a gene product. (A) The peptide substrate (GARASVLSK-biotin), myristoyl-CoA and bacterial cell lysate containing NUS-zNMT1 or NUS tag were mixed and reacted at 30 °C for 1 hour. After collection of the reacted peptide, MS of the peptide were acquired. (B) The recombinant NUS-zNMT1 protein in E. coli lysate was purified by His tag affinity chromatography. The purified sample was reacted with HRV3C protease to removal of NUS tag. Purification of zNMT1 at each process was confirmed by SDS-PAGE followed by CBB staining. (C) The purified recombinant zNMT1 protein was reacted with substrate peptide labeled with biotin as indicated, after linkage between zNMT1 and its substrate peptide. The zNMT1-substrate complex in each samples were analyzed by SDS-PAGE followed by immunoblotting with anti-biotin antibody.

In conclusion, we have shown in this study that zebrafish NMT (zNMTs) has high homology to human or mouse NMTs and those NMTs are essential for their embryonic development like mammalians’ case. These findings clearly show that zebrafish can be a substitute for mammalian NMTs to study the role of NMTs in embryonic development.
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**Figure 7:** Detection of endogenous zNMTs during early development. (A) The embryos at 2-72 hpf were homogenized in 5×SDS-PAGE sample buffer and the protein in the extracts were separated by SDS-PAGE followed by immunoblotting with antiserum against zNMT1. Alternatively, the protein extracts from 6 hpf and 72 hpf were assessed by immunoblotting with antiserum against thiodexosin (Txn) as a negative control. Arrows showed specific bands detected by the antiserum. (B) The zNMT1-myc-His protein was expressed in 6 hpf embryos and total proteins were extracted. The zNMT1-myc-His was purified with Ni-Sepharose beads; the protein was assessed by immunoblotting with anti-myc antibody. (C) The protein extracts were prepared from embryos at 6 hpf. The embryonic lysate was reacted with biotinylated substrate peptide or biotinylated N-myristoylated substrate peptide in presence or absence of myristoyl-CoA (mCoA) as indicated. The substrate peptides linked to proteins were detected by SDS-PAGE followed by immunoblotting with anti-biotin antibody (asterisks).

model for mammalian when analyzing a function of NMTs during development. We have also found that N-terminal part of zNMT1 (29 kDa) appeared specifically during embryogenesis. These findings suggest that N-terminal part of zNMT1 is cleaved by post-translational N-myristoylation.

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