The *nme* gene family in zebrafish oogenesis and early development

T. Desvignes · C. Fauvel · J. Bobe

Received: 17 December 2010 / Accepted: 26 February 2011 / Published online: 11 March 2011
© Springer-Verlag 2011

Abstract After the recent report of the expression of several *nme* genes in the zebrafish gonads, the present study aimed at further analyzing the expression of *nme* genes in the ovary with special attention for the *nme* transcripts that are maternally inherited and could thus participate in the determination of oocyte developmental competence. The expression levels of all groups I and II *nme* genes were characterized by QPCR in a panel of zebrafish tissues. The *nme* genes exhibiting an ovarian expression were subsequently monitored throughout oogenesis and early development, and their expression sites characterized using in situ hybridization. Here, we show that *nme2b1*, *nme3*, *nme4*, and *nme6* are highly expressed in the ovary and present in the zebrafish oocyte throughout oogenesis. While the four transcripts are maternally inherited, *nme3* and *nme6* display a typical maternal profile and are detected in the zebrafish early embryo. In contrast to *nme3*, *nme6*, abundance exhibits a sharp decrease during early embryogenesis. After zygotic genome activation, we observed an increased expression of *nme2b1*, *nme2b2*, *nme3*, and *nme6*. The present study provides a comprehensive overview of the expression of *nme* family members during zebrafish oogenesis and early development. In addition, the maternal origin of two *nme* transcripts in the early embryo is reported here for the first time in any vertebrate species. Together, our observations suggest an important role of the *nme* family in oocyte and embryo development in vertebrates.

Keywords NDPK · *Nm23* · Oocyte · Maternal · Teleost · Vertebrate

Abbreviations

Hpf Hour post-fertilization
BSA Bovine serum albumin
MZT Maternal-to-zygotic transition
MBT Mid-blastula transition

Introduction

The *Nme* gene family is involved in multiple physiological and pathological processes such as cellular differentiation, development, metastatic dissemination, and cilia functions (Boissan et al., 2009; Biggs et al., 1990). Nme proteins can be separated in two different groups—groups I and II—based on their nucleoside diphosphate kinase (NDPK) activity and evolutionary history (Boissan et al., 2009; Desvignes et al., 2010). While genes of the group II did not undergo major evolutionary events in vertebrates, genes of the group I underwent several independent duplications in the vertebrate lineage. For instance, mammalian *Nme1*, *Nme2*, *Nme3*, and *Nme4* genes are co-orthologs of *Awd*, the only group I *Nme* gene found in the *Drosophila* genome (Desvignes et al., 2010). Similarly, both mammalian *Nme1* and *Nme2* genes are co-orthologs of zebrafish *nme2a*, *nme2b1*, and *nme2b2* genes (Desvignes et al., 2009). Despite the known importance of *Nme* genes in several human pathologies and their use as clinical markers of tumor aggressiveness, their role in non-cancerous physio-
logical processes has, in contrast, received far less attention. The major role of the group I Nme gene awd during *Drosophila* oogenesis and development has been thoroughly documented (Timmons and Shearn, 2000; Woolworth et al., 2009). In contrast, the role of Nme proteins in oogenesis and development has been poorly documented in vertebrates. However, the expression of specific nme genes during development has been reported in several mammalian (Lakso et al., 1992; Amrein et al., 2005; Carotenuto et al., 2006; Postel et al., 2009) and non-mammalian species (Ouatas et al., 1998; Murphy et al., 2000; Bilitou et al., 2009). More recently, we reported an intriguingly high expression of several nme genes in zebrafish gonads, and more specifically, in the ovary (Desvignes et al., 2009). In vertebrates, early development relies on maternal gene products stored into the egg during oogenesis, as the genome of the embryo does not start to produce its own RNAs until the maternal-to-zygotic transition (MZT) (see Tadros and Lipshitz, 2009 for review). In mammals, MZT occurs after a few embryonic divisions, while it occurs during mid-blastula transition (MBT) in teleost fish and amphibians. In zebrafish, MBT occurs around 3–4 h post-fertilization (Kane and Kimmel, 1993) but is more progressive than initially thought (Mathavan et al., 2005). Before this transition, all developmental processes are driven and supported by maternal gene products, RNA and proteins, that are stored into the oocyte during oogenesis (Pelegri, 2003; Dosch et al., 2004; Abrams and Mullins, 2009). Post-MBT development is also, but to a lesser extent, still dependent on maternally inherited gene products, (Wagner et al., 2004). In this context, fertilization and developmental success of zebrafish embryos are dependent on the storage and processing of RNA and proteins that occurs in the oocyte prior to fertilization throughout oogenesis. The dynamic of gene expression in the oocyte during oogenesis is thus important to study in order to gain insight into the molecular mechanisms that determine oocyte developmental competence (i.e., oocyte quality) (Bobe and Labbe, 2010).

After the recent report of the expression of several nme genes in the zebrafish gonads, the present study aimed at further analyzing the expression of nme genes in the ovary with special interest for the nme transcripts that are maternally inherited and could thus participate in the determination of oocyte developmental competence. The present study also aimed at characterizing the dynamic profiles of maternally inherited nme transcripts in the early embryo, prior to the activation of the embryonic genome. In order to achieve these goals, the expression levels of all groups I and II nme genes were characterized in a panel of zebrafish tissues. Nme genes exhibiting a significant ovarian expression were subsequently monitored throughout oogenesis and their expression sites characterized using in situ hybridization. For the nme transcripts exhibiting a maternal profile, transcript abundance was subsequently monitored during early embryonic development.

**Materials and methods**

**Animals and sampling**

Investigations were conducted according to the guiding principles for the use and care of laboratory animals and in compliance with French and European regulations on animal welfare (DSV approval #35-31). All zebrafish, *Danio rerio*, were obtained from the fish rearing facility at INRASCRIBE (Rennes, France). For all tissue collections, male or female zebrafish were anesthetized and subsequently killed by head sectioning. Tissues were then sampled, snap-frozen in liquid nitrogen and stored at −80°C until RNA extraction. For the tissue distribution study, tissues and organs were collected from three mature female zebrafish. In addition, testis was sampled from three mature males. For the oogenesis study, stages I to IV ovarian follicles were dissected from ovaries collected from four mature zebrafish females according to developmental stages defined by Selman et al. (1993). After removal from the body cavity, ovaries were subsequently incubated for 5 min in a Petri dish containing Hank’s full-strength solution (Westerfield, 2000) supplemented with Collagenase (0.35 mg/ml), then washed three times in large volume of Hank’s full-strength solution containing BSA (0.5 mg/ml). Ovarian follicles were then separated manually using forceps. Stage V oocytes were obtained from ovulating females by gentle manual stripping.

For the early development study, fertilized eggs were collected within 5–10 min of fertilization. For each batch, 15–20% of the eggs were kept to assess developmental success and used to monitor survival at 24 hpf and hatching rate. In order to analyze the developmental sequence of embryos originating from developmentally competent eggs only egg batches exhibiting hatching rates above 80% were kept for further analysis. Remaining eggs of each batch were serially sampled at fertilization (0.25 hpf), 1 cell (0.75 hpf), sphere stage (4 hpf), shield stage (6 hpf), and 24 hpf, according to previously described developmental stages (Kimmel et al., 1995). For in situ and whole-mount in situ hybridization, ovaries or embryos were fixed overnight in 4% paraformaldehyde (PFA) at 4°C and then transferred in 100% methanol after three successive short incubations in methanol solution of increasing concentrations and subsequently conserved at −20°C until further processing.

**PCR analysis**

For each sample, total RNA was extracted using Tri-Reagent (Molecular Research Center, Cincinnati, OH,
USA) according to the manufacturer's instructions. Reverse transcription (RT) was performed using 2 μg of RNA for each sample with M-MLV reverse transcriptase and random hexamers (Promega, Madison, WI, USA). Briefly, RNA and dNTPs were denatured for 6 min at 70°C, chilled on ice for 5 min before the RT master mix was added. RT was performed at 37°C for 1 h and 15 min followed by a 15-min incubation step at 70°C. Control reactions were run without reverse transcriptase and used as negative control in the real-time PCR study. Quantitative RT-PCR (QPCR) experiments were performed using an Applied Biosystems StepOne Plus. RT products, including control reactions, were diluted to 1/25, and 4 μl were used for each PCR. All QPCR were performed in triplicates. QPCR was performed using a real-time PCR kit provided with a Fast-SYBR Green fluorophore (Applied Biosystems) with 100, 200, or 300 nM of each primer in order to keep PCR efficiency between 90% and 100% for all target genes. In order to avoid unspecific signal due to genomic DNA contamination, primers (Table 1) were designed on exon boundaries whenever possible. The relative abundance of target cDNA within a sample set was calculated from serially diluted cDNA pool (standard curve) using Applied Biosystem StepOne™ V.2.0 software. After amplification, a fusion curve was obtained to validate the amplification of a single PCR product. The fusion curves obtained showed that each primer pair used was specific of a single nme transcript. The negative control reactions were used to estimate background level. Genes were considered significantly expressed when measured level was significantly above background at $p<0.05$ and within the range of the standard curve.

Tissue specificity

For each studied tissue, cDNA originating from three individual fish were pooled and subsequently used for real-time PCR. Before further analysis, real-time PCR data were collected using the same detection threshold for all studied genes. Data were subsequently normalized using the $ΔΔ\text{Ct}$ method to 18S transcript abundance in samples diluted to 1:2,000.

Oogenesis and early development specificity

For oogenesis and early development samples, 9 pg of exogenous luciferase RNA (Promega) was added per oocyte/embryo to the sample prior to homogenization in Tri-reagent. QPCR data were analyzed using the same detection threshold for all studied genes. Data were subsequently normalized using the $ΔΔ\text{Ct}$ method to the exogenous luciferase transcript abundance in samples diluted to 1:25.

In situ and whole-mount in situ hybridization

Digoxigenin-labeled anti-sense RNA probes were produced using the Promega SP6/T7 RNA polymerase Riboprobe Combination System following manufacturer's instructions, using as DNA template a PCR product. Bacterial clones containing, nme2b1 (GenBank Acc# FDR202-P00003-BR_F18), nme3 (GenBank Acc# FDR103-P00026-BR_E16), nme4 (GenBank Acc# FDR306-P00036-BR_P09), and nme6 (GenBank Acc# FDR202-P00041-BR_C14) inserts were obtained from the Genome Institute of Singapore. For nme3, nme4, and nme6 the PCR product used to generate the probe corresponds to the full-length clone. Due to high similarities between the three nme2 transcripts, the nme2b1 anti-sense probe corresponds to the 3′UTR of the RNA sequence (nuc # 478 to 624 of RefSeq NM_130926) that was generated using a specific forward primer (GCACAGCAGTGACATCA) and M13 reverse primer. nme2b1 sense probe was synthesized on the full-length clone. For all plasmids, insert sequence was checked by sequencing. Digoxigenin-labeled riboprobes were then purified by precipitation in ammonium acetate 7.5 M/ethanol

### Table 1 QPCR primer sequences and accession numbers of target genes

<table>
<thead>
<tr>
<th>Target gene</th>
<th>GenBankAcc#</th>
<th>Forward sequence</th>
<th>Reverse sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S</td>
<td>XM_001922869</td>
<td>CGAGGAGTTCAAGACGATCA</td>
<td>GAGGTTTCCCTGTGTTGAGTC</td>
</tr>
<tr>
<td>nme2a</td>
<td>NM_199970</td>
<td>GACGCTCCATCAAACATCTCCC</td>
<td>AAGATCTATCTGCTGCTGATTA</td>
</tr>
<tr>
<td>nme2b1</td>
<td>NM_130926</td>
<td>CCAGACGCTGTCTGTTCTGT</td>
<td>TCATGGAAGAAAGACGAGATGA</td>
</tr>
<tr>
<td>nme2b2</td>
<td>NM_130927</td>
<td>TGAGCTGCTGCTGCTT</td>
<td>GGGTTCCTGTGTTGTTGTTG</td>
</tr>
<tr>
<td>nme3</td>
<td>NM_130928</td>
<td>TCCGGAACGAGAGATGAT</td>
<td>AACCTATCTGCTGCTGCTC</td>
</tr>
<tr>
<td>nme4</td>
<td>NM_201195</td>
<td>TGAGCTGCTGTGTTGAGTC</td>
<td>TAACTCCGACGGGAGTTCC</td>
</tr>
<tr>
<td>nme5</td>
<td>NM_001002516</td>
<td>GAGGCTCTGCTGCTGAGTTA</td>
<td>ATGGGGGAAATGAGGCTGTA</td>
</tr>
<tr>
<td>nme6</td>
<td>NM_131597</td>
<td>GCACACATGGGATCAGCTGAA</td>
<td>CATCAAGTGGGACAGAAAGGA</td>
</tr>
<tr>
<td>nme7</td>
<td>NM_130929</td>
<td>GGTTTGCTGCGATGACTCC</td>
<td>ATGCTGCGATGCTTGTCC</td>
</tr>
<tr>
<td>nme8</td>
<td>NM_001089475</td>
<td>ATCAAGAAGACGCTGAGAG</td>
<td>TCCCGCATAAAACGAGAAAGG</td>
</tr>
</tbody>
</table>
for 2 h at −20°C, and RNA concentrations were measured using a NanoDrop spectrophotometer. Samples dehydration (increasing ethanol: 15 min in 50% ethanol, twice 15 min in 70% ethanol, 15 min in 80% ethanol, 30 min in 96% ethanol, and 30 min in 96% ethanol/butanol v/v), clearing (butanol once for 30 min, and twice for 3 h each), and paraffin infiltration (once for 1 h and twice for 2 h, at 60°C) were performed in a Citadel 1000 tissue processor (Shandon, Pittsburgh, PA, USA). Dehydrated tissues were embedded in paraffin using a HistoEmbedder (TBS88, Medite, Germany). For in situ hybridization (ISH), serial cross sections of 5 μm were deparaffinized, re-hydrated in TBS (50 mM Tris, pH 7.4, 150 mM NaCl), and post-fixed in 4% PFA for 20 min. ISH was performed using the In situ Pro, Intavis AG robotic station. Incubation volumes for all ISH steps were set to 250 μl. Digestion was carried out for 20 min at 37°C with 2 μg/ml of proteinase K. Pre-hybridization (2 h, 60°C) and hybridization (12 h, 60°C) were carried out in 50% formamide, 2× SSC, 1× Denhardt, 10% dextran sulfate, and 250 μg/ml tRNA. For hybridization, the digoxigenin-labeled anti-sense RNA probes were diluted in hybridization buffer at a final concentration of 8 ng/μl. Washing steps (6×10 min, 60°C) were performed with 2× SSC followed by an RNase treatment at 37°C. The digoxigenin signal was then revealed with an anti-digoxigenin antibody conjugated with alkaline phosphatase (Roche Diagnostics Corp.) and a NBT/BCIP revelation system (Roche Diagnostics Corp.) as recommended by the manufacturer. Slides were mounted with Mowiol 4–88 (Calbiochem). Whole-mount in situ hybridization (WISH) was also performed using the In situ Pro, Intavis AG robotic station. Incubation volumes for all WISH steps were set to 700 μl. Digestion was carried out for 20 min at 37°C with 25 μg/ml of proteinase K. Samples were post-fixed in 4% PFA/0.2% glutaraldehyde for 40 min and then washed twice for 20 min in PBST. Embryo sample were not digested with proteinase K. Pre-hybridization (2×1 h, 65°C) and hybridization (16 h, 65°C) were carried out in 50% formaldehyde, 5× SSC, 0.1% Tween 20, 0.005% heparine, and 100 μg/ml tRNA. For hybridization, the digoxigenin-labeled anti-sense RNA probes were diluted in hybridization buffer at a final concentration of 2 ng/μl. Post-hybridization washing steps (3×40 min, 65°C) were performed in 50% formamide/2× SSC. Further washing steps (6×40 min, 55°C) were performed in 2× SSC and 0.2× SSC and (4×40 min, RT) in PBST. The digoxigenin signal was then revealed with an anti-digoxigenin antibody conjugated with alkaline phosphatase (Roche Diagnostics Corp.) and a NBT/BCIP revelation system (Roche Diagnostics Corp.) as recommended by the manufacturer. For each studied gene, the duration of revelation was kept identical for all developmental stages assayed. Samples were then rinsed overnight in PBS and subsequently observed under binocular (Zeiss, Stemi 2000-C).

Statistical analysis

Significant differences from negative sample and between samples were analyzed using Wilcoxon rank-sum test with superiority or inferiority alternatives hypothesis. All statistical analyses were performed using R v.2.6.2.

Results

Tissue distribution of nme genes

The tissue distribution study shows that most tissues exhibit very specific expression patterns of nme genes (Fig. 1). Among group I, nme genes (nme2–4), nme2a, nme2b1, and nme2b2 are the genes exhibiting the highest expression levels, while nme3 and nme4 are expressed at much lower levels. However, the copy of the nme2 gene exhibiting the highest expression is highly dependent on the tissue. For example, nme2a is the most highly expressed nme2 gene in eyes and testis. In contrast, nme2b1 is the most highly expressed nme2 gene in hepatopancreas, intestine, and ovary, while nme2b2 is the most highly expressed nme2 gene in brain, muscle, and gills. For all these tissues with the exception of eyes and gills, there is a clear predominant expression of one nme2 gene in comparison to the other nme2 genes. In most of these tissues, the predominant nme2 gene exhibits at least a ten-fold over expression in comparison to the two other nme2 genes. In contrast to group I, nme genes of group II exhibit a very specific tissue distribution with the exception of nme6 that is expressed in most studied tissues. The ovary is the expression site exhibiting the highest level of nme6, while nme5 and nme8 exhibit a testis-specific expression. In contrast to other tissues, the gonads exhibit a wide variety of nme gene expression with marked differences between male and female gonads. In the ovary, nme2b1, nme3, nme4, and nme6 are the most expressed genes, while nme5 and nme8 are the only ones that remain undetected. By contrast, nme5, nme2a, and nme8 are the most expressed nme genes in testis, while nme2b1, nme3 and nme4 exhibit lower expression levels. In the testis, the expression of all nme genes could be detected above background levels.

nme gene mRNA profiling during oogenesis

The expression of nme genes exhibiting an ovarian expression was monitored in the ovary throughout the oogenetic process. Among all ovarian nme genes, only nme2b1, nme3, nme4, and nme6 were significantly expressed in oocytes and ovarian follicles. In contrast, the expression levels of nme2a, nme2b2, and nme7 were extremely low in ovarian tissue (Fig. 1), ovarian follicles,
Fig. 1 Zebrafish tissue nme gene expression profile. nme gene expression in zebrafish eyes, brain, hepatopancreas, intestine, muscle, gills, ovary, and testis. Mean and SD are shown (n=4). Number sign indicates not detected or detected at extremely low levels. For all tissues, gene expression level is normalized by 18S gene expression using the $\Delta\Delta$Ct method so that gene abundance can be compared among tissues.
and oocytes (data not shown). Among studied genes, nme2b1 is the most expressed gene with expression levels 9, 11, and 18 times higher than nme6, nme3, and nme4, respectively. As shown by the in situ analysis, the expression of nme2b1, nme3, nme4, and nme6 was shown to be restricted to the oocyte, as no signal was observed in follicular layers (Fig. 2). These four genes exhibited a similar expression profile during oogenesis (Fig. 2). The highest expression levels per oocyte were observed in stage I oocytes. The levels of transcript subsequently decreased throughout oogenesis. A drop of mRNA levels of nme2b1, nme3, and nme4 was observed in stage II oocytes in comparison to stage I, while this drop was not statistically significant for nme6. The mRNA levels of nme4 subsequently exhibited a step-wise decrease from stage III to stage V of oocyte development. A decrease in nme2b1, nme3, and nme6 mRNA levels was further observed in stage IV and stage V oocytes in comparison to stage III.

**nme** gene mRNA profiling during early development

During early development, only nme2b1, nme2b2, nme3, and nme6 were found to be significantly expressed in the zebrafish embryo (Fig. 3). In contrast, nme2a, nme4, nme5, and nme7 were either not detected or expressed at very low levels (data not shown). Significant levels of nme3 and nme6 transcripts were detected in fertilized zebrafish eggs, thus demonstrating their maternal origin. In contrast, nme2b1 and nme2b2 were not significantly detected in fertilized eggs. The transcripts of nme2b1, nme2b2, and nme3 were weakly detected before MBT but were actively transcribed after zygotic genome activation as shown by their high expression level at 24 hpf. In contrast, nme6 displayed a different pattern as corresponding mRNA levels decreased very quickly after fertilization. Similarly to nme2b1, nme2b2, and nme3, nme6 was actively expressed after MBT. When comparing expression levels of those four genes, nme2b2 was the most expressed gene at 24 hpf.

**Early development whole-mount in situ hybridization**

As indicated above, nme3 and nme6 are the only nme mRNAs maternally inherited by the zebrafish embryo. Using WISH, we observed that nme3 transcript labeling can be detected in the zebrafish embryo at one cell and 4 hpf but not at 6 hpf (Fig. 4b). At 24 hpf, nme3 transcript was
localized in the eyes, the brain with a strong signal in midbrain and hindbrain. nme3 transcript was also detected all along the neural tube with a stronger labeling in the tail bud region (Fig. 4b). Weak nme3 signal could also be observed along the pronephric duct. nme6 transcript labeling was moderate and ubiquitous from one-cell stage to 6 hpf (Fig. 4b). At 24 hpf, a moderate labeling was observed in the eyes and the brain, and a weak signal was present in the somites (Fig. 4b).

Discussion

Zebrafish nme gene expression patterns

In mammals, the Nme gene family has been extensively studied, mostly because of its role in metastatic process (Boissan et al., 2009). The amount of available information is, however, very different depending on the member of the family. While specific Nme family members such as Nme1 and Nme2 have been thoroughly studied, other Nme genes (e.g., Nme6) have received far less attention. In addition, the analysis of existing data is further complicated by the numerous lineage specific gene duplications of group I Nme genes that have been found in all metazoans, including mammals and teleost (Desvignes et al., 2009; Desvignes et al., 2010). For instance, mammalian Nme1, Nme2, Nme3, and Nme4 genes are co-orthologs of Awd, the only group I Nme gene found in the Drosophila genome (Desvignes et al., 2010). Similarly, mammalian Nme1 and Nme2 genes are co-orthologs of zebrafish nme2a, nme2b1, and nme2b2. In contrast, orthology relationships of Nme3 and Nme4 genes were clearly established among vertebrates, including zebrafish and mammals (Desvignes et al., 2009). Similarly, a single ortholog of each group II Nme gene (Nme5, Nme6, Nme7, and Nme8) could be found in all metazoan species with some exceptions in species in which some genes highly diverged or were lost (Desvignes et al., 2010). Together, these observations suggest that the expression patterns and tissue distribution of group II Nme genes can be compared among Metazoans. Similarly, the expression patterns and tissue distribution of Nme3 and Nme4 can be compared among vertebrates. In contrast, interpretation of expression data regarding Nme1/Nme2 genes among vertebrates and metazoans is much more difficult due to independent lineage-specific duplications.

In mouse, Nme2 is widely and ubiquitously expressed among tissues and organs whereas Nme1 expression can be
highly variable depending on tissues, despite a broadly distributed expression (Barraud et al., 2002). Within a specific mammalian tissue, the Nme1/Nme2 expression ratio is, however, highly variable (Tsuchiya et al., 1998; Barraud et al., 2002). Together, these observations are consistent with the widely distributed expression of nme2 genes reported here in zebrafish. It has been shown that all three nme2 zebrafish paralogous genes originate from a unique vertebrate nme2 ancestral gene. Similarly, mammalian Nme1 and Nme2 originate from the same unique nme2 ancestral gene (Desvignes et al., 2009). It is thus tempting to postulate that ancestral nme2 functions could have been ubiquitous and multifunctional among tissues, and that Nme2 genes may have undergone subsequent sub-functionalization, at least in some organs, after successive gene duplication events (Zhang, 2003). For example, it has been shown that Nme1 is preferentially expressed in the nervous system of tetrapods and more specifically in the brain (Kimura et al., 1990; Ouatas et al., 1998; Dabernat et al., 1999a; Dabernat et al., 1999b; Barraud et al., 2002). This suggests that Nme1 may have retained or developed a specific function in the nervous system that Nme2 may not have. In zebrafish, we show that nme2b2 is the most expressed gene in the brain, thus also suggesting that following duplication events in teleosts, nme2b2 has also retained this cerebral function in contrast to nme2a and nme2b1 that are poorly expressed in the brain. In this context, we can therefore speculate that the nme2 ancestor gene has undergone multiple functional changes in vertebrates following the successive duplication events. Among the very specific tissue distribution of the zebrafish nme2 genes, the very high expression of nme2b2 in muscle should be noted. In addition to being expressed in all assayed tissues at moderate levels, nme2b2 exhibits a highly predominant expression in muscular tissue that has never been described in any other vertebrate species. This expression pattern is in striking contrast with existing Northern blot data reporting a low or moderate expression of Nme1 and Nme2 in mammalian skeletal muscle (Tsuchiya et al., 1998; Dabernat et al., 1999b; Masse et al., 2002). Similar to nme2b2, nme2b1 is also widely distributed with a predominant expression in the ovary. In contrast to nme2 genes, other nme genes (i.e., nme3-8) display more specific tissue distributions that are also observed in other vertebrate species. For instance, nme5 and nme8 exhibit a strict testis-specific expression in agreement with existing data in mammals (Munier et al., 1998; Sadek et al., 2001; Hwang et al., 2003; Miranda-Vizuete et al., 2003). Similarly, nme3 and nme6 are widely distributed in zebrafish tissues and organs in agreement with existing data in mammals (Mehus et al., 1999; Masse
et al., 2002). However, the predominant ovarian expression of nme3 and nme6 has never been documented in mammals, birds, or amphibians. Finally, it is noteworthy that in comparison to other studied tissues, reproductive organs express a wide variety of nme genes. While nme4 and nme7 are both expressed at comparable levels in ovary and testis, nme2b1, nme3, and nme6 are expressed at higher levels in the ovary, whereas nme2a, nme5, and nme8 are strongly expressed in the testis.

Nme genes in zebrafish oocyte and early embryo development

Nme gene expression during oogenesis

During zebrafish oogenesis, nme2b1, nme3, nme4, and nme6 are expressed at significant levels in the oocyte. These observations are consistent with the tissue distribution study reported here. Similarly, the lack of nme2a, nme2b2, and nme7 expression is also consistent with low ovarian expression level reported in Desvignes et al. (2009) and the present study. It is noteworthy that all ovarian nme genes display a decreasing expression profile during oogenesis, the decrease in oocyte expression levels being more progressive for nme6 and nme3 in comparison to nme2b1 and nme4. This suggests that nme2b1 and nme4 are translated during oogenesis. It is thus possible that the corresponding proteins are present in the mature oocyte and are subsequently maternally inherited. The strong decrease in mRNA levels in the oocyte between stages III and V would be in favor of this hypothesis. This would also be consistent with the identification of an Nme2b-related protein in zebrafish and gilthead seabream (Sparus aurata) oocytes during maturation (Ziv et al., 2008), an Nme2-related protein identified in Persian sturgeon (Acipenser persicus) mature oocytes (Keyvanshokhooh and Vaziri, 2008), and an Nme2-related protein in sea bass (Dicentrarchus labrax) eggs (Crespel et al., 2008). Using ISH, we confirmed that the transcripts are expressed in the oocyte and not in surrounding follicular layers. The labeling is predominant in small oocytes and undiscernable in stage IV oocytes. The absence of labeling in the last stages of oogenesis can be explained by a dilution effect due to the dramatic increase in oocyte volume due to major yolk accumulation (Lubzens et al., 2010). In addition, QPCR data clearly demonstrated that full-grown oocytes contain nme2b1, nme3, nme4, and nme6 transcripts. Together, our observations indicate that nme2b1, nme3, nme4, and nme6 mRNAs are maternally inherited.

Nme gene expression during early development

During zebrafish early development, only nme2b1, nme2b2, nme3, and nme6 could be detected at significant levels. At pre-MBT stages, only nme3 and nme6 could be detected, in agreement with expression levels in stage V oocytes (see above). In contrast, the lack of detection of nme4 and nme2b1 transcripts in fertilized eggs suggests a drop of transcript abundance either during post-ovulatory aging (Aegerter et al., 2005) and/or during fertilization and activation, before the formation of the first embryonic cell. The lack of nme2a expression is consistent with previous reports of predominant expression in eyes and testis (Desvignes et al., 2009), organs that are not fully developed at 24 hpf. In addition, nme2b1 and nme2b2 display low levels of expression before 24 hpf in comparison to nme3 and nme6 that are detected from fertilization to 6 hpf. Because of this very low expression level of nme2b1 and nme2b2 before 24 hpf, these genes were not studied by whole-mount in situ hybridization. This observation is consistent with the report of an nme2b-related transcript in Atlantic halibut during embryonic development (Hippoglossus hippoglossus) (Bai et al., 2007). The very high expression of nme2b1 at 24 hpf, a period when organogenesis occurs, is consistent with the wide distribution of nme2b1 transcript in zebrafish tissues (Desvignes et al., 2009). This observation is also consistent with the observations made in Atlantic salmon (Salmo salar) embryos in which an nme2 transcript corresponding to an nme2b was cloned and detected only after gastrulation (Murphy et al., 2000). Similarly, this is also consistent with prior report of the knock-down of nme2b1 and nme2b2 expression in zebrafish embryos that did not induce any apparent phenotype before 24 hpf (Hippe et al., 2009). Furthermore, in Xenopus laevis, no nme2-related transcript could be detected before MBT (Ouatas et al., 1998). In the 24 hpf embryo, nme2b2 is the most abundant nme transcript in agreement with the high expression level observed in adult muscle.

nme3 in zebrafish development

As described above, nme3 displays a typical maternal RNA profile as it is expressed in the oocytes during oogenesis and present in the embryo during early development. During the first hours of development, abundance levels are stable. Using WISH, we confirmed that nme3 transcript was present in the embryo from one-cell stage to 4 hpf. At 6 hpf, a lack of signal can be observed that may result from a dilution effect of the RNA within the embryo. At 24 hpf, a weak expression signal can be observed along the pronephric duct. The expression of nme3 in fish kidney was, however, previously unreported and would require further analysis. Interestingly, nme3 transcript labeling is predominant along the nervous systems as revealed by a strong signal in brain and eyes and a moderate signal all along the neural tube. This localization is in total agreement with
expression patterns of nme3 observed in adult mouse tissues and during mouse organogenesis with highest expression in nervous system (Masse et al., 2002; Amrein et al., 2005). Such a similar expression pattern between mouse and zebrafish strongly suggest that nme3 could be an important factor common to all vertebrates for nervous and sensory system development.

nme6 in zebrafish development

As indicated above, nme6 also display a typical maternal mRNA profile during zebrafish early development. However, in contrast to nme3 that is stable during the first 6-h post-fertilization, we show here using both QPCR and WISH that nme6 abundance displays a rapid decrease during the first cell cycles. This decrease of nme6 transcript abundance suggests an important translational activity and an important role in the very first steps of development. At 24 hpf, nme6 is actively transcribed and its expression is mainly localized in the brain, the eyes, and in the somites. This localization is consistent with expression data of nme6 among zebrafish tissues.

Conclusion

The present study provides new insights into the fish nme gene family by providing a complete expression survey of the entire family during zebrafish oogenesis and early development. We show that nme2b1, nme3, nme4, and nme6 are highly expressed in the ovary and present in the oocyte throughout oogenesis. While the abundance of the four transcripts exhibit a continuous decrease throughout oogenesis, nme3 and nme6, clearly display a typical maternal mRNA profile as they are also present in the embryo before the activation of the embryonic genome. The maternal origin of several nme transcripts in the early embryo is reported here for the first time in any vertebrate species. Together, our observations suggest an important role of the nme family in oocyte and embryo development in vertebrates.

Acknowledgments The authors thank the Singapore Genome Institute for providing bacterial clones, Thaovi Nguyen for technical assistance, INRA-SCRIBE histological platform, and Frederic Borel and Amélie Patinote for zebrafish care. This work was supported by the European Community’s Seventh Framework Program (FP7/2007-2013) under grant agreement no. 222719-LIFECYCLE.

References


