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

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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 2 different groups - group I and group 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 physiological 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 MZT (Maternal-to-Zygotic Transition) (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 to 4 hours 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 group I and group 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 (DDSV approval #35-31). All zebrafish *Danio rerio* were obtained from the fish rearing facility at INRA-SCRIBE (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 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) 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). Briefly, RNA and dNTPs were denatured for 6 min at 70°C, chilled on ice for 5 min before the reverse transcription master mix was added. Reverse transcription 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 reaction. All QPCR reactions 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^{TM} 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 ΔΔCt method to *18S* transcript abundance in samples diluted to 1/2000.

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 ΔΔCt 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 (GCACAGCAGTGGATCTACGA) 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 for 2 hours 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/butanolvol/vol), 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). 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, 2X SSC, 1X 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 x 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 x 1 h, 65°C) and hybridization (16 h, 65°C) were carried out in 50% formamide, 5X 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 x 40 min, 65°C) were performed in 50% Formamide / 2X SSC. Further washing steps (6 x 40 min, 55°C) were performed in 2X SSC and 0.2X SSC and (4 x 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 10-fold over expression in comparison to the two other *nme2* genes. In contrast to group I, *nme* genes of the 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 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 4 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 4 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 1cell 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 1cell 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 *Nme*2 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). Similarly 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 stage III and stage 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 (Keyvanshokooh 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 not-discernable 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 ageing (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 1 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 hour 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.

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Tables

Table1. QPCR primer sequences and accession numbers of target genes.

Target gene	GenBankAcc#	Forward sequence	Reverse sequence
18S	XM_001922869	CGGAGGTTCGAAGACGATCA	GAGGTTTCCCGTGTTGAGTC
nme2a	NM_199970	GACTGCCATCAAACAATTCCAA	AAGATCCTCATCTGCCTGGATTA
nme2b1	NM_130926	CCAGACGGGTTCTGTTCTGT	TGCTTGAGAAAAGACGAGATGA
nme2b2	NM_130927	TGGAGCTCAGAGTCCCTGTT	GGGTTCTGCTGTGTGTGTGT
nme3	NM_130928	TCCTGCACGGAGAAGATGAT	AACTCCATCCGGCTTCACT
nme4	NM_201195	TCAGCTGTTTGTGTGGCATT	TAACCCTCGGACCGTTACAC
nme5	NM_001002516	GACGCATCCTGACTGTCTGA	ATGGGGAAACATGAACCTGA
nme6	NM_131597	GCACACATGAGGATCACTGG	CATCAAGTGCAGCAGAAGGA
nme7	NM_130929	GGTTGTTGCCGAGTACCCTA	CATCTGGGGCATGTATTTCC
nme8	NM_001089475	ATCAAGAACGAGCTGGGAGA	TCCCGCATAAAACAGAAAGG

Legends

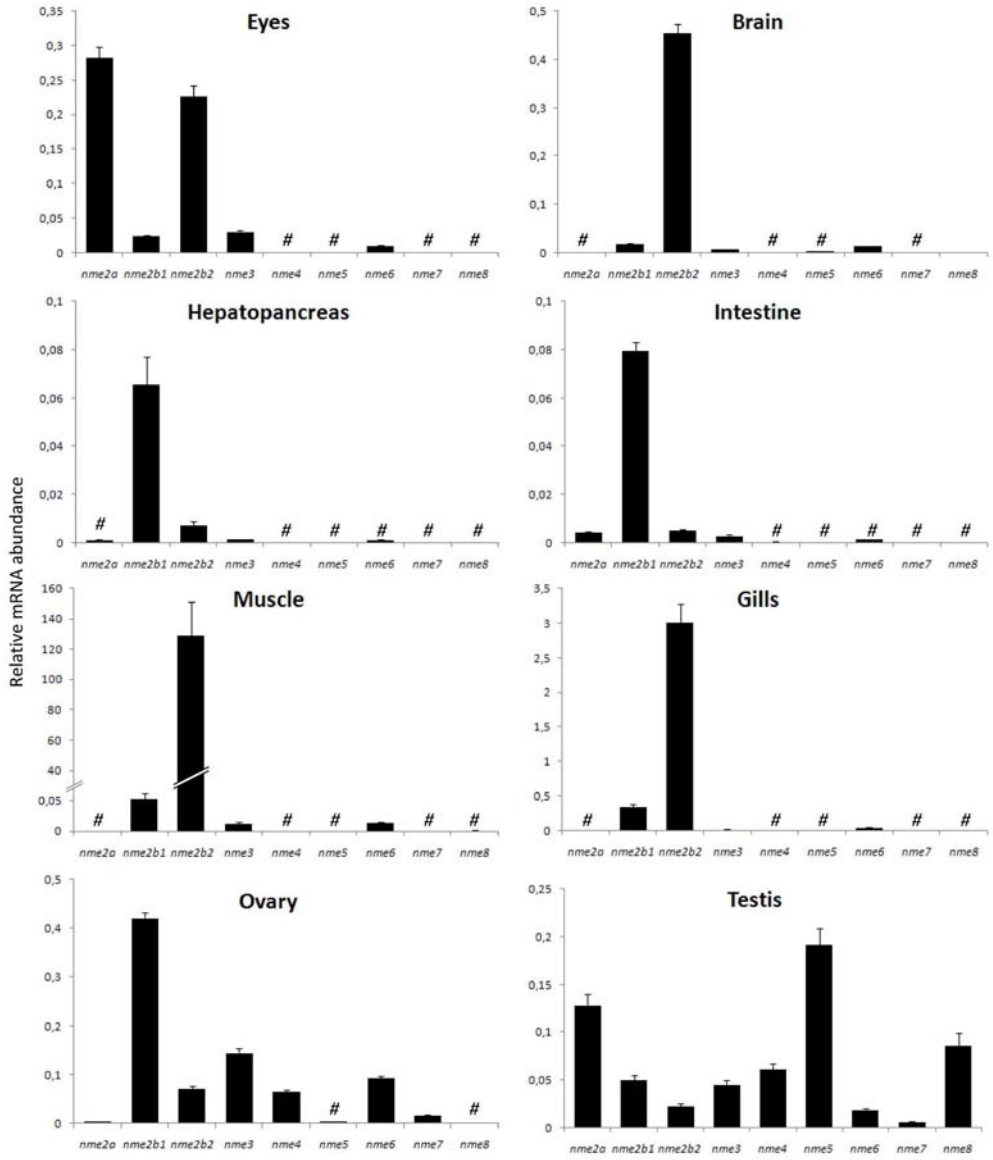
Fig.1 Zebrafish tissue *nme* gene expression profile. *nme* gene expression in zebrafish eyes, brain, hepatopancreas, intestine, muscle, gills, ovary and testis. Mean and SD are shown (n = 4). #, not detected or detected at extremely low levels. For all tissues, gene expression level is normalized by 18S gene expression using the ΔΔCt method so that gene abundance can be compared among tissues.

Fig.2 Zebrafish *nme2b1, nme3, nme4* and *nme6* gene expression during oogenesis. Oocyte developmental stages according to Selman *et al* (1993). Mean and SD are shown (n = 4 for all stages I, II, III, and V, n=5 for stage IV). Different letters indicate significantly different levels of expression at $p < 0.05$. For all stages, gene expression level is normalized by exogenous *luciferase* RNA abundance using the ΔΔCt method so that gene abundance can be compared among stages. Expression level was arbitrarily set to 1 for the lowest value of the data set (*nme4* stage V). For each gene, an ISH picture of an ovary section is displayed to demonstrate the expression of the corresponding *nme* gene in the oocytes and the lack of expression in follicular layers. fl, follicular layers ; n, nucleus ; o, oocytes, the oocyte developmental stage is shown in brackets.

Fig.3 Zebrafish *nme2b1, nme2b2, nme3* and *nme6* gene expression during early development. Embryonic developmental stages according to Kimmel *et al* (1995). Mean and SD are shown (n = 4 for zyg, 4hph, 6hpf and 24hpf, n=3 for 1-cell stage). Different letters indicate significantly different levels of expression at $p < 0.05$. For all stages, gene expression level is normalized by exogenous *luciferase* RNA expression using the ΔΔCt method so that gene abundance can be compared among stages. Expression level was arbitrarily set to 1 for the lowest value of the data set (*nme2b1*, Zyg). Zyg, correspond to the fertilized egg, before the formation of the first embryonic cell.

Fig.4 In situ hybridization and whole-mount in situ hybridization of *nme3* and *nme6* transcripts during zebrafish oogenesis and early development. A. Corresponding zone hybridized with sense probe is shown in smaller size. I, stage I oocytes; II, stage II oocytes; IIa, stage IIa oocytes; IIb, stage IIb oocytes; III,

stage III oocytes; IV, stage IV oocytes. B. Early development localization of *nme3* and *nme6* transcripts examined by whole-mount in situ hybridization. b, brain; hb, hindbrain; mb, midbrain; nt, neural tube; pd, pronephric duct; s, somites ; tb, tail bud; e, eyes.



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). #, not detected or detected at extremely low levels. For all tissues, gene expression level is normalized by 18S gene expression using the ΔΔCt method so that gene abundance can be compared among tissues

173x205mm (600 x 600 DPI)

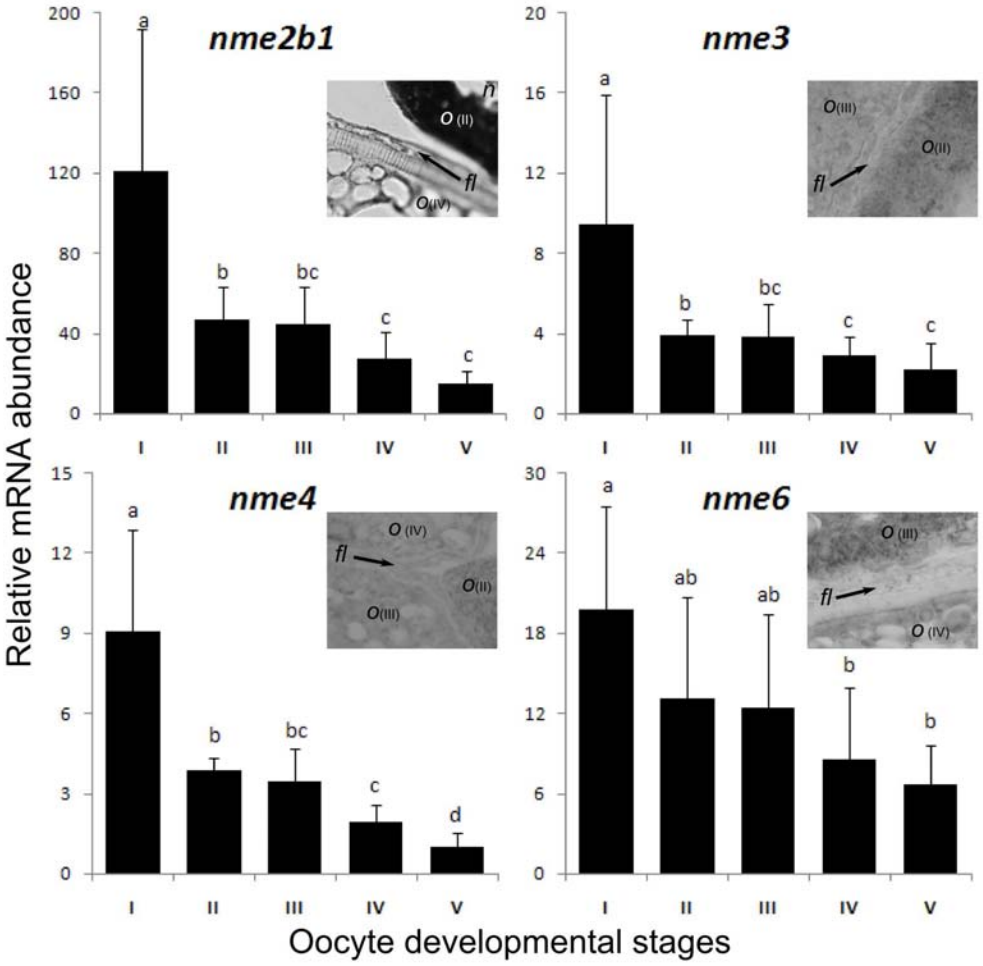


Fig.2 Zebrafish nme2b1, nme3, nme4 and nme6 gene expression during oogenesis. Oocyte developmental stages according to Selman et al (1993). Mean and SD are shown (n = 4 for all stages I, II, III, and V, n=5 for stage IV). Different letters indicate significantly different levels of expression at $p < 0.05$. For all stages, gene expression level is normalized by exogenous luciferase RNA abundance using the ΔΔCt method so that gene abundance can be compared among stages. Expression level was arbitrarily set to 1 for the lowest value of the data set (nme4 stage V). For each gene, an ISH picture of an ovary section is displayed to demonstrate the expression of the corresponding nme gene in the oocytes and the lack of expression in follicular layers. fl, follicular layers ; n, nucleus ; o, oocytes, the oocyte developmental stage is shown in brackets.
83x81mm (600 x 600 DPI)

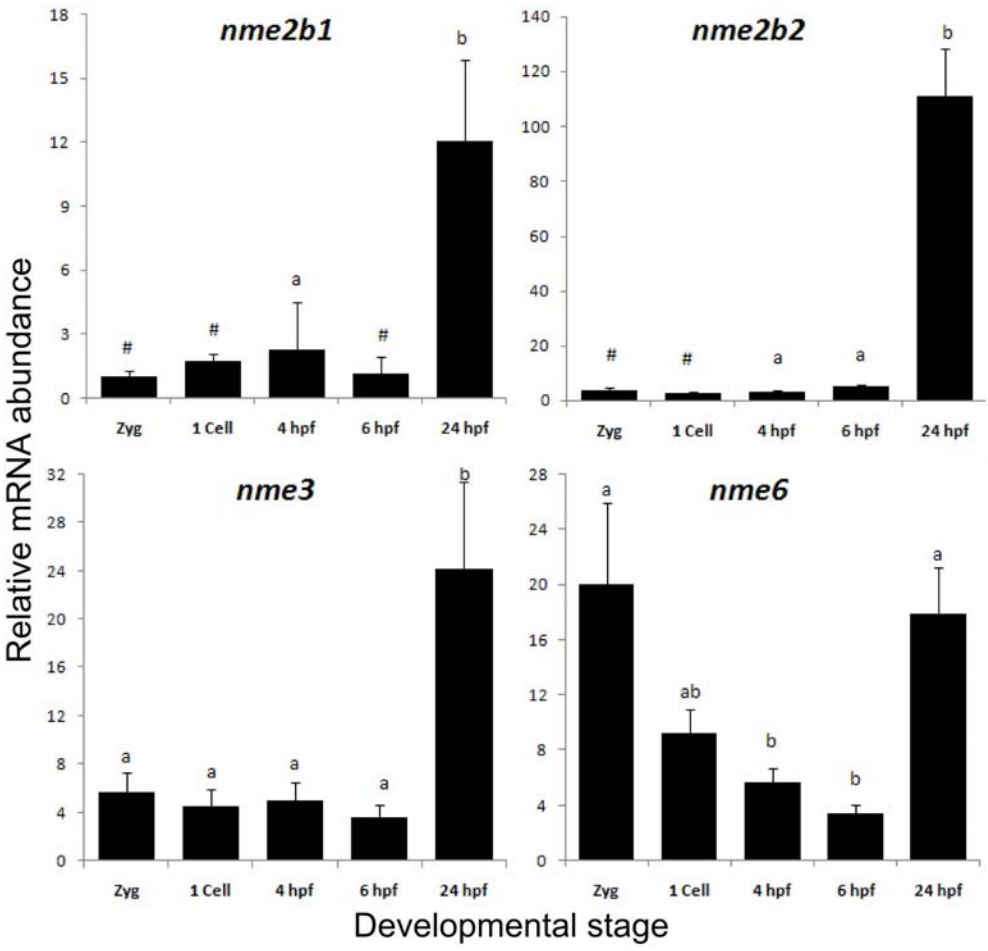


Fig.3 Zebrafish nme2b1, nme2b2, nme3 and nme6 gene expression during early development. Embryonic developmental stages according to Kimmel et al (1995). Mean and SD are shown (n = 4 for zyg, 4hph, 6hpf and 24hpf, n=3 for 1-cell stage). Different letters indicate significantly different levels of expression at $p < 0.05$. For all stages, gene expression level is normalized by exogenous luciferase RNA expression using the ΔΔCt method so that gene abundance can be compared among stages. Expression level was arbitrarily set to 1 for the lowest value of the data set (nme2b1, Zyg). Zyg, correspond to the fertilized egg, before the formation of the first embryonic cell.
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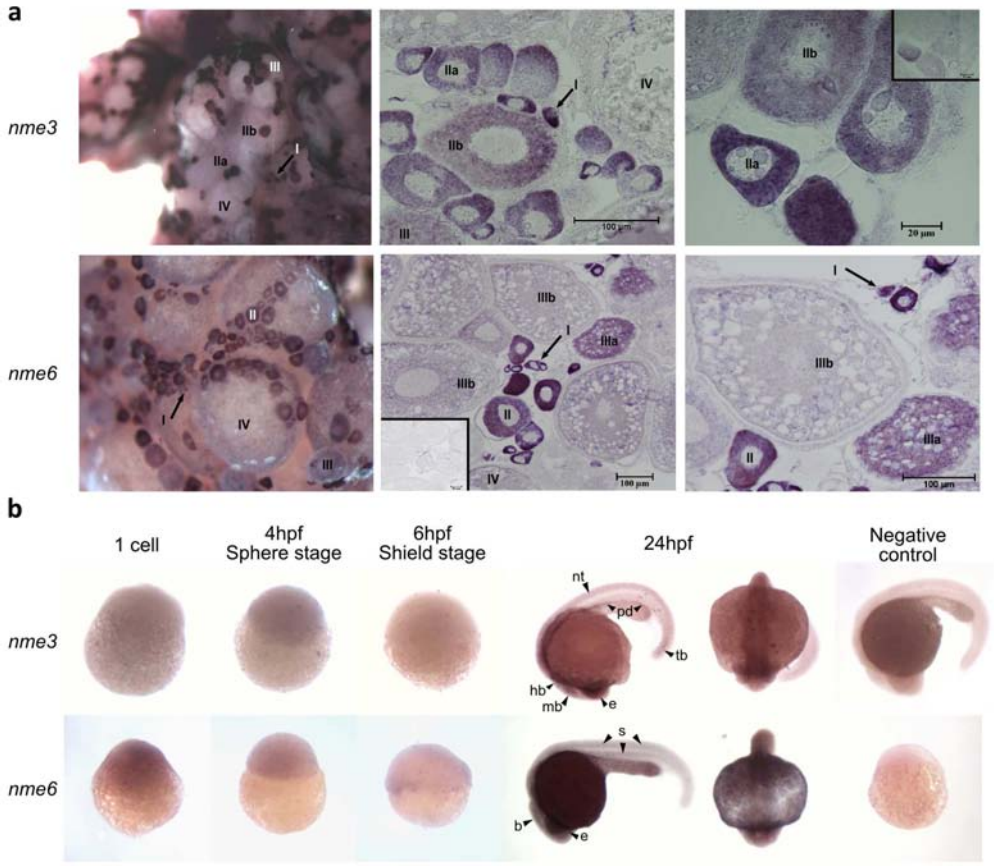

In situ hybridization and whole-mount in situ hybridization of nme3 and nme6 transcripts during zebrafish oogenesis and early development. A. Corresponding zone hybridized with sense probe is shown in smaller size. I, stage I oocytes; II, stage II oocytes; IIa, stage IIa oocytes; IIb, stage IIb oocytes; III, stage III oocytes; IV, stage IV oocytes. B. Early development localization of nme3 and nme6 transcripts examined by whole-mount in situ hybridization. b, brain; hb, hindbrain; mb, midbrain; nt, neural tube; pd, pronephric duct; s, somites ; tb, tail bud; e, eyes.
173x151mm (300 x 300 DPI)