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Zinc transporter expression in zebrafish (*Danio rerio)* during development⋆

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Abstract

Zinc is a micronutrient important in several biological processes including growth and development. We have limited knowledge on the impact of maternal zinc deficiency on zinc and zinc regulatory mechanisms in the developing embryo due to a lack of in vivo experimental models that allow us to directly study the effects of maternal zinc on embryonic development following implantation. To overcome this barrier, we have proposed to use zebrafish as a model organism to study the impact of zinc during development. The goal of the current study was to profile the mRNA expression of all the known zinc transporter genes in the zebrafish across embryonic and larval development and to quantify the embryonic zinc concentrations at these corresponding developmental time points. The SLC30A zinc transporter family (ZnT) and SCL39A family, Zir-,Irt-like protein (ZIP) zinc transporter proteins were profiled in zebrafish embryos at 0, 2, 6, 12, 24, 48 and 120 hours post fertilization to capture expression patterns from a single cell through full development. We observed consistent embryonic zinc levels, but differential expression of several zinc transporters across development. These results suggest that zebrafish is an effective model organism to study the effects of zinc deficiency and further investigation is underway to identify possible molecular pathways that are dysregulated with maternal zinc deficiency.

Introduction

The role of zinc in a wide range of cellular processes, including cell proliferation, reproduction, immune function, and defense against free radicals, has been well established. Zinc is considered to be the most abundant trace intracellular element, and there exists increasing evidence that zinc plays an important role in fetal growth and development. It is

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estimated that 82% of women worldwide have inadequate intakes of zinc and may be at risk for zinc deficiency (Caulfield et al. 1998). In humans, suboptimal zinc intake is associated with poor pregnancy outcomes, increased premature birth, low birthweights and increased congential malformations (Meadows et al. 1983; Garg et al. 1993; Scholl et al. 1993; Shah et al. 2006; Hess et al. 2009). In mammalian experimental models, maternal zinc deficiency also results in increased embryonic cell death and increases in numerous developmental defects in the offspring (Oteiza et al. 1990; Peters et al. 1991; Liu et al. 1992; Keen et al. 1993; Jankowski et al. 1995; Lopez et al. 2008). In addition to these acute effects, maternal marginal zinc deficiency is also associated with longer term health consequences including impaired glucose tolerance (Padmavathi et al. 2009), increased susceptibility to diabetic stress (Uriu-Hare et al. 1989), impaired learning and memory (Halas et al. 1986) and compromised immune system (Vruwink et al. 1991).

Zinc homeostasis is maintained by the activities of a family of zinc transporters in the cell plasma membrane and intracellular organelles. The SLC30A zinc transporter family (ZnT) act to decrease intracellular zinc levels through transport of zinc from the cytoplasm to the extracellular space or into organelles. In contrast the second SCL39A family, Zir-,Irt-like protein (ZIP) act in a opposing manner to increase intracellular zinc levels. At least ten ZnT and fourteen Zip family members have been identified in mammals, and their tissue expression, cellular localization and regulation are very different (see (Lichten et al. 2009) for detailed review). The critical role of zinc and zinc transporters during developmental processes has been clearly been established with molecular and genetic approaches in model systems. For example, knockdown of ZIP6, a LIV1 family zinc transporter, results in early embryonic malformations in zebrafish and is critical for epithelial-mesenchymal transition (EMT) during gastrulation (Yamashita et al. 2004). In rodents, knockdown of ZnT1 results in early embryonic lethality (Andrews et al. 2004). Zinc also plays an important role in cell meiosis, and in during oocyte maturation (Bernhardt et al. 2010; Kim et al. 2010). Thus there is clear evidence supporting a key role of zinc and its regulatory proteins during development.

Although there has been intense study of zinc regulatory proteins, such as zinc transporters, to control zinc homeostasis, their function and regulation at the organism level is far less well understood. In particular the impact of maternal zinc status on fetal zinc homeostasis *in vivo* is virtually unknown because zinc depletion during pregnancy usually causes severe embryonic deformities (which are often lethal) or causes early fetal resorption. The use of zebrafish (*Danio rerio*) offers a unique model that allows us to directly study the effects of maternal zinc on embryonic development following implantation and gain an understanding of the mechanistic function and regulation of zinc during development at the organism level. During development, zinc levels rapidly increase after fertilization through 512-cell stage (~2.75 h post fertilization (hpf)) and plateaus at the mid-gastrula state (~ 6 hpf) (Riggio et al. 2003). The dynamics of zinc regulatory protein expression during the early stages of development are unknown. Orthologs for the zinc regulatory proteins, and many of the zinc transporters have also been identified in zebrafish (Chen et al. 2002; Chen et al. 2007; Zheng et al. 2008). The goal of the current study was to mRNA profile the family of zinc transporters in zebrafish across developmental time (from single cell at 0–2 h post fertilization through full development at 5 days of age) and establish the use of zebrafish as a model organism for the study of zinc metabolism and function.

Materials and Methods

Zebrafish Husbandry and Embryo Collection

Embryonic zebrafish (*Danio rerio*) were reared at Sinnhuber Aquatic Research Laboratory (SARL) at Oregon State University from adult AB strain fish 33 weeks of age. Adults were

maintained at 29°C with a light/dark cycle of 14/10 hours in reverse osmosis water supplemented with 0.6% Instant Ocean® salt solution. Zebrafish were spawned and embryos were collected at 0, 2, 6, 12, 24, 48 and 120 hpf. Samples were collected in triplicate, with each replicate consisting of 30 pooled embryos. Embryos collected for RNA extraction were stored in 500 μL RNAlater® and kept at −20°C. Embryos collected for ICP analysis were rinsed twice with Chelex-treated water, then stored dry at −20°C.

RNA extraction

RNA extractions were carried out according to the TRIzol method. Briefly, RNAlater® was removed from the embryonic samples and 1.0 mL of TRIzol was added to the sample. Embryos were homogenized for 30 seconds, incubated at room temperature for 5 minutes, and then 200 μL of chloroform was added. After another incubation at room temperature for 2–3 minutes, samples were centrifuged at max speed for 15 minutes at 4°C. The upper aqueous layer was transferred to a new tube and 500 μL isopropyl alcohol was added. After a 10 minute incubation at room temperature, samples were centrifuged at max speed for 10 minutes at 4°C. Supernatent was decanted and following an ethanol wash and spin step, the RNA pellet was resuspended in DEPC treated water. cDNA synthesis was conducted using Superscript® III First Strand Synthesis according to the manufacturer's suggestions.

qRT-PCR Analysis

Plasmid standards of the qRT-PCR gene targets were cloned using TOPO-TA cloning kit®. Ornithine decarboxylase 1 (*odc1*) was chosen as a reference gene for normalization. Primers for experimental gene targets and *odc1* are listed in Table 1. Each primer set has a melting temperature at or near 60°C and amplifies a 100–300 bp target. qRT-PCR reactions were set up as follows: 50 ng cDNA, 0.5 μM each primer, 10 μL 2X DyNAmo HS SYBR green ENZ mix and DEPC water to a final volume of 20 μL. Using a MJ Research thermocycler, the reaction proceeded as follows: 95°C for 10 minutes, 40 cycles of 94°C for 10 seconds, 58°C for 20 seconds and 72°C for 20 seconds. A final extension at 72°C for 10 minutes was used. A melting curve analysis was also conducted from 60–95°C, read every 0.5°C and held for 1 second. Opticon Monitor version 2.0 software was used to analyze qRT-PCR data following the standard curve method.

Zinc analysis

Embyronic zinc concentrations were determined by Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES; Teledyne Leeman Labs) with small modification of a previous described method (Verbanac et al. 1997). Samples were digested in 69%–70% OmniTrace nitric acid (VWR) overnight. Following digestion, samples were diluted 10 times with water treated with chelex 100 resin (Bio-rad) and analyzed by ICP-OES against known standards (Bruno et al. 2007).

Statistics

Statistical analysis was performed with the use of PRISM (version 4.0; GraphPad Software). Significantly differences between means across time were analyzed by one-way ANOVA followed by Dunnett's post-hoc test when appropriate.

Results

Zinc concentrations and all known zinc transporters from both the ZIP family and the ZnT family were analyzed over developmental time in *Danio rerio* embryos. Figure 1 depicts ICP data for zinc content in 30 pooled *D. rerio* embryos over developmental time. Zinc levels were relatively constant over developmental time (0 to 120 hpf).

Figure 2 illustrates expression patterns of ZIP transporters normalized to *odc1* mRNA transcript through 120 hpf. ZIP3, ZIP4, ZIP7, ZIP8 and ZIP10 showed a pattern of increasing transcript level, with maximal expression at 120 hpf compared to earlier time points. ZIP6, ZIP9, ZIP11 and ZIP13 transcript levels dropped following 0 hpf time point, with lowest expression level at 6 hpf. Transcript levels then increased again at 12 hpf with highest expression at 120 hpf. In contrast, there was a bimodal expression pattern for ZIP1 expression, a rise expression level at 6 hpf and 120 hpf. Table 2 shows ZIP and ZnT expression as a fold change from 0 hpf for each respective gene, after normalization to *odc1*. Relative transcript levels for ZIPs 1, 6, 9, 10, 11 and 13 was less than 5 fold for all time points. ZIPs 3 and 4 showed a greater increase relative to 0 hpf, with ZIP3 reaching 60 fold and 80 fold difference at 120hpf respectively. Among the ZIPs tested, ZIP8 exhibited the greatest fold change from 0hpf, culminating with an approximately 460 fold change.

Figure 3 illustrates ZnT transcript levels normalized to *odc1* mRNA transcript through 120 hpf. ZnT1, ZnT7 transcript levels increased over time, with highest expression at 120 hpf. ZnT2 expression was not detectable until 24 hpf with highest expression at 120hpf. ZnT4 and ZnT6 showed a similar trend as ZIP13, with a decrease in expression from 0–6 hpf followed by steady increase in expression through 120 hpf. Interestingly, ZnT8 expression was not detectable until 6 hpf and had its highest transcript levels at 48 hpf, and an insignificant but marked decrease in transcript levels at 120 hpf. As shown in Table 2, among the ZnTs analyzed, ZnTs 4, 6, 7 and 9 did not surpass a 7 fold change for all time points. nT5 maintained a fairly constant relative transcript level around 1 fold or less until 120 hpf, when relative expression reached nearly 12 fold that of 0hpf. ZnTs 1 and 2 reached even higher relative fold changes of roughly 80 and 615 at 120 hpf respectively. Lastly, ZnT8 had the greatest overall relative fold change from 0 hpf, reaching a nearly 5000 fold change at 48 hpf and dropping to roughly 2100 fold change at 120 hpf.

Discussion

Zinc and its transporters play key roles during embryonic development and many biological processes. Ablation of ZIP6 and ZnT1 results in developmental abnormalities in zebrafish and rodents, respectively (Andrews et al. 2004; Yamashita et al. 2004). ZIP6 also plays a role in the maturation of immune cells, including dendritic cells and the regulation of T-cell receptor signaling (Kitamura et al. 2006; Yu et al. 2011). ZIP13 knockout mice exhibit defects in connective tissue development, via dysregulation of bone morphogenic protein and TGF-β signaling (Fukada et al. 2008). ZIP14 appears to be required for systemic growth via control of G-protein coupled receptor-mediated signaling (Hojyo et al. 2011). ZnT3 is essential for pre-synaptic MAP kinase signaling and the development of hippocampus-dependent memory (Sindreu et al. 2011). Although a key role of zinc during growth and developmental processes has been clearly established in experimental models, in human studies, the data linking zinc to adverse fetal growth and developmental outcomes is conflicting (Hess et al. 2009). However, interpretation of human studies is hampered by the lack of specific and sensitive biomarkers for zinc status, especially during pregnancy and development (Shah et al. 2006). This is coupled with a lack of knowledge of the precise mechanisms that regulate zinc delivery from maternal stores to the fetus, especially at early stages of development, and a lack of understanding the mechanistic consequences of zinc deficiency on fetal outcomes. The development of an in vivo model, such as zebrafish, to study the zinc metabolism during embryo development would significantly aid in filling these knowledge gaps and aid in our understanding of the relationship between zinc & its regulatory proteins and developmental processes at a whole organism level.

The ZnT family of transporters functions in zinc efflux from the cytoplasm to either the extracellular space and/or intracellular organelles. The ZIP family of protein functions in

zinc uptake from the extracellular matrix and/or intracellular organelles into the cytoplasm (Lichten et al. 2009). These zinc transporters are expressed in a tissue-specific manner, and respond differentially to dietary zinc levels and physiological conditions. Therefore, a loss of function or dysregulation of certain zinc transporters would result in an impairment of zinc homeostasis and predispose the body to zinc-imbalance-related diseases, such as cancer, asthma, diabetes, and Alzheimer's disease (Lichten et al. 2009). For example, in humans, mutations of Zip4 gene is the cause of human acrodermatitis enteropathica, an autosomal recessively inherited disease (Kury et al. 2002), and polymorphisms in ZnT8 is associated with type 2 diabetes (Sladek et al. 2007). Dysregulation of ZnT1 has been associated with the genetic disorder epidermodysplasia verruciformia, where patients are highly susceptible to human papillomavirus (HPV) infection (Orth 2008). Mutations in ZnT2 has been linked to an inability for the mammary gland to secrete zinc during lactation (Seo et al. 2010). Over-expression of zinc transporters, such as ZIP6 and ZIP4 has also been associated with cancer cell growth in breast, liver and pancreatic cancers (Li et al. 2007; Shen et al. 2009; Weaver et al. 2010). We observed trends for differential zinc transporter expression patterns that were affected by developmental age. This data highlights our ability to monitor zinc transporter expression during zebrafish development and supports studies using this model organism for zinc metabolism and/or functional zinc transporter studies.

The tissue expression, cellular localization and regulation among each zinc transporter are very different. For example, ZnT1 is ubiquitously expressed, but highly expressed in tissues involved in zinc transfer, such as the basolateral side of enterocytes and kidney tubules (McMahon et al. 1998). ZnT1 in particular appears to play a critical role in dietary zinc absorption, controlling the efflux of zinc out of the enterocyte across the basolateral membrane (Andrews et al. 2004). It also localizes to the yolk sac membrane in rodents and may facilitate transfer of zinc between mother and fetus (Liuzzi et al. 2003). The severity lethality of ZnT1 knockdown in mammals also renders it more difficult to identify the precise role of ZnT1 and zinc during development. Relative to the other zinc transporters, ZnT1 and ZnT5 transcript copy number was most abundant at 0 hpf in zebrafish.

Large increases in expression of ZIP3,4,8 and ZnT2 and ZnT8 were apparent across development (Table 2). The greatest change in expression levels occurred in ZnT8 mRNA levels across development. We highlight that there is marked increase in expression of ZnT8 at 6 hpf (~646 fold increase) that corresponds with embryonic synthesis of proinsulin (Kinkel et al. 2009) and peaks at 48hpf (~5000 fold increase) that corresponds with organogenesis. ZnT8 appears to play a critical role in the synthesis and transport of zinc into secretory vesicles and formation of zinc-insulin complexes (Chistiakov et al. 2009). Loss of ZnT8 in pancreatic β cells reduces insulin content and loss of insulin release (Fu et al. 2009; Lemaire et al. 2009). Genome-wide association studies have found that a polymorphism variant in ZnT8, rs1226634 [C/T transition; Arg(325)- Trp (325)] is associated with increased risk of Type 2 diabetes (Saxena et al. 2007; Scott et al. 2007; Pound et al. 2009). Interestingly, ZnT8 is also an auto-antigen in Type I diabetes (Wenzlau et al. 2007). The insulin producing pancreatic β-cells contain some of the highest levels of zinc in the body. This high zinc requirement is largely due to the critical function of zinc for insulin synthesis, secretion and signaling (Tallman et al. 1999). High zinc levels in the pancreas may also be necessary to providing protection against oxidative stresses (Ho et al. 2001). Zinc deficiency may predispose individuals to diabetes and its cardiovascular complications (Mocchegiani et al. 2008). Interestingly, maternal zinc deficiency increases her offsprings' susceptibility to increased body mass, glucose intolerance and impaired insulin secretion (Padmavathi et al. 2009). The use of zebrafish as a model organism for zinc studies is powerful in that for the first time we could quantitatively track zinc transporter expression from the single cell zygote stage through all stages of vertebrate development. Since early stages of vertebrate development are remarkably conserved, these zebrafish studies are highly relevant to human

health. Importantly, similar to humans, zebrafish also require zinc and have in place similar zinc regulatory proteins to maintain zinc homeostasis (Feeney et al. 2005). Both family of zinc transports (ZIP and ZnT) and their control via Metal Transcription Factor and Metal Response Elements have been previously established to occur in zebrafish (Zheng et al. 2008). Studies in zebrafish gills, also suggest that zebrafish tissues respond to both zinc supplementation and zinc deficiency exposures (Zheng et al.; Zheng et al.). The zebrafish, especially the developing embryo, is an attractive model system for studies of zinc function. Beneficial attributes include its small size (<1 gram body weight), rapid embryonic development (less than 1 week) and short life cycle (Dodd et al. 2000; Wixon 2000; Udvadia et al. 2003). The zebrafish model enables assessment of integrative, whole animal effects. Importantly, fundamental processes and mechanisms of development are conserved across species (Lein et al. 2005).

Collectively, the current data confirms the expression of zinc transporter proteins in zebrafish, and that they are differentially expression during development. This sets the stage for using zebrafish to aid in defining the specific molecular targets that control zinc homeostasis during development and characterize mechanisms by which zinc status or alterations in transporter expression affects fetal health. Future studies using purified zebrafish diets containing adequate or deficiency levels of zinc will be performed to closely examine the impact of dietary zinc status on embryonic developmental processes. This mechanistic work in zebrafish will provide the framework for translating results to humans and gain a better understanding of importance of zinc during pregnancy and development.

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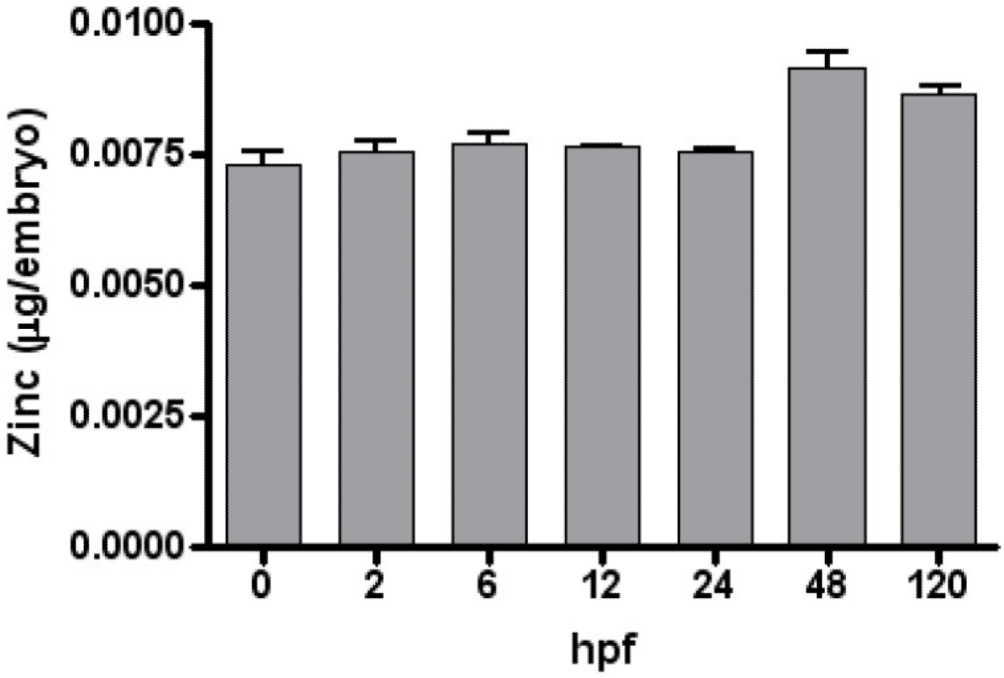

Figure 1.
Embryonic zinc levels across development. Zinc levels were determined by inductively-coupled plasma spectroscopy. Bars represent means ± SEM (n= 3, 30 pooled embryo samples). Zinc levels were calculated per embryo.

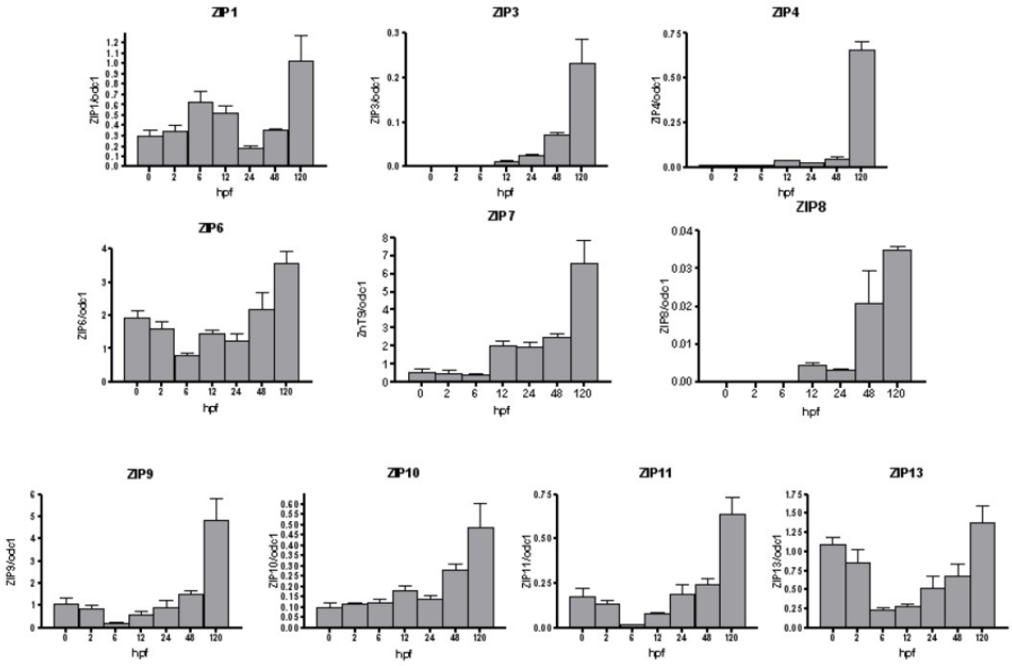

Figure 2.

ZIP (SLC39A) mRNA abundance profiles in zebrafish embryos across development. mRNA levels were measured as described in Material and Methods. Results are transcripts copy number normalized to ornithine decarboxylase (ODC1) transcripts. Values are means ± SEM (n= 3).

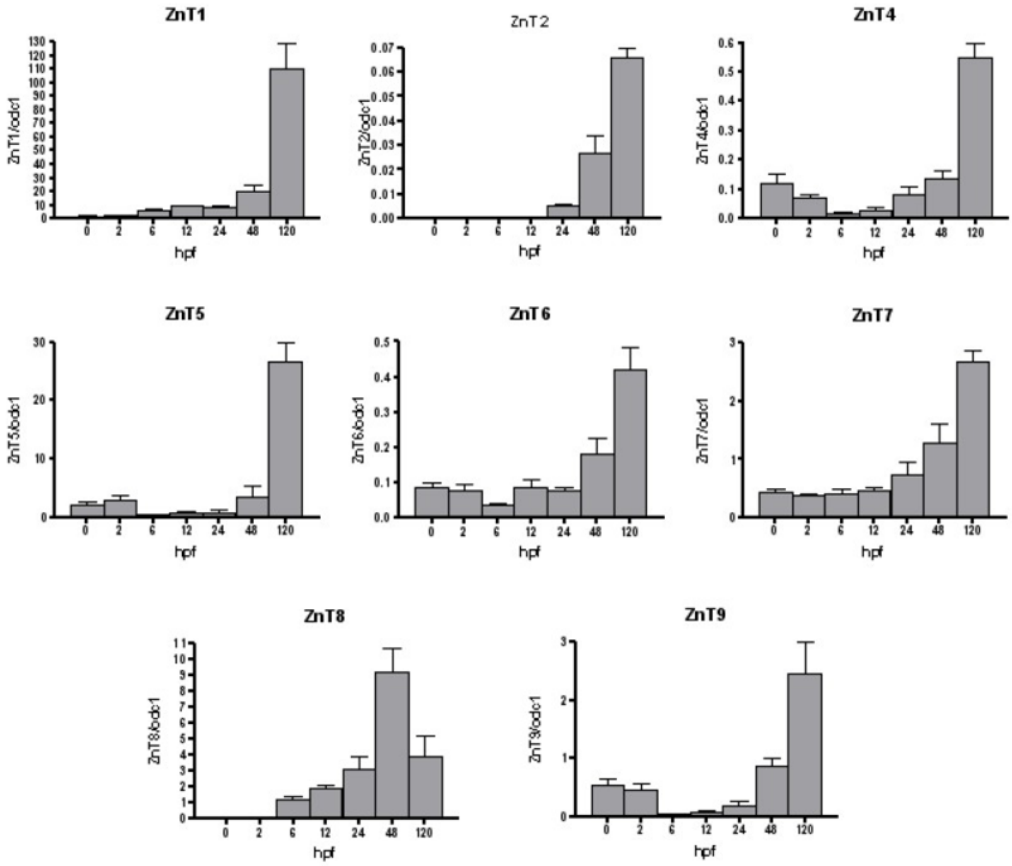

Figure 3.

ZnT (SLC30A) mRNA abundance profiles in zebrafish embryos across development. mRNA levels were measured as described in Material and Methods. Results are transcripts copy number normalized to ornithine decarboxylase (ODC1) transcripts. Values are means ± SEM (n= 3).

Table 1

primers for qRT-PCR analysis.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Product Size (bp)
odc1	GTGGGCGACTGGCTGCTGTT	CCGCAGTGGGATGGCACGTT	200
ZIP1	GGTGAGAGTTGGAGCTCTGG	AGTGGGAAGCCATCATCAAG	243
ZIP3	CGTATACGGCTGATGTGGTG	AGGCCTGCTGTAAACCACTG	299
ZIP4	CAGACATGCTTCCTACGCTG	GCCCGATCTGGTCTTCATAA	132
ZIP6	GTCATCATGGGAGACGGACT	GGCAAAATCACCGAGTTCAT	141
ZIP7	AAGAAAGTTGTGGAAGCAGGCA	CCACCAGATGCAAAACTCAAGAG	176
ZIP8	TCCCCGCCTGCCCTTACACTT	AGTGTCCCGATGGCCAGTCCAA	199
ZIP9	TCGGAATGTGACGAGCCTTCGC	ACATGTATCCTCGGAGATCGCGTG	226
ZIP10	TCACCTGCACATGGTGTTCT	ACATCCAAACCCATCCTGAA	223
ZIP11	TCAGGCCCTGCTGGGGACTC	GCCCACAGCCACTGGGAGGA	226
ZIP13	GGAGACCAACCCAAGGAACT	GTCTTTGGGAGGGTGACAAA	220
ZnT1	GAAGGCTGCCGATATGTGTC	AGGACATGCAGGAAAACACC	132
ZnT2	TCGGCTGGCACAGATCAGAGATT	ACCGTGGCCCACAGGACTCA	230
ZnT4	CATCCTGCTGGAGGGTGTA	CTGCAGTTGTACCGTGCAGT	247
ZnT5	TATCTCCAGTGGGAAGCTGG	ATCACTGCACACCCCATTTT	180
ZnT6	CCATCGCTCCGTCCTGGGGA	ACCGCCAGCACCTCGAAACG	282
ZnT7	CCCTTCCTGAATGCTACCAA	CACCGACCTGTGTGAAGATG	180
ZnT8	ATCGTCTTGATGGAAGGCAC	TTTCTCGAAGCACCTCCTGT	187
ZnT9	CCTGTTTTGGTTGGCAAAGT	GAATGCTCTCTGCCTTCGTC	240

Table 2

Normalized ZIP and ZnT gene copies expressed as a fold change with respect to 0hpf for each gene.

Gene	0 hpf	2 hpf	6 hpf	12 hpf	24 hpf	48 hpf	120 hpf
ZIP1	1.0 ± 0.2	1.17 ± 0.17	2.12 ± 0.34 *	1.75 ± 0.22	0.59 ± 0.1	1.21 ± 0.02	3.44 ± 0.85 *
ZIP3	1.0 ± 0.04	1.08 ± 0.11	1.05 ± 0.17	3.16 ± 0.56 *	6.79 ± 0.29 *	18.58 ± 1.42 *	60.07 ± 14.3 *
ZIP4	1.0 ± 0.16	1.38 ± 0.16	0.79 ± 0.21	4.26 ± 0.47 *	2.68 ± 0.04 *	5.60 ± 1.31 *	80.7 ± 6.02 *
ZIP6	1.0 ± 0.13	0.83 ± 0.13	0.41 ± 0.04 *	0.76 ± 0.06	0.64 ±0.12	1.14 ± 0.27	1.86 ± 0.20
ZIP7	1.0 ± 0.3	0.87 ± 0.35	0.74 ± 0.09	3.63 ± 0.55 *	3.53 ± 0.50 *	4.54 ± 0.27 *	11.91 ± 2.41 *
ZIP8	1.0 ± 0.26	1.84 ± 0.94	4.51 ± 0.15 *	60.0 ± 6.61 *	40.4 ± 4.81 *	273.3 ± 115.0 *	456.29 ± 14.50 *
ZIP9	1.0 ± 0.26	0.80 ± 0.12	0.19 ± 0.02 *	0.53 ± 0.14	0.82 ± 0.33	1.38 ± 0.16	4.49 ± 0.92 *
ZIP10	1 ± 0.27	1.22 ± 0.046	1.24 ± 0.20	1.89 ± 0.23 *	1.47 ± 0.13	2.89 ± 0.34 *	5.04 ± 1.23 *
ZIP11	1.0 ± 0.24	0.76 ± 0.13	0.10 ± 0.01 *	0.46 ± 0.02 *	1.08 ± 0.28	1.39 ± 0.17	3.64 ± 0.52 *
ZIP13	1.0 ± 0.09	0.79 ± 0.16	0.22 ± 0.02 *	0.26 ± 0.04 *	0.48 ± 0.16 *	0.62 ± 0.15	1.27 ± 0.21
ZnT1	1.0 ± 0.32	1.38 ± 0.15	4.73 ± 0.46 *	6.65 ± 0.038 *	5.88 ± 0.72 *	14.78 ± 3.18 *	82.47 ± 13.27 *
ZnT2	1.0 ± 0.30	2.037 ± 0.62	0.78 ± 0.04	1.07 ± 0.18	50.16 ± 6.24 *	247.67 ± 68.88 *	614.52 ± 32.02 *
ZnT4	1.0 ± 0.30	0.58 ± 0.10	0.15 ± 0.01 *	0.24 ± 0.09 *	0.70 ± 0.23	1.17 ± 0.21	4.63 ± 0.43 *
ZnT5	1.0± 0.18	1.33 ± 0.36	0.17 ± 0.04	0.35 ± 0.16	0.36 ± 0.21	1.61 ± 0.74	11.72 ± 1.48 *
ZnT6	1.0 ± 0.16	0.88 ± 0.24	0.41 ± 0.04	1.01 ± 0.25	0.91 ± 0.11	2.11 ± 0.52	4.92 ± 0.74 *
ZnT7	1.0 ± 0.17	0.87 ± 0.08	0.99 ± 0.14	1.12 ± 0.13	1.72 ± 0.51	3.04 ± 0.75 *	6.34 ± 0.41 *
ZnT8	1.0 ± 0.61	1.20 ± 0.13	646.18 ± 103.45 *	1047.62 ± 111.58 *	1681.94 ± 401.69 *	4957.78 ± 787.02 *	2097.04 ± 716.99 *
ZnT9	1.0 ± 0.20	0.83 ± 0.21	0.06 ± 0.01 *	0.16 ± 0.03 *	0.34 ± 0.12 *	1.57 ± 0.26	4.47 ± 0.96 *

* P<0.05 versus 0 hpf (1-way ANOVA, Dunnett's multiple comparison test)