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Maternal Transcriptome Analysis of Medaka (*Oryzias latipes*) Embryos by Use of mRNA–seq

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In this study, we investigated the transfer of maternal transcripts to embryos during the early stages of development in medaka (*Oryzias latipes*). Using mRNA–Seq, we analyzed gene expression levels in medaka embryos, focusing on maternal transcripts during embryonic development from immediately after fertilization to the 7th day post–fertilization. We found that 1,560 transcripts were detected only in the early development stage and that the levels of these transcripts decreased as development progressed. MKRN4, a known medaka maternal transcript, was among the detected transcripts, as were CYP26A1 and NANOG. Our results show that CYP26A1 and NANOG are maternally transported from female fish and play an important role in embryonic development. In this study, we confirmed that using an mRNA–Seq time–course following embryonic development can be used to investigate maternal transcript transfer to embryos.

Key words: mRNA–Seq, maternal transcript, CYP26A1, NANOG

INTRODUCTION

The early stages of embryonic development are important for subsequent development, and maternally transferred substances are believed to play an important role in these processes. Some reports have shown that embryos spawned from adult female fish exposed to chemicals show effects of that maternal exposure. We previously reported that in *ovo*–nanoinjection of 3–hydroxybenzo[*c*]phenanthrene into medaka embryos, which simulates maternal transfer, led to an acceleration of embryonic development and significant changes in gene expression in the early embryos (Chen *et al*., 2016). In addition, exposure to tributyltin and polychlorinated biphenyls (PCBs) caused a decrease in spawning performance, a reduction in survival rate, and an increase in malformation in the next generation (Nirmala *et al*., 1999; Qiu *et al*., 2019). Some reports have suggested that larvae malformation is linked to the concentration of RA (retinoic acid) in the embryo, which is regulated by CYP26A1 (cytochrome P450, family 26, subfamily A, polypeptide 1). RA is metabolized from maternal vitamin A in the early stages of embryonic development and plays an important role in anterior–posterior axis formation (Conlon, 1995); however, excessive RA levels can cause teratogenesis (Cahu *et al*., 2003; Gevers and Denuc, 1993). CYP26A1 has been reported to be a maternal transcript (McCaffery *et al*., 2003), but maternal expression of CYP26A1 has not been confirmed in medaka embryos. In zebrafish, CYP26A1 has been shown to rescue embryos from excess RA–induced teratogenesis (Hernandez *et al*., 2007).

Female fish are known to transfer some transcripts to their embryos to establish metabolism and protect their offspring from harmful effects caused by other maternal substances. Therefore, a clearer understanding of maternal transcripts should help us better understand the embryonic development cascades and effects caused by the environment of the embryos and adult fish.

Medaka (*Oryzias latipes*) is a fresh water fish that is native to Asia, primarily Japan, and is frequently used as a model organism for fresh water research (Wittbrodt *et al*., 2002). Its short life cycle, small size, and high reproduction rate makes the medaka fish and its embryos easy to observe in the laboratory. In addition, the genome of the medaka has been sequenced completely (Kasahara *et al*., 2007; Takeda, 2008), and the development stages of the medaka are well known (Iwamatsu, 2004). These advantages make the medaka a perfect model organism for many fields of study, including embryology, genetics, and ecotoxicology. Morphological observations of the medaka embryonic developmental stages have also been performed; however, time–course studies of changes in gene expression levels, including the expression of maternal transcripts, during embryonic development have not been reported.

Recent advances in mRNA–Seq analysis have facilitated the detection of the levels of enormous numbers of transcripts simultaneously. In our previous study, we used medaka embryonic mRNA–Seq to show that in ovo nanoinjection of 3–hydroxybenzo[*c*]phenanthrene into medaka embryos causes a significant change in the expression levels of many genes and accelerates embryonic development (Chen *et al*., 2016). These findings indicated that changes in gene expression in the early stages of development markedly affect embryonic devel-

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opment and suggested that the maternal transcripts were affected by the nanoinjected 3–hydroxybenzo[*c*] phenanthrene. An mRNA–Seq time–course following embryonic development should, therefore, be useful to investigate and detect maternal transcripts.

Accordingly, this study was performed to analyze gene expression levels in medaka embryos by means of mRNA–Seq, mainly focusing on maternal transcripts, during early embryonic development from immediately after fertilization to the 7th day post–fertilization (dpf). To our knowledge, this is the first report to confirm the expression of CYP26A1 and NANOG as maternal transcripts by using mRNA–Seq in the early developmental stages of the medaka embryo.

MATERIALS AND METHODS

Medaka fish and embryos

Embryos for this study were taken from 15 adult medaka fish (mating pairs, 10 female and 5 male) of the strain bred in our laboratory. The mating pair adult fish were put into a culture tank (60 cm × 30 cm × 30 cm) with artificial seawater (1‰ salinity, 45 l). The water temperature was 27 ± 1°C, and the illumination cycle was light 14 h and dark 10 h. The fish were fed *Artemia* nauplii juveniles (24–h incubation) twice per day (10:00 and 17:00). Fertilized embryos were taken within 5 min of spawning from the medaka mating pairs, and fertilized healthy embryos were selected under an anatomical microscope (SZX12, Olympus, Tokyo). The selected embryos were sterilized in 0.9% H_2O_2 for 10 min to minimize the risk of fungal or bacterial infections (Marking *et al*., 1994). The embryos were then cleaned with and cultured in embryo culture medium (ECM) solution (0.1% NaCl, 0.003% KCl, 0.004% $CaCl_2$–H_2O, 0.008% $MgSO_4$, 0.0002% Methylene Blue, pH 7.0). Embryos were cultured in petri dishes kept on a shaker (30 rpm) at 27 ± 1°C.

Total RNA extraction

At 0, 1, 3, 5, and 7 days post–fertilization (dpf), 10 embryos were randomly selected, pooled, and transferred into 1.5–ml tubes containing 500 μl of trizol–1%SDS, and homogenized (vortex, 20 s). The 0 dpf sample was treated within 30 min after fertilization. Samples were stored at –80°C until RNA extraction. Total RNA was extracted following the protocol developed by Piotr Chomczynski and Nicoletta Sacchi (Chomczynski and Sacchi, 1987), and by using an RNeasy Mini Kit (Qiagen, USA).

mRNA quality analysis and mRNA sequencing

mRNA was purified from total RNA by using the NEBNext Poly (A) mRNA Magnetic Isolation Module (New England Biolabs Inc., USA). The quality of the mRNA samples was assessed and a parameter called the RNA integrity number (RIN) was generated by a bioanalyzer (Agilent 2100 Bioanalyzer, Agilent Technologies, USA). The threshold for high quality mRNA is an RIN value > 8.0, and the mRNA RIN values of our samples were > 8.0. The mRNA was sequenced by using the NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs Inc.), the NEBNext Multiplex Oligos for Illumina (Index Primers Set 1, New England Biolabs Inc.), and the Miseq Reagent Kit v3 (300 cycles) (Illumina, MS–102–3003, USA) according to the manufacturers' instructions.

mRNA–Seq data analysis

The quality of reads from each file was checked with the software FastQC (version 0.11.8, https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Short–length fragments and low quality sections of mRNA reads were cut by using Trimmomatic (version 0.38, http://www.usadellab.org/cms/?page =trimmomatic). In this study, we focused on the chromosomal genome; therefore, non–chromosomal mRNA reads (ribosomal mRNA reads and mitochondrial mRNA reads) were removed by using RiboPicker (version 0.4.3, http://ribopicker.sourceforge.net). The cleaned mRNA reads were analyzed with HISAT2 (version 2.1.0, https://ccb.jhu.edu/software/hisat2/index.shtml), SAMtools (version 1.9, http://www.htslib.org), StringTie (version 1.3.5, https://ccb.jhu.edu/software/stringtie), and Ballgown (version 3.8, https://www.bioconductor.org/packages/release/bioc/html/ballgown.html). mRNA–Seq data analysis was performed according to the protocol developed by Pertea, *et al*. (2016). Gene expression was quantified as FPKM (fragments per kilobase of transcript per million fragments sequenced). The genome sequence and annotation information of Japanese medaka (*O. latipes*) were downloaded from Ensembl (http://asia.ensembl.org/index.html) in April 2019.

RESULTS

mRNA–Seq analysis findings

In this study, 1.1, 2.8, 3.1, 2.0, and 2.4 million paired–end reads (250 bp) were sequenced from the 0, 1, 3, 5, and 7 dpf samples, respectively. The data set of raw reads obtained in this study was submitted to the DDBJ Sequence Read Archive (https://www.ddbj.nig.ac.jp/dra/index.html) under the accession number DRA008340.

After the pipeline analysis, 59,429 transcripts were shown to be expressed in the medaka embryo. We removed the low expression transcripts (i.e., those for which the highest FPKM through embryonic development was < 16.00) in keeping with the protocol of Pertea, *et al*. (2016). This step enabled us to remove low or artifact mapped fragments including contaminating mRNA from the egg membrane or sperm cells. The expression levels of 36,751 transcripts were estimated to have markedly changed through the course of embryonic development. To identify maternal transcripts whose expression decreased during embryonic development, we categorized the transcripts into three types by using Ballgown: (1) 673 transcripts that were detected at only 0 dpf, (2) 887 transcripts whose expression continuously decreased during embryonic development, and (3)

841 transcripts whose expression continuously increased during embryonic development. In addition, we confirmed the expression of CYP26A1 and NANOG as transcripts of maternal origin.

Transcripts that were detected at only 0 dpf

We found that 673 transcripts were only detected at 0 dpf and were not expressed (FPKM = 0.00) on any other dpf in the embryos. Table 1a shows the top 20 transcripts that were detected at high levels at 0dpf. NANOG (Nanog protein; ENSORLT00000046319) and BTG4 (maternal B9.10 protein; ENSORLT00000019398) were annotated (Table 1a, Fig. 1a), but the other transcripts were not previously annotated and are, therefore, unknown transcripts.

Transcripts whose expression decreased continuously after fertilization

Our results show that 887 transcripts were detected at 0 dpf and had gradually decreasing expression levels (the FPKM at each dpf decreased) during embryonic development. Table 1b shows the top 10 transcripts that were detected at high levels at 0 dpf and whose expression levels then gradually decreased. As known transcripts, SUMO4 (small ubiquitin–related modifier 4; ENSORLT00000016957), MKRN4 (E3 ubiquitin–protein ligase makorin–1; ENSORLT00000008303), MT2 (metallothionein; ENSORLT00000019509), and NASP (nuclear

Table 1. Expression levels of the transcripts obtained in this study. FPKM (fragments per kilobase of transcript per million fragments sequenced) is used as a measure of the transcript expression levels. "–" indicates an unknown transcript that was not annotated

			FPKM				
Chromosome	Transcript ID	Transcript name	0 dpf	1 dpf	3 dpf	5 dpf	7 dpf
Transcripts whose levels significantly decreased after fertilization							
13	ENSORLT00000008566	Novel transcript	833.37	0.00	0.00	0.00	0.00
24	–	–	367.41	0.00	0.00	0.00	0.00
24	–	–	351.00	0.00	0.00	0.00	0.00
10	–	–	349.01	0.00	0.00	0.00	0.00
4	–	–	336.44	0.00	0.00	0.00	0.00
20	ENSORLT00000046319	NANOG	224.22	0.00	0.00	0.00	0.00
2	–	–	220.65	0.00	0.00	0.00	0.00
22	–	–	213.22	0.00	0.00	0.00	0.00
13	–	–	199.82	0.00	0.00	0.00	0.00
19	–	–	199.80	0.00	0.00	0.00	0.00
5	–	–	183.69	0.00	0.00	0.00	0.00
2	–	–	175.03	0.00	0.00	0.00	0.00
21	–	–	174.52	0.00	0.00	0.00	0.00
1	ENSORLT00000015064	Novel transcript	150.00	0.00	0.00	0.00	0.00
5	–	–	147.55	0.00	0.00	0.00	0.00
14	ENSORLT00000019398	BTG4	145.71	0.00	0.00	0.00	0.00
7	–	–	122.23	0.00	0.00	0.00	0.00
3	–	–	116.90	0.00	0.00	0.00	0.00
2	–	–	115.37	0.00	0.00	0.00	0.00
4	–	–	100.05	0.00	0.00	0.00	0.00
Transcripts whose levels gradually decreased after fertilization							
10	–	–	993.70	5.97	2.78	2.12	1.53
22	ENSORLT00000016957	SUMO4	733.21	455.33	290.76	203.89	163.59
17	–	–	674.36	83.46	40.41	11.82	7.80
10	ENSORLT00000001246	Novel transcript	427.38	15.15	9.74	3.03	2.17
4	ENSORLT00000022569	Novel transcript	403.36	153.61	122.75	113.19	95.36
15	ENSORLT00000003572	Novel transcript	371.25	288.10	147.16	80.57	77.69
7	ENSORLT00000008303	MKRN4	367.51	11.63	4.48	1.74	0.00
12	–	–	350.93	181.62	139.61	110.19	109.67
6	ENSORLT00000019509	MT2	347.29	237.88	186.94	134.30	65.38
17	ENSORLT00000013226	NASP	336.73	27.18	16.37	7.63	7.58
19	ENSORLT00000018195	CYP26A1	43.80	5.59	1.00	1.48	1.35
Transcripts whose levels continuously increased throughout embryonic development							
13	–	–	0.00	13.07	1419.83	4468.20	4721.81
2	ENSORLT00000003022	MYLZ3	0.00	0.34	227.28	1351.84	2931.78
8	–	–	0.00	1.27	131.94	816.62	1644.05
6	ENSORLT00000044115	TPM1	0.00	3.45	357.42	994.02	1404.60
6	–	–	0.00	0.34	326.85	751.01	899.30

autoantigenic sperm protein; ENSORLT00000013226) were observed (Table 1b, Fig. 1b). Three novel transcripts (ENSORLT00000001246, ENSORLT00000022569, and ENSORLT00000003572) were also observed (Table 1b, Fig. 1b).

We found that the expression of CYP26A1 (cytochrome P450, family 26, subfamily A, polypeptide 1) was similar to that of these transcripts, that is, it was detected at 0 dpf and then decreased from 1 dpf to 7 dpf (Table 1b, Fig. 1b).

Transcripts whose expression continuously increased throughout embryonic development

We found 841 transcripts that were not expressed at 0 dpf (FPKM = 0.00) but whose expression gradually increased during embryonic development. Table 1c shows the top 5 transcripts whose expression gradually increased and that were expressed at high levels until 7 dpf. As known transcripts, MYLZ3 (myosin light chain 3, skeletal muscle isoform; ENSORLT00000003022) and TPM1 (tropomyosin 1; ENSORLT00000044115) were observed (Fig. 2).

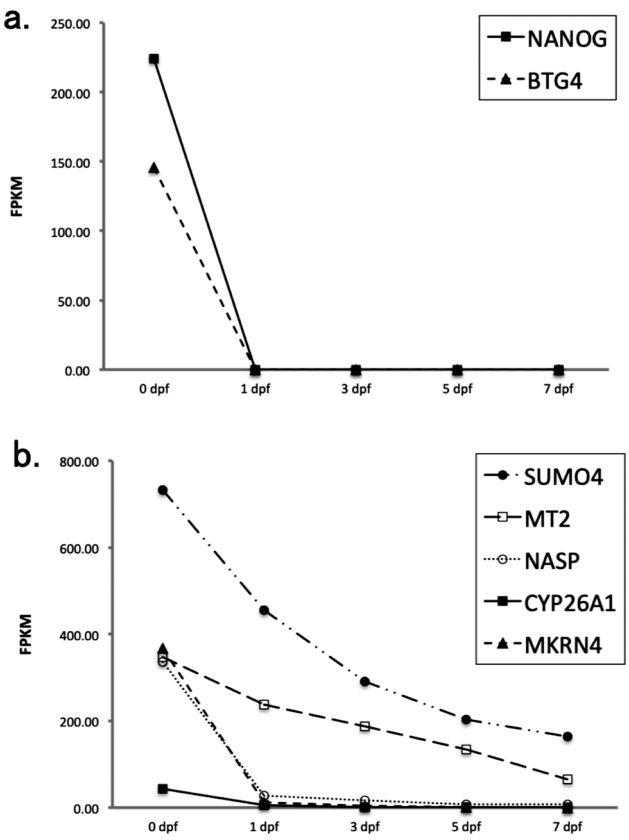

Fig. 1. Expression levels of candidate maternal transcripts.
FPKM (fragments per kilobase of transcript per million fragments sequenced) is used as a measure of transcript expression levels.

a. The expression levels of transcripts NANOG (ENSORLT00000046319) and BTG4 (ENSORLT00000019398); these transcripts were expressed at only 0 dpf (i.e., they were not expressed at other dpf; FPKM = 0.00).

b. The expression levels of transcripts SUMO4 (ENSORLT00000016957), MKRN4 (ENSORLT00000008303), MT2 (ENSORLT00000019509), NASP (ENSORLT00000013226), and CYP26A1 (ENSORLT00000018195); these transcripts were expressed at 0 dpf and their expression levels gradually decreased (FPKM decreased for each dpf) throughout embryonic development.

DISCUSSION

To our knowledge, this is the first report to investigate maternal transcripts in medaka embryos by using mRNA–Seq. Our results clearly identify candidate maternal transcripts in the medaka embryo. We found 36,751 transcripts whose expression levels markedly changed during embryonic development, and 1,560 transcripts, including the known maternal transcript MKRN4, were detected as candidate maternal transcripts. Hence, we confirmed that time–course mRNA–Seq that follows embryonic development can detect and identify maternal transcripts in the embryo.

In this study, we showed that CYP26A1 is present at high levels at 0 dpf and that its levels decrease slightly from 1 dpf to 7 dpf (Table 1b, Fig. 2). CYP26A1 regulates the level of RA, the active form of vitamin A, which has many critical roles in embryonic development, including embryonic organization (Cahu *et al.*, 2003). However, excessive levels of RA can cause teratogenesis (Cahu *et al.*, 2003; Gevers and Denuc, 1993). It has also been reported that RA degradation by CYP26A1 protects the embryo from teratogenesis caused by RA excess (Hernandez *et al.*, 2007). Moreover, CYP26A1 has been reported to play an important role in protecting the mouse embryo from teratogenesis caused by maternal RA (McCaffery *et al.*, 2003), although CYP26A has not yet been confirmed as a maternal transcript. In the present study, we used mRNA–Seq to show that CYP26A1 is present at 0 dpf and that its levels decrease markedly after fertilization in the medaka embryo. This finding suggests that CYP26A1 may be a maternal transcript that protects the early embryo from teratogenesis

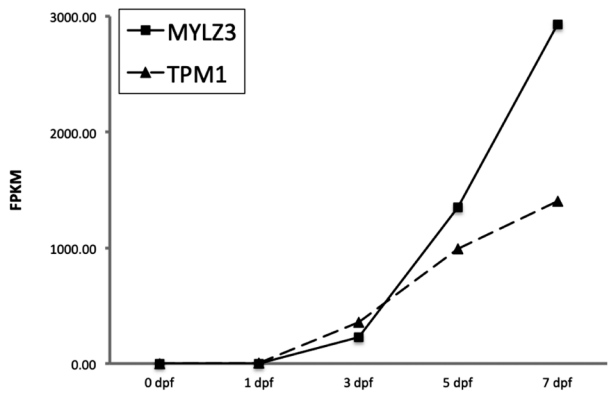

Fig. 2. Transcripts whose expression continuously increased throughout embryonic development.
The figure shows the expression levels of transcripts MYLZ3 (ENSORLT00000003022) and TPM1 (ENSORLT00000044115), which continuously increased through the course of embryonic development. FPKM (fragments per kilobase of transcript per million fragments sequenced) is used as a measure of transcript expression levels.

caused by excessive levels of RA in the early stage embryo.

In our study, we found that NANOG existed at high levels at 0 dpf and that its levels markedly decreased at 1 dpf (Table 1a, Fig. 1a). NANOG is key regulator of pluripotency in embryonic stem cells. Previously, it was reported that NANOG is expressed until stage 23/24 (1 day and 17–20 h post–fertilization) in the medaka embryo, and it was suggested that NANOG is a medaka maternal transcript (Camp *et al*., 2009; Iwamatsu, 2004). Here, we confirmed the presence of NANOG at 0 dpf, which confirms that NANOG is one of the maternal transcripts in medaka. This is the first report to confirm the maternal expression of NANOG in medaka. The details of NANOG functioning in medaka remain unclear, but it has been suggested that NANOG plays an important role in organizing pluripotency in medaka embryonic stem cells as a maternal transcript.

MT2 and other genes were detected at 0 dpf and their expression levels gradually decreased during development. MT2 is a metallothionein gene, and is a known stress–induced biomarker for metal exposure in fish since its expression is induced by exposure to metallic particles (Chae *et al*., 2009). Hence, MT2 may play an important role in detoxification of heavy metals during the early developmental stage. SUMO proteins, including SUMO4, regulate various cellular processes and are thought to have a role in medaka embryonic development (Seki *et al*., 2010). In most organisms, including *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, *Arabidopsis thaliana*, and mice, SUMO proteins function as protein modifiers, and SUMO modification is essential in these organisms (Geiss–Friedlander and Melchior, 2007). How SUMO4 functions in medaka is unclear, but it may play an important role in protein modification in the early stage of development in medaka. BTG4, a member of the BTG/TOB protein family, is thought to be involved in various physiological processes including regulation of embryonic development, cell differentiation, and apoptosis in mammals (Mauxion *et al*., 2009). The function of BTG4 in fish is unclear, but BTG4 may play some role in the regulation of medaka embryonic development. MKRN4 is a member of the makorin gene family of zinc–finger proteins with a typical C3HC4 motif called the RING domain. The function of makorins remains unclear, but it has been reported that MKRN4 is one of the maternal transcripts in medaka (Böhne *et al*., 2010). NASP is a chaperone protein that transports histone from the cytoplasm to the nucleus, and *Xenopus laevis* NASP is of maternal origin and is expressed in the early embryo (Kleinschmidt *et al*., 1985; Nabeel–Shah *et al*., 2014). The function of NASP in medaka is unclear; however, it may be involved in the regulation of embryonic development in medaka. Clearly the roles in medaka of many of the transcripts mentioned above have yet to be defined, but it is likely that these transcripts function as maternal transcripts playing important roles in organizing and protecting medaka embryonic development.

We confirmed that the expression of MYLZ3 and TPM1 increased during embryonic development. MYLZ3 and TPM1 are expressed in muscle, and the levels of transcripts involved in the construction of muscle increase from 0 dpf to 7 dpf. It makes sense that the levels of transcripts involved in muscle and tissue construction would increase during embryonic development.

AUTHOR CONTRIBUTIONS

Y. Takai designed the study, performed mRNA–Seq analysis and wrote the paper. K. Chen participated in the design of the study and performed mRNA–Seq analysis. S. Yoshitake participated in the design of the study and extracted total RNA. Y. Ogino supervised the study. K. Tashiro provided facilities and resources. Y. Shimasaki and Y. Oshima designed the study, supervised the work, wrote the paper and provided facilities and resources. All authors assisted in editing of the manuscript and approved the final version.

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