Profile analysis of expressed sequence tags derived from the ovary of tilapia, *Oreochromis mossambicus*

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Abstract

Precise regulation of gene expression is required for a proper transition through the different phases of the ovarian cycle. To study ovarian gene expression in teleosts, we constructed an adult ovary cDNA library of tilapia, *Oreochromis mossambicus*, and analyzed the gene expression profile using an expressed sequence tag-based strategy. Of 768 random clones, 530 clones, with insert lengths of >100 base pairs (bp), were assembled into 474 tentative unique genes (TUGs), including 34 contigs and 440 singlets. Among these, 230 TUGs were highly homologous to known genes in public databases, while others exhibited a low sequence homology or no significant matches to known sequences; these are called novel genes here. The abundance of each identified gene was assayed to evaluate its relative importance in ovarian functions. In addition, we analyzed the expression patterns for 6 development-and reproduction-related genes, including cofilin, fibulin-4, glucosamin-6-phosphate deaminase 2 (GNPDA), MCM-6, stathmin, and zona pellucida C5 (ZPC5), as well as 9 novel genes. Results showed that GNPDA, MCM6, ZPC5, and 7 of the novel genes, including O34, O141, O148, O149, O243, O258, and O319, were predominantly expressed in the ovary and/or testis. In addition, the expression patterns of gonad-specific novel genes in tilapia larvae were differentially affected by treatment with 17 β -estradiol and 17 α -methyltestosterone during the sex-sensitive period. © 2005 Elsevier B.V. All rights reserved.

Keywords: *Oreochromis mossambicus*; EST; Ovary; Cofilin; MCM-6; Stathmin

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1. Introduction

The ovary is a female reproductive organ with dual functions: the production of ova and the timely secretion of hormones for regulating growth, development,

and structural maintenance of animal life. Precise regulation of gene expression is required for the proper transition through different phases of the ovarian life cycle (Richards, 1994). Principally through the study of known genes, we have learned the basics of genes critical for the sequence of the hormone-dependent ovarian cycle. A primordial follicle is initiated in order to assemble extracellular coat proteins, such as zona pellucida 2 and zona pellucida 3, around the oocyte (Millar et al., 1991). An oocyte in the early growth stage expresses specific signaling factors, such as *c-kit*, a tyrosine kinase receptor (Packer et al., 1994). However, the factor which triggers the growth of primordial oocytes remains unclear. After initiation of early follicular growth, preovulatory follicular growth is mainly regulated by luteinizing hormone (LH) and follicular-stimulating hormone (FSH). LH alone or in cooperation with FSH elicits the growth, atresia, development, ovulation, and ultimately the formation and breakdown of the corpus luteum. The combination of LH and FSH induces highly synchronized and exquisitely timed gene expression for precise control of the ovarian cycle (Richards, 1994). Although, a few LH/FSH downstream effectors, such as adenylyl cyclase (Richards and Rolfes, 1980), are known, the regulatory mechanisms of the ovarian cycle are still far from being fully understood. It is very likely that other undiscovered genes may be involved in the control of ovarian function. In this regard, the expressed sequence tag (EST) strategy is remarkably suitable for the large-scale screening for novel genes and gene expression profile analysis.

EST cloning has been widely applied in a variety of organisms since its first use in the human genomic project (Adams et al., 1991). In addition to gene identification, one advantage of EST analysis is its ability to measure the relative abundance of expressed transcripts, which allows the evaluation of the relative importance of expressed genes. EST libraries of various organs and tissue from teleosts (Davey et al., 2001; Douglas et al., 1999; Gong et al., 1997; Hirono and Aoki, 1997; Inoue et al., 1997; Ju et al., 2000; Karsi et al., 2002; Kocabas et al., 2002; Martin et al., 2002; Zeng and Gong, 2002) have been constructed, including those for tilapia (Hamilton et al., 2000; Shiue et al., 2004). Of particular interest to us, an ovary library was produced for zebrafish (Zeng and Gong, 2002). The ovary cDNA library has yielded a

substantial amount of novel genes. In addition, several genes were shown to be specifically expressed in the ovary that may serve as useful molecular markers for the analysis of oogenesis and development.

Oreochromis mossambicus and its close relative, *O. niloticus*, are important fish species for both research and aquaculture. Tilapia are the second most important food fish, next to carp worldwide. They are also excellent laboratory animals because (1) they have a short generation time of 6–7 months, (2) the embryos and fry are easily obtained and reared in laboratory, and (3) transgenic animals can be produced relatively easily (Maclean et al., 2002). Despite their importance to fisheries, studies of their genome are still very preliminary and far behind other fish species like zebrafish or medaka. In addition, the feasibility of controlling the sexual differentiation by various methodologies, including genetics, changes in the environment, and drug treatments, make tilapia favorable models for the functional study of gonads. Therefore, in this study, we sequenced and analyzed 768 EST clones derived from an ovary cDNA library of adult *O. mossambicus* and identified 474 tentative unique genes. In addition, we also analyzed 6 development- and reproduction-related genes and 9 novel genes for their gene expression patterns. Furthermore, the expression patterns of 6 gonad-specific novel genes under the influences of 17 α -methyltestosterone and 17 β -estradiol were also analyzed during the sex differentiation-sensitive period from days 24 to 33 after fertilization. The establishment of the gene profile and its analysis of this tilapia ovary cDNA library are thus proving to be a fruitful gene identification source, which will benefit research of ovarian functions and sex differentiation of tilapia as well as other economically important cultured fishes.

2. Materials and methods

2.1. Construction of a cDNA library

Adults of *O. mossambicus* were obtained from the Mariculture Research Center, Fisheries Research Institute, Tainan, Taiwan. PolyA⁺ RNA was extracted using the NucleoTrap[®] mRNA Kit (BD Biosciences Clontech) from the ovaries of 3 females. One-half microgram of polyA⁺ RNA was utilized for the construction

of the library using a Creator™ SMART™ cDNA Library Construction Kit (BD Biosciences Clontech) according to the manufacturer's instructions. The titer of the primary cDNA libraries was about 1.5×10^6 cfu, and 5000 primary clones were randomly picked and stored in 96-well plates at -80°C .

2.2. Selection, sequencing, and size determination of cDNA clones

The cDNA clones of the primary library were randomly selected for single-pass 5' sequencing. Double-stranded DNA sequencing was carried out using the BaseStation™ DNA Fragment Analyzer (MJ Re-

search Base Station). Each clone was sequenced once from the 5' end using the vector sequencing primer pDNR-F (5'-TTATACGAAGTTATCAGTCGAC-3'). To evaluate the size of the insert in a cDNA clone, the inserted DNA fragments of plasmids isolated from each individual colony were amplified by PCR using the M13 sequencing primer pair (forward, 5'-GTAAAACGACGGCCAGT-3' and reverse, 5'-AAACAGCTATGACCATGTTCA). PCR amplification was performed in a 10- μl reaction volume with the thermostable DNA polymerase (*TaKaRa Taq*™, TaKaRa Biochemicals). PCR was performed using a thermal cycler (PTC-200, MJ Research) for 25 cycles according to the following protocol: denaturation at

Table 1

Primer sequences for each gene used in the analysis of the temporal and spatial gene expression patterns by RT-PCR

Group ID*	Gene annotation	Primer sequences
TO_TUS_217	cofilin	Forward: 5'-GATCATCGTGAAGAGGGAA-3' Reverse: 5'-GAGACATCACAGCTGCTTGC-3'
TO_TUS_293	fibulin-4	Forward: 5'-GAGTGTACGGAGACGCAGAACT-3' Reverse: 5'-AATGGACACCCATCTCCAGGTC-3'
TO_TUS_13	glucosamin-6-phosphate deaminase 2	Forward: 5'-GTCGACATGAGGCTGGTCATTCTC-3' Reverse: 5'-GGATTCTGGTCTTTATGCTGAGC-3'
TO_TUS_356	MCM6	Forward: 5'-TGGATGAGATCCGCAGATACCT-3' Reverse: 5'-GCGAAAGACAGACGCAGAGAT-3'
TO_TUS_437	stathmin	Forward: 5'-GTCGACATGGCAGCCGCTGAAGATA-3' Reverse: 5'-GGATTGCGCTCCATCTTCTGGTTGA-3'
TO_TUS_10	ZPC5	Forward: 5'-CCTCAGTCAGAGTTGGACAT-3' Reverse: 5'-GTGTAGCAGTCAGGAGCAGT-3'
TO_TUS_34	O34	Forward: 5'-GAAGGATGCAGAGATGCCTGTAC-3' Reverse: 5'-TGACAGACCGTGAGCTCGAATT-3'
TO_TUS_62	O62	Forward: 5'-AACGACAGTGTACGAAAAACCC-3' Reverse: 5'-GGTGAGTCCAGGTGAGGAAACATA-3'
TO_TUS_141	O141	Forward: 5'-ATCACTGGATGGAAAAATAGTA-3' Reverse: 5'-TGATGTGGGCCTTAAACC-3'
TO_TUS_148	O148	Forward: 5'-ACCTGAGGGAGAGAACGGGG-3' Reverse: 5'-GTAGCACCTGGAGGTGAAGGG-3'
TO_TUS_149	O149	Forward: 5'-GGGGGAAACTATTATTCT-3' Reverse: 5'-AGGAAGACAAATCCTGTTTAA-3'
TO_TUS_175	O175	Forward: 5'-GCAGGATCACAACCAGGTCAAT-3' Reverse: 5'-AGACGTTGACGAGATAGTGG-3'
TO_TUS_243	O243	Forward: 5'-GGAGGCTGAGAAACGAAAGC-3' Reverse: 5'-GATGTCGTCGTCGCCATCTG-3'
TO_TUS_258	O258	Forward: 5'-ACGGTGTATGGAGTGAGGTAATGC-3' Reverse: 5'-GGCTTATCCTGGAGTTAGGCTTC-3'
TO_TUS_319	O319	Forward: 5'-GCAAAGGATGCAGTGAGGAC-3' Reverse: 5'-CGAGCTGTCAGGATCACGTCA-3'
AB037865 (Genbank No.)	β -actin	Forward: 5'-ACCACCACAGCCGAGAGGGA-3' Reverse: 5'-TTGTTGGGCGTTTGGTTGGG-3'

* Group ID is the identification number used at the tilapia ovary sequence web site (http://140.109.18.216/~public/EST_Analysis/public/tilapia/ovary/index.htm).

Fig. 1. Insert size distribution of clones isolated from a cDNA library of the tilapia ovary. The cDNA clone insert size was determined by PCR as described in "Materials and methods". We successfully determined the insert sizes of 665 clones and found that most clones (643) had an insert size of between 0.1 and 2.5 kb. The most-abundant groups (55.6%) were clones with insert sizes ranging from 0.9 to 1.5 kb, and 14% clones had an insert size of 0.1–0.3 kb.

94 °C for 30 s, annealing at 45 °C for 30 s, and elongation at 72 °C for 1.5 min.

2.3. Sequence analysis and annotation

After elimination of the vector sequences and correction of obvious reading errors, all sequences larger than 100 bp were submitted to the Bioinformatics Center (National Yang-Ming University, Taipei, Taiwan) for further analysis. Identification of a homologous cDNA clone was based on the e-value reported in the blast results against selected NR databases, including Non-redundant GenBank CDS translations, PDB, SwissProt, PIR, and PRF. An identified clone generally has a matching sequence e-value of $<10^{-10}$. A clone containing the complete coding region was identified from the 5'EST sequence covering the start and stop codons of a matching sequence. Reverse sequencing from the 3'end was performed as necessary to obtain the complete coding region sequence. Using the BGSSJ20040204 software developed by the Institute of Information Science (Academia Sinica, Taipei, Taiwan), we first collected UniGene accession numbers for known TUGs from the NCBI UniGene database (available at <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene>). Second, we obtained gene ontology information of each TUG according to its UniGene accession number or symbol in the Gene Ontology Consortium (available at <http://www.geneontology.org/>). If no gene ontology information was available for the matching sequence with the highest score, the

gene ontology was determined according to the next matching sequence and so on until the matching sequence's e-value exceeded 10^{-10} .

2.4. Animal husbandry and hormonal treatments of tilapia larvae

Adult tilapia (*O. mossambicus*) were reared in a freshwater tank at 24–28 °C. Fertilized eggs were collected from mouth-brooding females and kept in a 28 °C freshwater tank with sufficient aeration. Five

Fig. 2. Increase in identifiable tentative unique genes (TUGs) with the number of clones sequenced in the tilapia ovary cDNA library. Numbers of tentative unique contigs (TUCs), tentative unique singlets (TUSs), and TUGs are plotted against the numbers of clone sequenced. TUGs and TUSs increased rapidly with an increase in the number of clones sequenced, but the TUCs increased much more slowly.

days after hatching, larvae were subdivided into 3 groups. Larvae were fed regular feed with the following ingredients: 16% fish meal, 30% rice bran, 25% lupin seed meal, 20% wheat flour, 3.4% fish oil, 1% vitamin mix, 4% mineral mix, 0.5% choline chloride, and 0.1% vitamin E (50% purity). For the 2 experimental groups, larvae received additives of 100 mg 17 β -estradiol (E_2) per kg feed during days 7–20 or 100 mg 17 α -methyltestosterone (MT) per kg feed during days 10–20, respectively.

2.5. Gene expression analysis using RT-PCR

Six development-and reproduction-related clones and 9 novel genes were selected for analysis of tissue-specific and temporal gene expression. The tissue expression patterns of the selected genes were determined by RT-PCR using the primers listed in Table 1. Total RNAs were prepared using the Trizol reagent (Invitrogen) from different adult tilapia tissues and fry at designated stages. For synthesizing single-stranded

Table 2

List of 25 identified clones containing the complete coding sequences from an *Oreochromis mossambicus* ovary cDNA library

Tilapia ovary ESTs			Matching sequences				
Accession number	Putative annotation	Functional class	Accession number	Species	E-value	Identity (%)	Overlap/total amino acids
AY737028	CCR4-NOT transcription complex, subunit 7	1	AAH07315.1	<i>Homo sapiens</i>	6e-38	90	84/246
AY737029	Unnamed protein product/ CKN1 protein	1,5	CAG10886.1	<i>Tetraodon nigroviridis</i>	1e-127	89	221/247
AY737031	Copper chaperone	1	AAF01286.1	<i>Canis familiaris</i>	5e-17	58	68/68
AY737032	CUTL1 protein	1,3,4	AAH66592.1	<i>Homo sapiens</i>	8e-72	91	104/1516
AY737033	Cytochrome c oxidase subunit IV isoform 2, mitochondrial precursor	1	AAF79934.1	<i>Thunnus obesus</i>	3e-61	62	110/176
AY737034	D7-like protein	13	AAC63308.1	<i>Perca flavescens</i>	4e-52	56	150/152
AY737035	Unnamed protein product/similar to DNA polymerase delta subunit 3	1,2	CAF97746.1	<i>Tetraodon nigroviridis</i>	1e-120	53	250/467
AY737036	Niemann-Pick disease, type C2	13	NP_775331.1	<i>Danio rerio</i>	2e-53	66	148/149
AY737037	F-box only protein 36	13	NP_079662.1	<i>Mus musculus</i>	1e-18	50	82/188
AY737038	G10 protein	1	AAT68045.1	<i>Danio rerio</i>	9e-72	93	126/135
AY737040	Hypothetical protein LOC90624	13	NP_859056.1	<i>Homo sapiens</i>	2e-25	54	103/104
AY737041	Hypothetical protein MGC64043	13	NP_956664.1	<i>Danio rerio</i>	4e-31	53	118/124
AY737043	glucosamine-6-phosphate deaminase 2	1,4	NP_612208.1	<i>Homo sapiens</i>	2e-95	79	211/276
AY737044	Microsomal glutathione S-transferase	13	AAL12230.1	<i>Oryctolagus cuniculus</i>	3e-48	68	139/152
AY737045	Mitochondrial carrier homolog 2	2	NP_571457.1	<i>Danio rerio</i>	1e-148	83	300/300
AY737046	Unnamed protein product/similar to myo-inositol monophosphatase	1	CAG05496.1	<i>Tetraodon nigroviridis</i>	1e-128	80	227/283
AY737047	Myosin light chain (19.9 kD)	4,7	NP_999864.1	<i>Danio rerio</i>	9e-91	94	163/172
AY737048	Proteasome (prosome, macropain) subunit, alpha type 1	1,2	NP_036095.1	<i>Mus musculus</i>	1e-82	93	153/263
AY737049	Selenoprotein W2a	13	NP_919398.1	<i>Danio rerio</i>	1e-34	72	92/92
AY737051	Similar to small acidic protein	13	NP_956488.1	<i>Danio rerio</i>	3e-35	74	74/100
AY737053	Unnamed protein product /Tm4sf3-prov protein	1	CAG12382.1	<i>Tetraodon nigroviridis</i>	6e-51	46	224/225
AY737054	Trappc3-prov protein	2	AAH53802.1	<i>Xenopus laevis</i>	1e-18	87	49/180
AY737059	SI:bZ1F1.7 (novel protein similar to human AKAP-associated sperm protein (ASP)	13	CAE17582.1	<i>Danio rerio</i>	5e-70	65	198/212
AY737060	Unnamed protein product (BAB27540)/ CDC28 protein kinase 1	1,2	BAB27540.1	<i>Mus musculus</i>	2e-28	78	74/49
AY737062	zgc:55453/ compass (complex proteins associated with Set1p) component	1,2	NP_955845.1	<i>Danio rerio</i>	1e-104	90	180/198

cDNAs, 3 µg of total RNA, oligo dT primers, and the M-MLV Reverse Transcriptase (Promega) were applied in a total reaction volume of 25 µl. RT-PCR was performed with respective primers for 25–30 cycles following the PCR protocols described previously at the optimal annealing temperatures of 46–60 °C.

3. Results

3.1. Summary of EST clones in the tilapia ovary cDNA library

In total, 768 random clones from the tilapia, *O. mossambicus*, ovary cDNA library were partially sequenced from the 5' end, and the insert sizes were determined by PCR, among which, 665 clones were successfully determined. Most clones (643) contained an insert of between 0.1 and 2.5 kb. The most-abundant clone insert sizes ranged 0.9–1.5 kb (364, 55.6%) and 0.1–0.3 kb (92, 14%) (Fig. 1). After vector sequence trimming, 69% (530) of the EST sequences were found to exceed 100 bp in length. These EST sequences were then subjected to further analysis and assembled into the tentative unique contigs (TUCs) and singlets (TUSs). The assembly of TUCs and TUSs forms the tentative unique genes (TUGs). To evaluate the potential of this ovary library to supply identifiable TUGs, we analyzed the efficiency of gene discovery for every 100 clones as shown in Fig. 2. For the first 500 clones, numbers of TUSs and TUGs rapidly increased with a slight rise in the number of TUCs. The percent TUGs of sequenced clones gradually decreased from 86.0% to 70.4%, which was due to the repeat sequences of the same genes. The percent TUCs of sequenced clones remained relatively constant from 6.8% to 8.0%. In total, 530 EST sequences were assembled into 34 TUCs and 440 TUSs. These TUCs and TUSs were compared by blasting against all non-redundant GenBank CDS translations, PDB, SwissProt PIR and PRF, which resulted in 230 TUGs (274 clones, with an expected threshold value of $<10^{-10}$). The cDNA identification rate was 52% (274 of 530 clones). Among the identified cDNAs, 10.9% (25 of 230) TUGs contained the complete protein coding region with identified start and stop codons when compared to known genes. Sequences of

those clones have been submitted to GenBank as listed in Table 2. Table 2 shows the gene accession numbers with details of the most-closely matched sequence species along with homology values and functional classifications as described below. All other sequences are available at http://140.109.18.216/~public/EST_Analysis/public/tilapia/ovary/index.htm, which is built and maintained at the Institute of Information Science, Academia Sinica, Taipei, Taiwan.

3.2. Functional categorization of the identified tilapia ovary TUGs

In total, 230 identified TUGs were classified into 13 broad biological process categories according to the Gene Ontology Consortium (available at <http://www.geneontology.org/>), and numbers of TUGs in the various categories are presented in Table 3. Because a gene may have multiple possible functions, some TUGs were assigned to more than 1 category. Apart from those genes with unclassified functions (30%, 69/230), the largest group of TUGs was allocated to metabolism (41.7%, 96/230), followed by cell growth and/or maintenance (25.7%, 59/230), cell communication and differentiation (7.8%, 18/230), development and reproduction (6.5%, 15/230),

Table 3
Functional distribution of identified tentative unique genes (TUGs) in the ovary library

Number	Category ^a	Number of TUGs
1	Metabolism	96
2	Cell growth and/or maintenance	59
3	Cell communication and differentiation	18
4	Development and reproduction	15
5	Response to stress or stimulus	6
6	Homeostasis	4
7	Cell motility	3
8	Behavior	3
9	Death	2
10	Secretion	2
11	mRNA splicing	1
12	Obsolete biological process	1
13	Unclassified	69

^a The category classification was based on the Gene Ontology Consortium (available at <http://www.geneontology.org/>) as described in “Materials and methods”.

responses to stresses or stimuli (2.6%, 6/230), homeostasis (1.7%, 4/230), cell motility (1.3%, 3/230), behavior (1.3%, 3/230), death (0.8%, 2/230), secretion (0.8%, 2/230), and single appearances of a gene involved in mRNA splicing and a gene for an obsolete biological process, respectively.

3.3. Analysis of gene abundances of the identified tilapia ovary TUGs

The most-abundant TUGs, which appeared at least twice among the cDNA clones analyzed, in the ovary library are summarized in Table 4. These abundant

Table 4
Most-abundant ovary cDNA clones

Tilapia ovary ESTs				Matching sequences				
Accession number	Putative annotation	Frequency	Functional class	Accession number	Species	E-value	Identity (%)	Overlap/total amino acids
<i>Housekeeping genes</i>								
AY737014	Quinone reductase	7	1	AAD38913.1	<i>Oryzias latipes</i>	2e-36	65	117/270
AY737036	Niemann-Pick disease, type C2	5	13	NP_775331.1	<i>Danio rerio</i>	2e-53	66	148/149
AY737015	Arylamine N-acetyl transferase	3	1	P13913	<i>Gallus gallus</i>	6e-60	50	222/290
AY737055	Ubiquitin	3	1	EAK88214.1	<i>Cryptosporidium parvum</i>	2e-77	92	154/167
AY737016	XNop56 protein	3	2	CAC44272.1	<i>Xenopus laevis</i>	4e-35	85	85/532
AY737044	Microsomal glutathione S-transferase	2	13	AAL12230.1	<i>Oryctolagus cuniculus</i>	3e-48	68	139/152
AY737017	Proteasome endopeptidase complex chain XC8	2	1	S38529	<i>Xenopus laevis</i>	1e-71	88	133/151
AY737018	Pyruvate dehydrogenase lipoamide alpha 1	2	1	AAH60928.1	<i>Danio rerio</i>	1e-100	88	117/393
AY737019	Unknown protein for MGC:77119/pan-epithelial glycoprotein	2	2,3,5	AAH66716.1	<i>Danio rerio</i>	4e-73	61	214/302
AY737020	Zgc:77127 protein/thioredoxin 2	2	2,6	AAH65316.1	<i>Danio rerio</i>	3e-51	67	104/155
<i>Non-house keeping genes</i>								
AY737021	Ferritin, middle subunit Ferritin M	4	2,6	AAB34576.1	<i>Salmo salar</i>	7e-52	89	97/108
AY737022	Unknown protein for MGC:56095/Ferritin lower subunit	2	2,5,6	AAH45905.1	<i>Danio rerio</i>	3e-66	68	172/179
AY737060	Unnamed protein product (dbj BAB27540.1)/CDC28 protein kinase 1	4	1,2	BAB27540.1	<i>Mus musculus</i>	2e-28	78	74/49
AY737023	G2 mitotic-specific cyclin B	2	2	P07818	<i>Xenopus laevis</i>	8e-63	86	115/255
AY737024	Calmodulin	2	2,3	AAO17827.1	<i>Paralichthys olivaceus</i>	3e-17	100	43/65
AY737047	myosin light chain (19.9 kD)	2	4,7	NP_999864.1	<i>Danio rerio</i>	9e-91	94	163/172
AY737034	D7-like protein/KLRL1	2	13	AAC63308.1	<i>Perca flavescens</i>	4e-52	56	150/152
AY737025	Interleukin-12 p40	3	3	BAC81423.1	<i>Takifugu rubripes</i>	4e-23	51	105/359
AY737026	Coded for by human cDNAs W37389(gb AAB46345.1)/ocular development associated gene	2	13	AAB46345.1	<i>Homo sapiens</i>	3e-26	88	58/140
AY737027	ZPC1	3	13	AAN31188.1	<i>Oryzias latipes</i>	1e-47	61	82/134
AY737039	Hypothetical protein BC012173/ Elongator associated protein	2	1,2	CAG13120.1	<i>Tetraodon nigroviridis</i>	2e-52	80	95/118

TUGs are comprised of 59 clones representing 11.1% (59/530) of the analyzed clones. About half of these abundant TUGs (47.6%, 10/21) are housekeeping genes, such as quinone reductase, arylamine N-acetyl transferase, Niemann-Pick disease type C2 protein, an ubiquitin precursor, XNop56 protein, proteasome endopeptidase complex chain XC8, pyruvate dehydrogenase lipoamide alpha 1, pan-epithelial glycoprotein, microsomal glutathione S-transferase, and thioredoxin 2. The remaining 11 genes (called non-housekeeping genes), including ferritin and others, may play functional roles in the ovary and are discussed in the “Discussion” section.

3.4. Tissue distribution analyses of selected development-and reproduction-related genes

We were particularly interested in genes which may be involved in the regulation of ovarian functions. Therefore, 6 genes allocated to the development and reproduction category were selected from this ovary library in order to analyze their expression patterns in different adult tilapia tissues by RT-PCR (Fig. 3). Cofilin was expressed in all tissues, but was much lower in the liver. Fibulin-4 was mainly expressed in the heart and only slightly in the skeletal muscle, kidney, spleen, and gill. Glucosamin-6-phosphate deaminase 2 (GNPDA/oscillin) was strongly expressed in the testis and was much weaker in

other tissues except the intestine. MCM6 was strongly expressed in the ovary and testis where strong replication activities occur and was also strongly expressed in eye. Stathmin was strongly expressed in the brain, eye, ovary, testis, and heart but showed lower expression in the skeletal muscle, kidney, and spleen. ZPC5 was specifically expressed in the ovary, but not in other tissues, which further confirms its role as an oocyte-specific component. An RT-PCR of β -actin, a housekeeping gene, was also performed to serve as an expression control among different tissues.

3.5. Spatial and temporal expression of novel ovary-derived genes

More than half of the TUGs identified in this tilapia ovary library lacked a significant match with known gene sequences. To study the expression of those potential novel genes, we selected 9 clones with an insert size exceeding 500 bp for the RT-PCR analysis in different adult tissues. As shown in Fig. 4, all genes were strongly expressed in the ovary except for O62, which was only slightly expressed; most genes were expressed in the testis as well except for O34; genes were also sparingly expressed in other tissue with wider expressions exhibited by O149 and O319.

We further examined the expression patterns of 6 gonad-specific novel genes during the sex-sensitive

Fig. 3. RT-PCR analysis of development-and reproduction-related genes in adult tissues of *Oreochromis mossambicus*. RT-PCR was performed using respective primers and protocols as described in “Materials and methods” against products from 11 different tissues and analyzed on agarose gels stained with ethidium bromide. The PCR product sizes are listed on the right of each panel. In the bottom panel, β -actin expression was analyzed to show the homogeneity of different tissues.

Fig. 4. RT–PCR analysis of the potential novel genes in adult tissues of *Oreochromis mossambicus*. RT–PCR was performed using the respective primers for 9 potential novel genes as designated and protocols as described in “Materials and methods” against products from 12 different tissues, and these PCR products were analyzed on agarose gels stained with ethidium bromide. The PCR product sizes are listed on the right of each panel. In the bottom panel, β -actin expression was analyzed to show the homogeneity of different tissues.

period of tilapia larvae on days 24–33 (Tsai et al., 2000). For those larvae receiving regular feed (Fig. 5, control), most genes were expressed throughout the entire period examined, except for O141 and O148. The expression of O141 decreased on days 26 and 27, then rose again on days 30 and 33. In contrast, expression of O148 was not observed until day 30. Furthermore, O149 exhibited the strongest expression.

Treatments with steroids during sex-sensitive periods are established means to control the sexual differentiation of tilapia (Tsai et al., 2000). To examine the effects of steroids on gene expression during sex-sensitive periods, we treated larvae with 17 β -estradiol (E_2) or 17 α -methyltestosterone (MT) to drive the sex of the fry towards being female or male, respectively, and the expression patterns of 6 gonad-specific

Fig. 5. RT–PCR analysis of the temporal expression of potentially novel genes in tilapia larvae during the sex-sensitive period. RT–PCR was performed as described in Fig. 4 against cDNAs from *Oreochromis mossambicus* larvae treated without (Control) or with 17 β -estradiol (E_2) or 17 α -methyltestosterone (MT). PCR products were analyzed on agarose gels stained with ethidium bromide. The PCR product sizes are listed on the right of each panel. In the bottom panel, β -actin expression was analyzed to show the homogeneity of different tissues.

novel genes were examined. For E₂-treated larvae, the expressions of O141, O149, O258, and O319 were downregulated, whereas those of O148 and O243 were upregulated (Fig. 5; E₂). For MT-treated larvae, the expressions of O141 and O258 were downregulated, whereas those of O148, O149, and O243 were upregulated (Fig. 5; MT).

4. Discussion

In this study, 768 random clones were sequenced and 474 tentative unique genes (TUGs) were identified, including 230 known genes. Among the known genes, only 6 of them have previously been reported in *O. mossambicus*. We were unable to annotate 244 (51.5%, 244/474) unique sequences due to the low sequence homology or no significant match (44 clones) compared to existing known sequences in public databases. The high percentage of un-annotated sequences implies that the genetic diversity of tilapia is high compared to those species whose genomes have been well characterized. Those potentially novel genes may therefore be functionally important in fish in an evolutionary context.

These identified tilapia TUGs were classified into 13 broad biological process categories. The functional diversity of TUGs coincides well with the roles of the ovary in hormonal regulation of a variety of biological processes (Richards, 1994). Among the most-abundant genes, ferritin is widely distributed among vertebrates, plants, and bacteria, and functions mainly in iron metabolism (Hentze and Kuhn, 1996). Its role in reproduction is intriguing due to the importance of iron metabolism in both male and female fertility. Ferritin can be transported to gonads via the gonad intestinal loop (Beninger et al., 2003) or may be expressed in the gonads or oocytes as evidenced by the presence of ferritin genes in the *Xenopus* germinal vesicle oocytes (Huang et al., 2003). However, ferritin mRNA is not expressed in adult *Galleria mellonella* gonads (Kim et al., 2001) which argues against it having a role in the gonads. In this study, 2 independent TUCs were found, which represent the middle and lower subunits of ferritin, respectively (Table 3). Ferritin was also abundant in an adult tilapia testis library (our unpublished data). These results suggest that ferritin may play an important role in tilapia

gonads. CDC28 protein kinase 1 and cyclin B are well-known cell cycle regulatory molecules. Calmodulin and the myosin light chain are also known as cell motility regulatory molecules. D7-like protein is an ovary-specific protein expressed in yellow perch and is homologous to KLRL1, a novel killer cell lectin-like receptor (Han et al., 2004). Lectins have been suggested to be involved in egg maturation and fertilization (Tateno et al., 2001). Two lectins have also been shown to be abundant in a zebrafish ovary library (Zeng and Gong, 2002). The abundance of lectin-like receptor genes in our tilapia ovary library is complementary to previous data and suggests a critical role of lectin signaling in the ovary. Cytokines are enriched in normal ovarian tissue and are important in the physiology of ovarian function and ovulation (Nash et al., 1999). The presence of interleukin-12 in follicular fluid appears to negatively regulate the success of in vitro fertilization (Gazvani et al., 2000). The abundance of interleukin-12 in the tilapia ovary implies its conservative functions in fish. ZPC1 is homologous to a member of the egg envelope gene ZPC identified in medaka (Kanamori et al., 2003) and it has also been demonstrated to be rich in a zebrafish ovary library (Zeng and Gong, 2002). The last abundant TUG is homologous to a hypothetical protein, which has been predicted to be a nucleotide kinase involving nucleotide transport and metabolism. However, its role in regulating ovarian function is unclear.

To study genes which may be related to ovarian functions, 6 genes classified into the regulation of development and reproduction category were selected from this ovary library for a gene expression assay in different adult tilapia tissues. Cofilin is a molecule known to mediate actin depolymerization, which is important during embryogenesis (Ono, 2003; Ono et al., 2003). Fibulin-4 is a member of a versatile extracellular matrix protein family (Timpl et al., 2003) and mainly exists in the walls of capillaries and large blood vessels (Giltay et al., 1999). Glucosamin-6-phosphate deaminase 2 (GNPDA/oscillin) is a protein of sperm extract, which was originally claimed to mediate the sperm-induced calcium oscillation in mammalian oocytes, but that was later proven to be wrong (Amireault and Dube, 2000). MCM6 is a member of the MCM2-7 proteins, which are important for chromatin assembly during DNA replication (Maiorano et al., 2000; Sible et al., 1998). Stathmin is

a ubiquitous, neuron-enriched gene mediating tubulin depolymerization (Sobel et al., 1989). It was also demonstrated that maternal stathmin accumulates in oocytes and is redistributed in pre-implantation embryos (Koppel et al., 1999). ZPC5 is another egg envelope gene ZPC identified in medaka (Kanamori et al., 2003). Results show in tilapia that 1) GNPDA is predominantly expressed in the testis, 2) MCM6 is an ovary- and testis-specific gene, and 3) ZPC5 is exclusively expressed in the ovary. These 3 genes may therefore be useful markers for functional studies in gonads.

We also examined the spatial and temporal gene expression patterns of several potentially novel genes. All novel genes examined except 1 were strongly expressed in the ovary, thus confirming their ovarian origin. Four genes, including O141, O148, O243, and O258, exhibited a trend of increasing expression during the sex-sensitive period, and their expression patterns could be altered by steroid treatments (Fig. 5). These results suggest that these genes may be involved in sexual differentiation. However, determining the exact roles of these genes in sex differentiation requires further investigation.

From our analysis of ESTs from the tilapia ovary, it is very likely that a more-intensive sequencing effort will result in more gene sequences being identified. The functional characterization of ESTs from the adult ovary library of tilapia, *O. mossambicus*, will further our understanding of the molecular and physiological nature of the ovary. Expression profile analyses will also be important for comparative genomic and evolutionary research of the gonads. More importantly, these data may provide a rich source for gene identification which is important for the sex manipulation technology that is frequently used in the culture of tilapia as well as other fishes.

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