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Analysis of Expressed Sequence Tags from the Placenta of the Live-Bearing Fish *Poeciliopsis* (Poeciliidae)

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Matrotrophic fish in the genus *Poeciliopsis* (Poeciliidae) have a placenta-like structure used in postfertilization maternal provisioning of the developing embryo. To understand better the structure and function of the *Poeciliopsis* placenta, we derived cDNA libraries from the maternal follicular placenta of 2 matrotrophic *Poeciliopsis* sister species, *P. turneri* and *P. presidionis*. These species inherited their placenta from a common ancestor and represent one of 3 independent origins of placentas in *Poeciliopsis*. Expressed sequence tags (ESTs) were generated and putative function was determined using BLASTX homology searches and Gene Ontology (GO) annotation. Reverse transcription–polymerase chain reaction was used to verify placenta tissue expression of a putative candidate gene, alpha-2 macroglobulin. In total, 1956 (71.5% of the total submitted ESTs) and 924 (71.0% of the total submitted ESTs) unique transcripts were identified for the *P. turneri* and *P. presidionis* placenta, respectively. Homology search and GO annotation revealed putative genes whose products may be involved in specific transport functions of the maternal follicle. These putative genes are excellent candidates for future research on the evolution of the placenta. We discuss our results in light of the parent–offspring conflict theory of placental evolution and in terms of the Poeciliid placenta structure and function.

Key words: *alpha-2 macroglobulin, expressed sequence tag, parent–offspring conflict, placenta, Poeciliid fishes, viviparity*

Although the placenta has evolved independently in live-bearing vertebrates, including bony and cartilaginous fishes, amphibians, and reptiles (Wourms et al. 1988; Wake and Dickie 1998; Blackburn 1999; Hamlett 1999; Reznick et al. 2002), the mammalian placenta has primarily been studied. Mammalian placental adaptations include structures that facilitate the transfer of nutrients, gas exchange, and suppression of immunological interactions. Recently, work has started to identify the genes expressed in the mammalian placenta (Sood et. al. 2006). A review (Rossant and Cross 2001) identified 50 loci that regulate the development of the placenta and others have found expressed fetal genes that produce protein hormones and hemoglobin (Haig 1993; Handwerger 1999). Knox and Baker (2008) reported placental transcriptome comparisons between mouse and human, and a recent review by Frost and Moore (2010) detailed the relatively large number of imprinted genes expressed in the mouse and human placenta. Currently, genetic expression studies from nonmammalian placental organisms are missing from the literature, yet we might expect that placental organisms ranging from fish to mammals will exhibit similar functional adaptations to meet the demands of their extensive postfertilization maternal provisioning (Reznick et al. 2002). Studies of nonmammalian placental taxa will, therefore, contribute to our understanding of the evolution of this complex trait (Reznick et al. 2002).

Fish in the genus *Poeciliopsis* (Poeciliidae) are viviparous (live bearers), and during gestation, embryos develop within an individual maternal ovarian follicle (Turner 1940; Grove and Wourms 1983). These fish vary in their degree of maternal provisioning after fertilization (Thibault and Schultz 1978). Some *Poeciliopsis* species are lecithotrophic and retain yolk-rich eggs after fertilization without continued maternal nourishment. Other species are matrotrophic and display varying degrees of postfertilization maternal provisioning (Reznick et al. 2002). The structures that facilitate extensive maternal provisioning during embryonic development are the maternal follicle and the embryonic pericardial membrane (or sac) (Turner 1940). During embryonic development in matrotrophic species, the maternal follicle specializes to become a nutrient transport tissue (Turner 1940; Grove and Wourms 1983) and the embryonic pericardial membrane becomes highly vascularized (Turner 1940). Due to the functional similarity to the mammalian placenta, these structures have been termed the "pseudo-placenta" (Turner 1940). The *Poeciliopsis* placenta

has evolved relatively rapidly, within less than 750 000 years, and independently at least 3 times within this genus (Reznick et al. 2002). This independent evolution makes *Poeciliopsis* an excellent system for investigating the evolution of the placenta and the genes involved in its structure and function.

To develop tools for studies on the molecular evolution of *Poeciliopsis* placental genes, we set out to determine the expressed genes in the *Poeciliopsis* placenta. A cDNA library was constructed from the maternal follicle tissue and expressed sequence tags (ESTs) were generated from 2 highly matrotrophic placental sister species, *P. turneri* and *P. presidionis*. As has been suggested for other EST projects (Parton et al. 2010; Sadd et al. 2010), these *Poeciliopsis* placenta EST libraries will be a valuable resource for future identification of placenta candidate genes, genes involved in the evolution of the Poeciliidae placenta, and a comparative expression analysis of candidate genes across species with varying degrees of matrotrophy.

We performed homology searches with the EST sequences using BLASTX against UniProt databases and 3 custom proteome databases. Gene Ontology (GO) annotation was used to categorize BLASTX gene names by cellular components, molecular functions, and biological processes. Tissue expression was verified by reverse transcription–polymerase chain reaction (RT-PCR) of a putative candidate gene, alpha-2 macroglobulin (A2M). We discuss our results in terms of the parent–offspring conflict theory of placental evolution (Haig 1993; Crespi and Semeniuk 2004) and with respect to the Poeciliid placenta structure and function. This is the first survey of genes expressed in the Poeciliidae placenta. The only prior work on the genetics of placentation in the Poeciliidae focused on a single candidate gene (IGF2) (Lawton et al. 2005; O'Neill et al. 2007).

Material and Methods

Fish Stocks

Poeciliopsis turneri and *P. presidionis* fish are sister species with high levels of matrophy (embryos display a 41.4 to 21.5-fold increase, respectively, in dry mass between fertilization and birth) and well-defined extensive maternal follicular placenta tissue (Reznick et al. 2002). Reznick et al. (2002) concluded that *P. turneri* and *P. presidionis* inherited their placenta from a common ancestor and that they represent one of the 3 independent origins of placentas in the genus *Poeciliopsis*. Both species came from well-established breeding colonies at the University of California, Riverside (Reznick laboratory).

Tissue Collection and cDNA Library Construction

For both species, maternal follicle tissue was dissected from embryos and frozen in liquid nitrogen. Follicle tissue from mixed stages of embryonic development was pooled and homogenized in TRIzol reagent (Invitrogen). Total RNA was isolated using an acid guanidinum thiocyanate–phenol–chloroform extraction (Chomczynski and Sacchi 1987). An aliquot of total RNA was used to isolate mRNA using the Micro Fast-Track kit (Invitrogen, V2.0). cDNA library construction was carried out with the CloneMiner cDNA Library Construction Kit (Invitrogen, Version B). To ensure success in future gene classification by protein homology search and use in downstream evolutionary analysis, we did not perform normalization or subtraction methods on the cDNA library as these methods compromise the cDNA length making it more difficult to obtain full-length sequences (Bonaldo et al. 1996; Rubin et al. 2000).

Plasmid DNA Preparation and DNA Sequencing

Each species' cDNA library was provided to Functional Biosciences, Inc. (Madison, WI) for sequencing. Colonies were grown overnight in 1 ml cultures and purified using a 96-well plate plasmid purification vacuum protocol. EST directional sequencing was performed with M13F (-20) primers and standard cycle sequencing conditions with Big Dye v3.1. Electrophoresis was done on an ABI 3730xl instrument.

Processing of Raw Sequence Data

The raw sequences from both cDNA libraries were processed with the Phred/Phrap/Consed suite (Gordon et al. 1998). The Perl script PhredPhrap, that is part of the software, was used to run the analysis pipeline with its default parameters. This included automated base calling and quality trimming with PHRED vector masking with Crossmatch and sequence assembly with PHRAP. Sequence assembly was used to form contiguous sequences (contigs) in order to reduce redundancy of our ESTs and to come closer to the full-length transcripts. Before the assembly, the ESTs were further postprocessed by removing their vector contaminants and low-quality areas at the sequence ends. This conservative strategy was chosen to maintain data consistency among the final EST set and the resulting contigs. The quality curation step was performed by identifying the longest possible sequence window that contained at most one low-quality base call with a Phred score of less than 20 per 100 hundred nucleotides. Vector and quality trimmed ESTs with less than 150 bp in length were excluded from the assembly and the downstream annotation steps. The resulting EST set was submitted to NCBI under the GenBank accession numbers H0910775-HO914683 and HO914684-HO914810.

Sequence Annotations

Singlet (ESTs that do not assemble into contiguous sequences) and contig sequences (ESTs assembled into contiguous sequences) were functionally annotated using translated BLASTX searches against the UniProt database (release 15.14) with an E value of 1×10^{-6} as the cutoff (Altschul et al. 1997; Leinonen et al. 2004). Similarly,

ortholog sequences in other fish species were identified by BLASTX searches against the proteomes of the fully sequenced genomes of *Takifugu rubripes* (Fugu, Ensembl Version v4.56), *Danio rerio* (Zebrafish, Ensembl Version v8.56) and the proteome encoded by the mitochondrial genome from *Fundulus heteroclitus* (downloaded from NCBI). Preliminary GO annotations were obtained by using the corresponding annotations from SwissProt. This database was chosen for this step because it is well annotated and a representable number of ESTs in our libraries showed matches in this database. The GO terms from the best ranking BLAST hit were assigned to the ESTs. GO slim term analysis was performed to provide a summary by counting statistics of sequences falling into general functional, cellular, and biological categories. A slightly modified version of the GO slim term set from *Drosophila* (http://www.geneontology.org/GO.slims.shtml) was used for this analysis. This GO slim set was chosen because it included many daughter terms of interest.

Confirmation of Expression in Placenta Tissue

To confirm follicular tissue EST expression, we performed a 2-step RT-PCR. Total RNA was extracted from the maternal follicle of pregnant adult females. Tissue was homogenized with a rotor–stator homogenizer and lysed in a lysis buffer plus β-mercaptoethanol (GE Healthcare illustra RNASpin Mini kit). Total RNA isolation and cleanup was carried out using the illustra RNASpin Mini protocol following homogenization. An aliquot of RNA was treated with DNAse enzyme (Ambion DNA-*free*; Applied Biosystems, Austin, TX). Random hexamer and Oligo(dT) primed cDNA was synthesized using RNase H^+ MMLV Reverse Transcriptase following the iSCRIPT protocol (BioRad, Hercules, CA). A PCR reaction was run with gene-specific primers (see below) and cDNA template.

Primers were designed to amplify a 271 bp region of the putative A2M gene identified from BLASTX results for the assembled libraries. A2M primers included the oligonucleotides, 5′-GACCAAAGCGCTCACTCTTC-3′ (forward) and 5′-GAGACCAGATCCCAGATCCA-3′ (reverse) and were designed from the *P. turneri* contig that showed significant (E value $< 1 \times 10^{-6}$) homology to A2M. A reference gene, β-actin, was chosen as a positive control for tissue expression (Lawton et al. 2005). The β-actin primers were designed for quantitative RT-PCR research involving *Poeciliopsis prolifica*, a close relative to both *P. turneri* and *P. presidionis* (see Lawton et al. 2005). RT-PCR products were visualized on a 2% agarose ethidium bromide gel and sequence verified for a subset of samples. To ensure the amplified product is from cDNA, and not genomic DNA contamination, we treated total RNA with DNAse enzyme (Ambion DNA-*free*; Applied Biosystems, Austin, TX) prior to cDNA synthesis and ran the appropriate negative reverse transcriptase control reactions (reverse transcription reaction with tissue-specific RNA minus the reverse transcriptase enzyme) for each tissue and primer pair.

Results

P. turneri and *P. presidionis* Sequence Assembly and Annotation

For the *P. turneri* library, a total of 3168 ESTs were processed and 2735 of these processed ESTs were submitted sequences after trimming and filtering (as described in Materials and Methods). For the *P. presidionis* library, 1548 ESTs were processed and 1301 sequences were submitted after trimming and filtering. An overview of the EST assembly for both species is summarized in Table 1. The percent of ESTs (of the total submitted) that did not assemble into contigs (singletons) is similar for both species—63.9% and 60.8% for *P. turneri* and *P. presidionis*, respectively.

For both species, BLASTX searches against the UniProt database (release 15.14; $E \leq 1 \times 10^{-6}$) and published fish proteomes were used for functional annotation and ortholog analysis of singlets and contigs (see Materials and Methods) and the results are summarized in Table 1. Of the submitted ESTs, the percent of identified putative gene transcripts was similar for both species (*P. turneri* = 71.5% and *P. presidionis* = 71.0%). The number of unique transcripts with an E value BLASTX result of smaller than or equal to $\times 10^{-100}$ was 163 for *P. turneri* and 74 for *P. presidionis* (Supplementary Table 1). The percent of gene transcripts (singlets plus contigs) to map to the custom databases (UniProt and published fish proteomes) was 51.7% for *P. turneri* and 44.4% for *P. presidionis*. Of the total putative gene transcripts, 48.3% (945 of 1956 sequences) and 55.6% (514 of 924 sequences) were identified as unknown sequences (no BLASTX match) for *P. turneri* and *P. presidionis*, respectively (Table 1). The dissimilarity in the percent of unknown sequences between the 2 species is likely due to a difference in the total number of ESTs analyzed per library. The percent of unknown sequences we found for both libraries is slightly higher but close to what has been reported for other EST libraries (e.g., *Bombus terrestris* thorax and abdomen EST library, 41.2% no "hits," Sadd et al. 2010). In our case, this result may represent the tissue-specific nature of the libraries and the constraint of using species in the reference databases that are, relatively, not very closely related.

Gene Ontology Analysis

Preliminary GO annotations and GO slim term analysis was performed as described in the Materials and Methods. The number of BLASTX hits in the preliminary GO annotation was 1308 for *P. turneri* and 568 for *P. presidionis*. These were further designated to 3 standard parent categories (biological processes, molecular functions, and cellular components) and summarized according to GO slim terms. Overall, there was considerable similarity between the 2 species in the number of represented sequences in the GO annotations and functional analysis (Tables 2, 3, and 4). Table 2 summarizes assignments for the classification of molecular function. The highest represented category for both species

Table 1 An overview of the *Poceiliopsis* maternal follicle EST sequence assembly and functional annotation for *P. turneri* and *P. presidionis*

	P. turneri	*P. presidionis*
Sequence assembly		
Average EST length[a]	658.9 bp	566.7 bp
Contigs[b] assembled	208 from 983 ESTs	133 from 508 ESTs
Range in the number of ESTs that compose a contig	2 to 137	2 to 72
Range in contig length	436 to 5292 bp	301 to 2702 bp
Number of singletons[c]	1748	791
Sequence annotation[d]		
Number of unique sequences representing gene transcripts[e]	1956	924
Number unknown sequences (no BLASTX match)	945	514
Number singlets and contigs mapped to custom databases	830 and 181	303 and 107
Number singlets and contigs mapped to UniProt/SwissProt	477 and 86	171 and 52

These data are based on the total number of submitted EST sequences after trimming and filtering (2735 and 1301 sequences for *P. turneri* and *P. presidionis*, respectively).

[a] This is the average EST length after trimming and filtering of EST sequences (see Materials and Methods).

[b] Contig refers to ESTs that assembled into contiguous sequences.

[c] Singletons refer to ESTs that did not assemble into contiguous sequences.

[d] Functional annotation with BLASTX searches against UniPort databases and published fish proteomes.

[e] Combined Contig and Singlets.

was the binding process (*P. turneri*: 59.6% of 680 annotated sequences and *P. presidionis*: 48.9% of 317 annotated sequences). The main difference between the 2 species for this classification is found for the structural constituent of ribosome category, which has greater representation by *P. presidionis* sequences (46 of 317) than *P. turneri* sequences (27 of 680). Within the biological processes (Table 3), the highest number of sequences fell into the nucleic acid metabolic process for *P. turneri*. The highest number of sequences for *P. presidionis* appeared in the multicellular

Table 2 The number and percent of annotated sequences assigned to a GO molecular function classification for the *Poceiliopsis turneri* and *P. presidionis* maternal follicle cDNA library

		P. turneri		*P. presidionis*	
Molecular function	GO ID	Number from 680 annotated sequences	Percent of 680 annotated sequences	Number from 317 annotated sequences	Percent of 317 annotated sequences
Binding	GO: 0005488	405	59.6	155	48.9
Structural molecule activity	GO:0005198	44	6.5	54	17.0
Transporter activity	GO:0005215	34	5.0	13	4.1
Signal transducer activity	GO:0004871	32	4.7	9	2.8
Structural constituent of ribosome	GO:0003735	27	4.0	46	14.5
Protein kinase activity	GO:0004672	23	3.4	5	1.6
Peptidase activity	GO:0008233	22	3.2	3	0.9
Actin binding	GO:0003779	17	2.5	7	2.2
Transcription factor binding	GO:0008134	15	2.2	2	0.6
Transcription factor activity	GO:0003700	13	1.9	3	0.9
Enzyme activator activity	GO:0008047	13	1.9	3	0.9
Translation factor activity, nucleic acid binding	GO:0008135	12	1.8	7	2.2
Enzyme inhibitor activity	GO:0004857	7	1.0	4	1.3
Phosphoprotein phosphatase activity	GO:0004721	6	0.9	4	1.3
Receptor signaling protein activity	GO:0005057	6	0.9	1	0.3
Motor activity	GO:0003774	4	0.6	1	0.3

A slightly modified version of the GO slim term set from *Drosophila* (http://www.geneontology.org/GO.slims.shtml) was used in this analysis. GO ID represents the GO ID number.

Table 3 The number and percent of annotated sequences assigned to a GO Biological Process classification for the *Poceiliopsis turneri* and *P. presidionis* maternal follicle cDNA library

		P. turneri		*P. presidionis*	
Biological process	GO ID	Number from 801 annotated sequences	Percent of 801 annotated sequences	Number from 271 annotated sequences	Percent of 271 annotated sequences
Nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	GO:0006139	125	15.6	33	12.2
Transport	GO:0006810	110	13.7	30	11.1
Multicellular organismal development	GO:0007275	72	9.0	36	13.3
Organelle organization	GO:0006996	66	8.2	24	8.9
Transcription	GO:0006350	63	7.9	14	5.2
Response to stress	GO:0006950	53	6.6	12	4.4
Cell cycle	GO:0007049	45	5.6	18	6.6
Cell communication	GO:0007154	39	4.9	12	4.4
Cell death	GO:0008219	27	3.4	11	4.1
Lipid metabolic process	GO:0006629	23	2.9	8	3.0
Carbohydrate metabolic process	GO:0005975	21	2.6	8	3.0
Intracellular protein transport	GO:0006886	21	2.6	3	1.1
Cell proliferation	GO:0008283	20	2.5	15	5.5
Transmembrane transport	GO:0055085	20	2.5	6	2.2
Cellular component movement	GO:0006928	18	2.2	10	3.7
Generation of precursor metabolites and energy	GO:0006091	16	2.0	8	3.0
Cellular amino acid and derivative metabolic process	GO:0006519	13	1.6	8	3.0
Response to hormone stimulus	GO:0009725	12	1.5	3	1.1
Behavior	GO:0007610	10	1.2	2	0.7
Sensory perception	GO:0007600	9	1.1	3	1.1
Defense response	GO:0006952	8	1.0	3	1.1
Cellular ion homeostasis	GO:0006873	7	0.9	1	0.4
Angiogenesis	GO:0001525	3	0.4	3	1.1

A slightly modified version of the GO slim term set from *Drosophila* (http://www.geneontology.org/GO.slims.shtml) was used in this analysis. GO ID represents the GO ID number.

Table 4 The number and percent of total identified sequences assigned to a GO cellular component classification for the *Poceiliopsis turneri* and *P. presidionis* maternal follicle cDNA library

		P. turneri		*P. presidionis*	
Cellular component	GO ID	Number from 1385 annotated sequences	Percent of 1385 annotated sequences	Number from 576 annotated sequences	Percent of 576 annotated sequences
Intracellular	GO:0005622	430	31.0	181	31.4
Cytoplasm	GO:0005737	347	25.1	153	26.6
Nucleus	GO:0005634	164	11.8	57	9.9
Plasma membrane	GO:0005886	84	6.1	25	4.3
Cytosol	GO:0005829	68	4.9	36	6.3
Mitochondrion	GO:0005739	60	4.3	28	4.9
Cytoskeleton	GO:0005856	53	3.8	14	2.4
Endoplasmic reticulum	GO:0005783	49	3.5	9	1.6
Extracellular region	GO:0005576	39	2.8	16	2.8
Golgi apparatus	GO:0005794	35	2.5	6	1.0
Ribosome	GO:0005840	30	2.2	48	8.3
Lysosome	GO:0005764	15	1.1	1	0.2
Centrosome	GO:0005813	7	0.5	1	0.2
Peroxisome	GO:0005777	2	0.1	1	0.2
Synaptic vesicle	GO:0008021	2	0.1	0	0.0

A slightly modified version of the GO slim term set from *Drosophila* (http://www.geneontology.org/GO.slims.shtml) was used in this analysis. GO ID represents the GO ID number.

organismal development category. For both species, the transport biological process was highly represented (13.7% *P. turneri* and 11.1% *P. presidionis*). Several sequences for both species also appear in the biological processes of response to stress, lipid and carbohydrate metabolic process, transmembrane transport, response to hormone stimulus, defense response, and angiogenesis. All these categories are ones we might expect to be associated with tissues that serve a placental-like function (see Discussion). Table 4 reveals the assignments for the cellular component category. Overall, 1385 annotated sequences are identified for *P. turneri* and 576 annotated sequences are identified for *P. presidionis*. The main category represented by both libraries is that of intracellular proteins (*P. turneri* 31% and *P. presidionis* 31.4%). One term, synaptic vesicle, was represented in the *P. turneri* but not in the *P. presidionis* identified sequences. Similar to the GO molecular function analysis, sequences that correspond to a ribosome or ribosome-related protein appear more in the *P. presidionis* library than in the *P. turneri* library. It is important to note that because the GO terms are hierarchical the number of sequences will vary among parent classifications (Dean et al. 2009; Parton et al. 2010).

GO annotation is a highly utilized method for classifying gene products and identifying putative gene functions for gene products derived from an EST analysis (Ashburner et al. 2000; Conesa et al. 2005; Roche et al. 2005; Botton et al. 2008; Parton et al. 2010). Our goal in the GO analysis was to better understand the functions of the proteins found in the *Poeciliopsis* maternal follicle and to identify candidate genes for future analysis. It is important, however, to point out that any differences between the species may change with the addition of a larger pool of ESTs. Furthermore, although the maternal follicle tissue was derived from a similar span of developmental stages for both species, any differences between the 2 libraries in the representation of one developmental stage versus another could influence the percent of GO terms identified for each species.

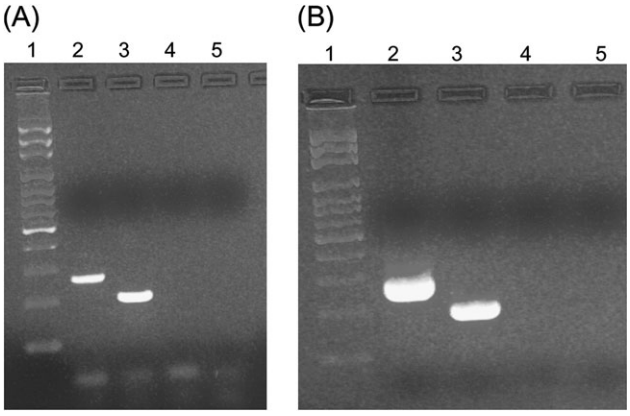

Figure 1. Verification of maternal follicle tissue expression for *Poeciliopsis turneri* (**A**) and *P. presidionis* (**B**). Lane 1 = 100 bp ladder; Lane 2 = RT-PCR of A2M amplicon; Lane 3 = RT-PCR of β-actin amplicon; Lane 4 and 5 = negative RT-PCR for A2M and β-actin, respectively (template is from a reverse transcription reaction with no reverse transcriptase, for details, see Materials and Methods).

Verification of Maternal Follicle Expression of A2M and β-Actin

To verify tissue expression of putative transcripts identified in the cDNA library, we subjected total RNA isolated from the maternal follicle to RT-PCR using primers designed to amplify a region of the A2M and the β-actin gene (Figure 1). We chose to amplify the putative A2M gene transcript for 2 reasons. 1) BLASTX searches returned contigs from both species that showed significant (E value $\leq 1 \times 10^{-6}$) homology to the A2M protein and 2) A2M has been shown to be involved in mammalian placentation and pregnancy and is, therefore, a candidate gene for future analysis of placenta evolution in Poeciliids. The β-actin gene was chosen as a positive control (Lawton et al. 2005), and a β-actin homolog is significantly (E value $\leq 1 \times 10^{-6}$) represented by contigs from both libraries. We found A2M and β-actin amplification product for the maternal follicle tissue in both species (*P. turneri* Figure 1A, lane 2 and 3; *P. presidionis* Figure 1B, lane 2 and 3).

Discussion

We have undertaken the first cDNA library construction and EST sequence annotations for the *Poeciliopsis* maternal follicular placenta (Table 1). This was carried out in 2 highly matrotrophic placenta species, *P. turneri* and *P. presidionis*. RT-PCR verified maternal follicle expression of 2 genes represented in both species (Figure 1). We used a BLASTX and GO annotation to reveal the putative functions of genes expressed in the maternal follicle for both species. Both species were comparable in their GO annotation (Tables 2–4) and GO terms likely to be related to the function and structure of the placenta were discovered (see below). To provide context to the GO and BLASTX results, we focus on gene annotations that represent potential candidate genes for future studies related to the function and evolution of the Poeciliid placenta. It should be noted that the functional assignments are not from direct assessment but derived from computational annotation, and it is likely that all potential functions of a gene are not completely described (Dean et al. 2009).

Candidate Genes Identified for Future Investigations Related to Maternal–Fetal Communication

Successful maintenance of pregnancy in organisms with placental-like structures will rely on signaling at the maternal–fetal interface between fetal and maternal cells (Haig 1993; Rossant and Cross 2001). In highly matrotrophic Poeciliids, the maternal–fetal interface is composed of the mother's hypertrophied follicular tissue and the embryonic pericardial membrane (or sac) (Turner 1940). In

some placental fish, there is an egg envelope that surrounds the embryo and is in contact with the follicular fluid that fills the space between the follicle and egg envelope (Grove and Wourms 1994). During gestation, the structures become specialized for maternal–fetal nutrient transport. Transport structures revealed through microscopy include finger-like villi or follicular pits on the internal surface of the maternal follicle which are in contact with the embryonic surface, apical microvilli on follicular cells, blood vessels, mitochondria, coated vesicles involved in endocytosis, and a folded basal plasma membrane (predicted to enhance the transport of amino acids, fatty acids, and monosaccharides) (Turner 1940; Knight et al. 1985; Grove and Wourms 1994). Preliminary scanning electron microscopy of *P. turneri* has also revealed similar villi and microvilli structures on the internal surface of the maternal follicle (Panhuis TM, Tuhela-Reuning L, unpublished data). Experimental evidence of maternal–embryonic nutrient transfer has also been reported for the placental fish *Heterandria formosa* (Grove and Wourms 1983; Wourms et al. 1988). In *H. formosa* maternal molecules are speculated to cross the maternal capillary endothelium by diffusion or vesicle transport and then cross through the follicular epithelium cells (Grove and Wourms 1983, 1994). The substances that enter the intrafollicular space can then be absorbed by the embryo (Grove and Wourms 1994). Given the structure and function of the follicular placenta, it is clear that constant communication at the maternal–fetal interface occurs (Grove and Wourms 1983, 1994; Knight et al. 1985) and we would predict the presence of proteins that facilitate this transport. For both *P. turneri* and *P. presidionis*, we find the expression of genes whose protein products may be directly or indirectly involved in transport. Examples of several predicted GO categories related to these activities include membrane transport, cell division promotion, angiogenesis, and energy production. Our results for both species revealed annotated sequences that appear in GO categories relating to these activities (Tables 2–4). More detailed analysis of several of these protein products will help determine their potential role in maternal–fetal communication.

Candidate Genes Identified for Future Investigations into the Evolution of the Poeciliid Placenta

Expression of a Putative A2M Gene Product in the Poeciliopsis Placenta

Homology to the protein A2M was found in the *P. turneri* Contig303 (composed of 108 ESTs; Contig303 TSA accession number HP608041) and 4 *P. presidionis* contigs (composed of 11, 12, and 14 ESTs and a longer Contig207 composed of 29 ESTs; TSA accession number HP608042). The large number of ESTs that represent these contigs suggests a high relative level of expression in the transcripts for this gene product(s) in both libraries. BLASTX results (E value $\leq 1 \times 10^{-6}$) for the *P. turneri* contig revealed A2M homology to published A2M sequences from several different taxa, including *D. rerio* (predicted A2M-like protein; accession number XP_001920744.2), *Xenopus laevis* (A2M-like 1; accession number NP_001128549.1), *Rattus norvegicus* (A2M, isoform CRA_a; accession number EDM02007), and *Homo sapiens* (A2M-like 1, isoform CRA_d; accession number EAW88605). *Poeciliopsis presidionis* contig BLASTX results were similar to the *P. turneri* homology searches. The putative A2M contig from both species (*P. turneri* Contig303 and *P. presidionis* Contig207) was computationally analyzed for the longest open reading frame (NCBI ORF Finder: http://www.ncbi.nlm.nih.gov/projects/gorf/, 4 November 2010) and shown to have the highly conserved thioester motif sequence (identified by Conserved Domains Database NCBI; http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml, 4 November 2010) typical of A2M (see below; Ma et al. 2010). A preliminary comparison (NCBI BLASTN 2 Sequences, 4 November 2010) of *P. turneri* Contig303 versus *P. presidionis* Contig207 revealed 48% nucleotide coverage (Contig303 is longer, 5291 bp, than Contig207, 2695 bp) with 98% maximum nucleotide identity (E value $< 1 \times 10^{-6}$). The expression of A2M in the maternal follicle of both species was verified with RT-PCR (Figure 1A,B). A2M is part of the thioester-containing protein superfamily and has been isolated and characterized in many vertebrates (Starkey and Barrett 1982; Sottrup-Jensen et al. 1989; Borth 1992; Huang et al. 2000; Chang et al. 2005; ; Li and Lu 2006; Padhi et al. 2008) and invertebrates (Quigley and Armstrong 1983; Spycher et al. 1987; Iwaki et al. 1996; Zhang et al. 2007; Rosa et al. 2008; Fujito et al. 2010; Ma et al. 2010). The A2M protein is a component of the innate immune system and is a universal protease inhibitor that can also bind cytokines, growth factors, lectins, ions, and lipopolysaccharides (Gu et al. 1992; He et al. 2005; Siu et al. 2006; Ma et al. 2010). With respect to the placenta, this protein is present in both human and rat decidual tissue, and in rats, it has been shown to be a key player in limiting trophoblast invasion of the mesometrial decidua (Gu et al. 1992). In humans, the uteroplacental role for A2M is still unclear, but it is suggested to be involved in tissue remodeling (Siu et al. 2006). At this point, we can only speculate as to the role of A2M in the *Poeciliopsis* maternal follicle. One function may be similar to the tissue remodeling seen in human mesometrial decidua. The maternal follicle of placenta poeciliids undergoes extensive hypertrophy and specialization during gestation and becomes an important site of maternal–fetal nutrient transfer (Turner 1940; Grove and Wourms 1994). These changes could be facilitated by the presence of A2M. There is no indication of maternal tissue invasion by the conceptus in placental fish species studied so far (Knight et al. 1985; Grove and Wourms 1994), suggesting it may be unlikely that A2M is limiting physical invasion of embryonic cells; however, this awaits more detailed study in *Poeciliopsis*. Another possible role for A2M may be through its binding properties to cytokines, growth factors, lectins, and other substances (Gu et al. 1992, He et al. 2005; Siu et al. 2006; Ma et al. 2010). This binding may be essential for proper communication at the maternal–fetal interface and may regulate fetal manipulation that is predicted by the parent–offspring conflict (Haig 1993;

see detailed discussion below). Future work on this placenta candidate gene will benefit from a complete characterization of the A2M gene product, analyzing the evolution of A2M within the poeciliid fishes, and an investigation into the expression profile of A2M in different tissue samples.

Candidate Genes Predicted to be Involved in a Parent–Offspring Conflict

The evolutionary forces driving placenta structure and function are not fully understood. One prevailing hypothesis is parent–offspring conflict, which considers the opposing interests between the maternal, embryonic, and paternal genomes (Haig 1993; Zeh DW and Zeh JA 2000; Crespi and Semeniuk 2004). For instance, the amount of resources that are in the best interest of the offspring to obtain from its mother may exceed what is in the best interest of the mother to provide to the offspring (Trivers 1974), while the fitness of the father will depend on the mating system (Haig 1993; Vrana et al. 1998). This conflict may result in a coevolutionary arms race between the genomes and has been implemented in the rapid evolution of genes associated with viviparity and placenta tissues (Zeh DW and Zeh JA 2000; Crespi and Semeniuk 2004). Although well documented in placental mammals, the parent–offspring conflict (Trivers 1974) is predicted to be a selective force in the evolution of viviparity and placentation in other taxa as well, such as reptiles, amphibians, sharks, and fish (Reznick et al. 2002; Crespi and Semeinuk 2004; O'Neill et al. 2007; Schrader and Travis 2008, 2009). Recently, work in nonmammalian placenta species has revealed that embryonic hormones may influence maternal physiology, and mothers may inflict antagonistic immune reactions onto embryos (reviewed in Crespi and Semeniuk 2004; Schrader and Travis 2009). In poeciliid fishes, experimental evidence suggests that offspring genotype influences the degree of maternal investment and that there is a trade-off between the degree of resources a mother invests into individual offspring and maternal fecundity (Schrader and Travis 2009). Both observations are consistent with a role for parent–offspring conflict in the evolution of the poeciliid placenta (Schrader and Travis 2009). Furthermore, O'Neill et al. (2007) found rapid adaptive evolution of the insulin-like growth factor II (*IGF2*) gene among poeciliid lineages that have evolved recent placentation. *IGF2* is an imprinted gene in eutherian mammals and marsupials and this imprinted gene, along with others, potentially influences embryonic acquisition of maternally-supplied nutrients during gestation (Haig 1993; O'Neill et al. 2007; Schrader and Travis 2009; reviewed in Tycko and Morison 2002; Reik et al. 2003; Burt and Trivers 2006; Frost and Moore 2010). One hypothesized maternal counter-adaptation to the antagonistic effects of IGF2 is the expression of the insulin-like growth factor–binding protein-1 (IGFBP-1), which is a major constituent of the decidual tissue in mammals (reviewed in Haig 1993). Haig (1993) speculates that IGFBP-1 may compete with IGF2 for receptors on the trophoblast and regulate its influence on maternal tissues. In our *P. turneri* library, a homologue to IGFBP-1 and IGFBP-2 (also known to bind IGF2) was found (accession numbers HO912389 and HO912734, respectively). We plan to explore these candidate genes further with respect to their potential role in a parent–offspring conflict. Protein partners that are involved in a coevolutionary arms race, like IGF2 and IGFBP, are predicted to be evolving rapidly under Darwinian selection (Panhuis et al. 2006). Further screening of the *P. presidionis* maternal follicle tissue will also be done to determine the expression of IGFBP-1 and -2 in this species.

Other gene products in our libraries are also candidates for a potential involvement in a parent–offspring conflict. For instance, the maternal immune system plays a vital role in placentation and potentially in countering antagonistic fetal adaptations (Haig 1993). Work by Paulesu (1997) and Paulesu et al. (2008) revealed that the production of different cytokines by both the maternal and fetal placenta tissue is not unique to mammals and is present in several nonmammalian taxa as well, highlighting a potential role for cytokines in the evolution of viviparity and placentation. In both of our libraries, several annotated sequences were identified as immune/defense response genes (Table 3). In *P. turneri*, homologs to interleukin-8 and interleukin-6 receptor subunit beta were identified, along with MHC class I and II antigens, and several antigen processing and presenting proteins (accession numbers, HO913679, HO911132, HO911440, HO911028, HO911221, and HO913058). The identified immune products from this study will be important for future studies on the immunological mechanisms and their potential role in a parent–offspring conflict in the poeciliid placenta.

Supplementary Material

Supplementary material can be found at http://www.jhered.oxfordjournals.org/.

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References

Altschul SF, Madden TL, Schaffer AA, Zhang JH, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25(17): 3389–3402.

Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, et al. 2000. Gene ontology: tool for the unification of biology. Nat Genet. 25(1):25–29.

Blackburn DG. 1999. Placenta and placental analogs in reptiles and amphibians. In: Neill EKJD, editor. Encyclopedia of reproduction. San Diego (CA): Academic Press. p. 840–847.

Bonaldo MDF, Lennon G, Soares MB. 1996. Normalization and subtraction: two approaches to facilitate gene discovery. Genome Res. 6(9):791–806.

Borth W. 1992. Alpha 2-macroglobulin, a multifunctional binding-protein with targeting characteristics. FASEB J. 6(15):3345–3353.

Botton A, Galla G, Conesa A, Bachem C, Ramina A, Barcaccia G. 2008. Large-scale gene ontology analysis of plant transcriptome-derived sequences retrieved by AFLP technology. BMC Genomics. 9:347–355.

Burt A, Trivers R. 2006. Genes in conflict: the biology of selfish genetic elements. Cambridge (MA): The Belknap Press of Harvard University Press.

Chang MX, Nie P, Liu GY, Song Y, Gao Q. 2005. Identification of immune genes in grass carp Ctenopharyngodon idella in response to infection of the parasitic copepod Sinergasilus major. Parasitol Res. 96(4):224–229.

Chomczynski P, Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate phenol chloroform extraction. Anal Biochem. 162(1):156–159.

Conesa A, Gotz S, Garcia-Gomez JM, Terol J, Talon M, Robles M. 2005. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics. 21(18):3674–3676.

Crespi B, Semeniuk C. 2004. Parent–offspring conflict in the evolution of vertebrate reproductive mode. Am Nat. 163(5):635–653.

Dean MD, Clark NL, Findlay GD, Karn RC, Yi XH, Swanson WJ, MacCoss MJ, Nachman MW. 2009. Proteomics and comparative genomic investigations reveal heterogeneity in evolutionary rate of male reproductive proteins in mice (Mus domesticus). Mol Biol Evol. 26(8):1733–1743.

Frost JM, Moore GE. 2010. The importance of imprinting in the human placenta. PLoS Genet. 6(7):e1001015.

Fujito NT, Sugimoto S, Nonaka M. 2010. Evolution of thioester-containing proteins revealed by cloning and characterization of their genes from a cnidarian sea anemone, Haliplanella lineate. Dev Comp Immunol. 34(7):775–784.

Gordon D, Abajian C, Green P. 1998. Consed: a graphical tool for sequence finishing. Genome Res. 8(3):195–202.

Grove BD, Wourms JP. 1983. The role of the follicle in maternal-embryonic nutrient exchange in the viviparous fish, *Heterandria formosa*. Am Zool. 23(4):1017–1017.

Grove BD, Wourms JP. 1994. Follicular placenta of the viviparous fish, *Heterandria formosa*: II. Ultrastructure and development of the follicular epithelium. J Morphol. 220(2):167–184.

Gu Y, Jayatilak PG, Parmer TG, Gauldie J, Fey GH, Gibori G. 1992. Alpha 2- macroglobulin expression in the mesometrial decidua and its regulation by decidual luteotropin and prolactin. Endocrinology. 131(3):1321–1328.

Haig D. 1993. Genetic conflicts in human pregnancy. Q Rev Biol. 68(4):495–532.

Hamlett WC. 1999. Placenta and placental analogs in elasmobranchs. In: Neill EKJD, editor. Encyclopedia of reproduction. San Diego (CA): Academic Press. p. 831–840.

Handwerger S. 1999. Placental and decidual protein hormones, human. In: Knobil E, Neill JD, editors. Encyclopedia of reproduction. San Diego (CA): Academic Press. p. 855–863.

He H, McCartney DJ, Wei QX, Esadeg S, Zhang JH, Foster RA, Hayes MA, Tayade C, Van Leuven F, Croy BA. 2005. Characterization of a murine alpha 2 macroglobulin gene expressed in reproductive and cardiovascular tissue. Biol Reprod. 72(2):266–275.

Huang W, Dolmer K, Liao XB, Gettins PGW. 2000. NMR solution structure of the receptor binding domain of human alpha(2)-macroglobulin. J Biol Chem. 275(2):1089–1094.

Iwaki D, Kawabata S, Miura Y, Kato A, Armstrong PB, Quigley JP, Nielsen KL, Dolmer K, SottrupJensen L, Iwanaga S. 1996. Molecular cloning of Limulus alpha(2)-macroglobulin. Eur J Biochem. 242(3): 822–831.

Knight FM, Lombardi J, Wourms JP, Burns JR. 1985. Follicular placenta and embryonic growth of the viviparous four-eyed fish (Anableps). J Morphol. 185(1):131–142.

Knox K, Baker JC. 2008. Genomic evolution of the placenta using co-option and duplication and divergence. Genome Res. 18(5): 695–705.

Lawton BR, Sevigny L, Obergfell C, Reznick D, O'Neill RJ, O'Neill MJ. 2005. Allelic expression of IGF2 in live-bearing, matrotrophic fishes. Dev Genes Evol. 215(4):207–212.

Leinonen R, Diez FG, Binns D, Fleischmann W, Lopez R, Apweiler R. 2004. UniProt archive. Bioinformatics. 20(17):3236–3237.

Li FL, Lu CP. 2006. Purification and characterization of alpha(2)-macroglobulin from grass carp Ctenopharyngodon idellus: cloning a segment of the corresponding gene. Fish Shellfish Immunol. 20(4): 474–481.

Ma HM, Wang B, Zhang JQ, Li FH, Xiang JH. 2010. Multiple forms of alpha-2 macroglobulin in shrimp *Fenneropenaeus chinesis* and their transcriptional response to WSSV or *Vibrio* pathogen infection. Dev Comp Immunol. 34(6):677–684.

O'Neill MJ, Lawton BR, Mateos M, Carone DM, Ferreri GC, Hrbek T, Meredith RW, Reznick DN, O'Neill RJ. 2007. Ancient and continuing Darwinian selection on insulin-like growth factor II in placental fishes. Proc Natl Acad Sci U S A. 104(30):12404–12409.

Padhi A, Buchheim MA, Verghese B. 2008. Dynamic evolutionary pattern of alpha(2)-macroglobulin in a model organism, the zebrafish (Danio rerio). Mol Immunol. 45(11):3312–3318.

Panhuis T, Clark N, Swanson W. 2006. Rapid evolution of reproductive proteins in abalone and Drosophila. Philos Trans R Soc Lond B Biol Sci. 361(1466):261–268.

Parton A, Bayne CJ, Barnes DW. 2010. Analysis and functional annotation of expressed sequence tags from in vitro cell lines of elasmobranchs: spiny dogfish shark (*Squalus acanthias*) and little skate (*Leucoraja erinacea*). Comp Biochem Physiol Part D. 5:199–206.

Paulesu L. 1997. Cytokines in mammalian reproduction and speculation about their possible involvement in nonmammalian viviparity. Microsc Res Tech. 38(1–2):188–194.

Paulesu L, Jantra S, Letta F, Brizzi R, Bigliardi E. 2008. Interleukin-1 in reproductive strategies. Evol Dev. 10:778–788.

Quigley JP, Armstrong PB. 1983. An endopeptidase inhibitor, similar to mammalian alpha-2 macroglobulin, detected in the hemolymph of an invertebrate, Limulus polyphemus. J Biol Chem. 258(13):7903–7906.

Reik W, Constancia M, Fowden A, Anderson N, Dean W, Ferguson-Smith A, Tycko B, Sibley C. 2003. Regulation of supply and demand for maternal nutrients in mammals by imprinted genes. J Physiol (Lond). 547(1):35–44.

Reznick DN, Mateos M, Springer MS. 2002. Independent origins and rapid evolution of the placenta in the fish genus Poeciliopsis. Science. 298(5595):1018–1020.

Roche JP, Wackym PA, Cioffi JA, Kwitek AE, Erbe CB, Popper P. 2005. In silico analysis of 2085 clones from a normalized rat vestibular periphery 3' cDNA library. Audiol Neurootol. 10(6):310–322.

Rosa RD, Perazzolo LM, Barracco MA. 2008. Comparison of the thioester domain and adjacent regions of the alpha2-macroglobulin from different South Atlantic crustaceans. Fish Shellfish Immunol. 24(2):257–259.

Rossant J, Cross JC. 2001. Placental development: lessons from mouse mutants. Nat Rev Genet. 2(7):'538–548.

Rubin GM, Hong L, Brokstein P, Evans-Holm M, Frise E, Stapleton M, Harvey DA. 2000. A Drosophila complementary DNA resource. Science. 287(5461):2222–2224.

Sadd BM, Kube M, Klages S, Reinhardt R, Schmid-Hempel P. 2010. Analysis of a normalized expressed sequence tag (EST) library from a key pollinator the bumblebee *Bombus terrestris*. BMC Genomics. 11: 110.

Schrader M, Travis J. 2008. Testing the viviparity-driven-conflict hypothesis: parent–offspring conflict and the evolution of reproductive isolation in a Poeciliid fish. Am Nat. 172(6):806–817.

Schrader M, Travis J. 2009. Do embryos influence maternal investment? Evaluating maternal-fetal coadaptation and the potential for parent–offspring conflict in a placental fish. Evolution. 63(11): 2805–2815.

Siu SSN, Choy MY, Leung TN, Lau TK. 2006. Lack of site-specific production of decidual alpha-2 macroglobulin in human pregnancy. J Soc Gynecol Investig. 13(7):491–496.

Sood R, Zehnder JL, Druzin ML, Brown PO. 2006. Gene expression patterns in human placenta. Proc Natl Acad Sci U S A. 103(14): 5478–5483.

Sottrup-Jensen L, Sand O, Kristensen L, Fey GH. 1989. The alpha-2 macroglobulin bait region. Sequence diversity and localization of cleavage sites for proteinases in five mammalian alpha-2 macroglobulin. J Biol Chem. 264(27):15781–15789.

Spycher SE, Arya S, Isenman DE, Painter RH. 1987. A functional, thioester-containing alpha-2 macroglobulin homolog isolated from the hemolymph of the American lobster (Homarus americanus). J Biol Chem. 262(30):14606–14611.

Starkey PM, Barrett AJ. 1982. Evolution of alpha-2 macroglobulin: the demonstration in a variety of vertebrate species of a protein resembling human alpha-2 macroglobulin. Biochem J. 205(1):91–95.

Thibault RE, Schultz RJ. 1978. Reproductive adaptations among viviparous fishes (Cyprinodontifores poeciliidae). Evolution. 32(2):320–333.

Trivers RL. 1974. Parent–offspring conflict. Am Zoo. 14(1):249–264.

Turner CL. 1940. Pseudoamnion, pseudochorion, and follicular pseudoplacenta in poeciliid fishes. J Morphol. 67:59–87.

Tycko B, Morison IM. 2002. Physiological functions of imprinted genes. J Cell Physiol. 192(3):245–258.

Vrana PB, Guan X-J, Ingram RS, Tilghman SM. 1998. Genomic imprinting is disrupted in interspecific Peromyscus hybrids. Nat Genet. 20:362–368.

Wake MH, Dickie R. 1998. Oviduct structure and function and reproductive modes in amphibians. J Exp Zool. 282(4–5):477–506.

Wourms JP, Grove BD, Lombardi J. 1988. The maternal-embryonic relationship of viviparous fishes. In: Hoar WS, Randall DJ, editors. Fish physiology. Orlando (FL): Academic Press. p. 1–134.

Zeh DW, Zeh JA. 2000. Reproductive mode and speciation: the viviparity-driven conflict hypothesis. BioEssays. 22(10):938–946.

Zhang H, Song LS, Li CH, Zhao JM, Wang H, Gao Q, Xu W. 2007. Molecular cloning and characterization of a thioester-containing protein from Zhikong scallop Chlamys farreri. Mol Immunol. 44(14):3492–3500.

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