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Transcriptional signature of progesterone-induced germinal vesicle breakdown in the fathead minnow ovary (*Pimephales promelas*)

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Abstract

A growing number of studies have examined transcriptional responses to sex steroids along the hypothalamic-pituitary-gonadal axis in teleost fishes. However, data are lacking on the molecular cascades that underlie progesterone signaling. The objective of this study was to characterize the transcriptional response in the ovary of fathead minnows (*Pimephales promelas*) in response to progesterone (P4) using transcriptomics. Fathead minnow ovaries were exposed *in vitro* to 500 ng P4/L. Germinal vesicle migration and breakdown (GVBD) was observed and microarrays were used to identify gene cascades affected by P4. Microarray analysis identified 1,702 differentially expressed transcripts after P4 treatment. Functional enrichment analysis revealed that transcripts involved in the molecular functions of protein serine/threonine kinase activity, ATP binding, and activity of calcium channels were increased after P4 treatment. There was an overwhelming decrease in levels of transcripts of genes that are structural constituents of ribosomes with P4 treatment. There was also evidence for gene expression changes in steroid and maturation-related transcripts. Pathway analyses identified cell cycle regulation, insulin action, hedgehog, and B cell activation as pathways containing an over-representation of highly regulated transcripts. Significant regulatory sub-networks of P4-mediated transcripts included genes regulated by tumor protein p53 and E2F transcription factor 1, suggesting that downstream expression targets of these two proteins may be preferentially involved in P4-mediated GVBD. These data provide novel insight into the molecular signaling cascades that underlie P4-signaling in the ovary and identify

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genes and processes that may indicate premature GVBD due to environmental pollutants that mimic progestins.

Keywords

microarrays; calcium signaling; germinal vesicle breakdown; ribosomes; progestins

Introduction

Growth and maturation of ovarian oocytes are under the control of multiple stimulatory and inhibitory endocrine signals that initiate molecular cascades within the oocyte. As oocytes progress towards final maturation, there is increased DNA replication and active uptake of vitellogenin, followed by the arrest of meiotic cell division. The cessation of cell division is followed by germinal vesicle breakdown (GVBD), a process that is characterized by the resumption of meiosis, yolk rearrangement, nucleus migration, breakdown of the germinal vesicle membrane and ultimately, ovulation from the germinal layer of the follicle. Ovulation and GVBD are caused by a surge of gonadotropin and progesterone (P4) over a short time period (hours). Yolk and GV migration under low levels of progesterone are slow and take weeks to occur. Reviews of this process have been described by Lessman [24] and Nagahama and Yamashita [35].

Hormonal control of oocyte maturation involves the pituitary-derived hormones follicle-stimulating hormone (FSH), to mediate growth and development, and luteinizing hormone (LH), to mediate final maturation and ovulation. Sex steroids (e.g. 17 β -estradiol (E2), testosterone (T), and progestins) also play significant roles in the ovary during sexual maturation. The role of progestins as pheromones and maturation inducing molecules has been well established [1,33,49] and includes the ovarian maturation-inducing substance (MIS) 17-alpha-20-beta-dihydroxyprogesterone, a dominant biologically active progestin in fish [34]. Progestins, including MIS, have been shown to promote GVBD during final oocyte maturation in fish [11,30,40]. Hormones, drugs, and environmental pollutants such as fungicides and pesticides have been found to be potent P4 mimics and suppressors (Table 1).

Although not as potent as other progestins, there is evidence that progesterone (P4) is metabolized into metabolites such as MIS; these metabolites have a significant role in oocyte development and final maturation as P4 has been shown to initiate GVBD in teleost fish [59]. Plasma levels of P4 in white-spotted char (*Salvelinus leucomaenis*) also correlate with developmental stages of ovarian oocytes and there is a preovulatory rise in plasma progesterone levels [18]. At the molecular level, both membrane (*pgrm*) and nuclear P4 receptor (*pgr*) expression are associated with morphological and physiological changes in the oocytes [54]. Kazeto et al. [21] showed that the alpha isoform of *pgrm* peaked prior to spawning in adult female channel catfish (*Ictalurus punctatus*) while the gamma isoform appeared to peak in expression at earlier stages of reproduction. Moreover, there is evidence that the alpha isoform of *pgrm* in Atlantic croaker (*Micropogonias undulatus*) at both the message and protein level of regulation are induced with gonadotropin treatments [58]. The nuclear P4 receptor in the teleost ovary has only recently been characterized in

zebrafish (*Danio rerio*) [6,13] and is expressed in many tissues including the ovary and brain. Based on data presented by Hanna and colleagues [13], zebrafish *pgr* is expressed strongly in follicular layers of oocytes at all stages of development and *pgr* immunoreactive protein is located predominately in the follicular layer at later stages (IV) of maturation. Using a dual luciferase transactivation /transcription assay, these same authors demonstrated that MIS, 17,20b-DHP, and P4 were the most potent activators of *pgr* transcription compared to other steroids tested. These studies indicate that P4, in addition to other well-studied progestins, plays a key role in regulating receptor signaling that mediates oocyte growth, development, and maturation.

There are growing data describing physiological and biochemical changes occurring in the ovary during final oocyte maturation and GVBD. However, molecular data are lacking on global expression changes in the teleost ovary in response to P4. Experiments conducted in mammals demonstrate that P4-regulated signal transduction pathways in follicles within the ovary involve calcium mobilization [10], G-protein kinases [42], and phospholipase C signaling [38]. However, due to the differences in folliculogenesis between mammal and fish, there are likely molecular differences underlying final oocyte maturation.

This study utilized a fathead minnow (*Pimephales promelas*, FHM) microarray to characterize the response of the ovarian transcriptome to P4 in order to explore these molecular events. In the context of ecotoxicology, Scippo et al. [47] demonstrated that some environmental pollutants of concern such as organochlorine pesticides (DDT, aldrin, etc) have a higher affinity for the hPR than hER and synthetic progestins found in birth control pills, for example Norethindrone, have recently been shown to decrease cumulative egg production in FHM after 21d exposure at concentrations of 1.2 and 85 ng/L [41]. These data underscore the need to increase understanding of the cell processes mediated by progestins in the ovary. This is important because adverse effects due to aquatic contaminants that mimic progestins may contribute to an increased risk of impaired oocyte maturation and early GVBD in teleost fish. The objective of this study was to simulate the natural P4 surge associated with ovulation to induce GV migration and GVBD in order to identify the genes associated with these events and to characterize potential maturation biomarkers.

2. Materials and Methods

2.1. Fathead minnow exposure to P4

Ovaries were collected from four reproductive FHM and prepared for in vitro exposure. The gonad was cut into two pieces (10 mg), and transferred to culture plates (Costar) containing 50 ml of Zebrafish Ringers media (116 mM NaCl, 2.9 mM KCl, 1.8 mM CaCl₂, 5 mM HEPES, pH 7.2) after rinsing. The ovaries were exposed to either media containing 500 ng/L progesterone (Sigma) or a control consisting of 0.05% of the carrier solvent (95% ethanol). Exposures were run at 25°C for 4 hours. A small subsample (~2 mg) of the ovary of each female was used for clearing and GV staging. The remaining tissue was flash frozen using liquid nitrogen, stored at -80 °C, and used for gene arrays. The egg maturation response for each female and treatment was verified by clearing the ovary tissue post-treatment (control & P4) using Pankurst solution (ethanol:formalin:acetic acid; 6:3:1) for 3–5 minutes under a microscope. Digital photographs were taken of cleared eggs from all

females and treatments. Maturation response was verified by comparing the position of the germinal vesicle of the largest eggs (maturation competent) in the control and progesterone treatments. All procedures involving live fish were reviewed and approved by the University of Florida Institutional Animal Care and Use Committee (IACUC).

2.2. GVBD in FHM ovary

Ovarian development in the FHM is asynchronous resulting in oocytes in many stages of development. Fathead minnows spawn small clutches (50–100 eggs) weekly. Upon the completion of vitellogenesis, the egg becomes “competent” and reactive to maturation hormones such as progesterone and its metabolites. Exposure to exogenous progesterone *in-vitro* simulates natural spawning conditions, induces germinal vesicle migration, breakdown of the germinal vesicle membrane (GVBD) in the most advanced oocytes and has been validated in many fish species.

Four different stages of egg development are visible after egg clearing: 1) pre-vitellogenic; 2) vitellogenic; 3) early maturation and 4) late maturation (GVBD). Previtellogenic eggs are the smallest and contain no vitellogenin or visible nucleus. Vitellogenic eggs have a centrally located GV. Eggs that have completed egg growth and have entered the maturation phase will have GV's that are off-center or near the oocyte periphery. Eggs ready for oviposition contain no GV and the nuclear membrane has broken down (GVBD). GVBD is P4-inducible in the most advanced oocytes and found in females in the act of spawning.

2.3. Gene expression analysis in the FHM ovary

Gene expression analysis was performed with four biological replicates for each treatment (control and P4). However one microarray in the control group did not satisfy quality control and was removed from the analysis. Total RNA for microarray analysis was extracted from FHM ovary using RNA STAT-60 reagent (TEL-TEST Inc., Friendswood, TX, USA) as per manufacturer's protocol. RNA quantity was measured using the NanoDrop ND-1000 (Nanodrop Technologies, Wilmington, DE) and RNA quality was evaluated using the Agilent 2100 BioAnalyzer with the RNA 6000 Nanochip. RNA integrity values (RIN) were > 8.6 for all samples used in the microarray analysis with an average of 9.3. Microarray hybridizations were performed using a 22,000-gene array designed by EcoArray (4×44k format, GPL7282). Array hybridizations were performed using a reference design. The reference material consisted of equal amounts of RNA from both female and male tissues (liver, brain, and gonad). The cDNA synthesis, cRNA labeling and hybridization were performed following the manufacturer's kits and protocols (Agilent Low RNA Input Fluorescent Linear Amplification Kit and Agilent 60-mer oligo microarray processing protocol; Agilent, Palo Alto, CA). After hybridizing for 17 h, microarrays were washed and then scanned with a laser-based detection system (Agilent, Palo Alto, CA). Text versions of the Agilent raw data have been deposited at the Gene Expression Omnibus website (GEO: <http://www.ncbi.nlm.nih.gov/geo/>; Accession series record number GSE43601).

2.4. Microarray analysis and functional enrichment

Raw expression data were imported into JMP[®] Genomics v3.2 (SAS, Cary, NC) using the ratio analysis procedure and normalized using the within array Loess normalization with a

smoothing factor of 0.2. Data quality analysis after normalization verified that microarray signal intensities of Cy5 and Cy3 were sufficiently normalized based on signal histogram and box-plots of each microarray. Differentially regulated transcripts were identified using a one-way analysis of variance (FDR=5%). All FHM probes identified as differentially expressed and used for further bioinformatics analyses had minimum annotation confidence (e-value) of $<10^{-04}$ and all duplicate probes were removed from further analysis.

For functional enrichment analysis, JMP® Genomics v3.2 was used to identify molecular functions that were over-represented in the list containing regulated transcripts compared to a second list of all gene probes on the FHM microarray. Multiple hypothesis testing correction was based on the method by Benjamini and Hochberg [2].

2.5 Pathway analysis and identification of sub-networks within regulated gene list

Pathway Studio® v7.1 and ResNet 7 (Ariadne Genomics, Rockville, MD, USA) were used to identify cell pathways and processes that were enriched or over represented in the list of highly regulated P4-mediated transcripts ($p < 0.01$). The closest human homologues were manually obtained from National Center for Biotechnology Information (NCBI) using BLAST for FHM genes. Human homologues were mapped using GenBank identifiers using the ID mapping service in Pathway Studio®. For these highly regulated transcripts, a pathway was built using direct interactions that included all parameters (e.g. binding, expression, direct regulation, promoter binding, and protein modification). In addition, both the membrane bound and nuclear progesterone receptors were included in the pathway to better discern direct effects of the nuclear and membrane bound progesterone receptors on gene expression in the ovary.

Regulated FHM genes ($p < 0.01$) were compared to metabolic and signaling pathways and groups annotated in Pathway Studio® to determine whether there were pathways over-represented by regulated transcripts. Only those pathways that involved greater than 5 entities were considered in further analysis and discussion. In addition, to explore further key pathways affected by P4 in the ovary, sub-networks enriched with select entities were identified. The sub-networks within the highly regulated transcripts included network targets based on expression regulation, protein binding, and downstream protein modifications. For this analysis, only those networks that had greater than 5 entities in the network were considered.

Further network analysis was performed using Ingenuity Pathway Analysis (IPA, Redwood City, CA). The network score was based on the hypergeometric distribution and was calculated with the right-tailed Fisher's Exact Test.

2.6. Real-time PCR

A total of 800 ng RNA were first reverse transcribed into cDNA in a 20- μ l reaction containing 250 ng random primers and SuperScript™ III reverse transcriptase (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. The synthesized cDNA was diluted to 10 ng/ μ l. Quantitative real-time polymerase chain reaction (QPCR) assays were performed on an ABI Sequence Detector 7900 (Applied Biosystems, Foster City, CA). Each 20- μ l reaction was run in triplicate and contained 6 μ l of synthesized cDNA template along with

2 μ l of each forward and reverse primer (5 μ M/ μ l) and 500 nM Sybr Green PCR Master Mix (Applied Biosystems). Cycling parameters were 95 °C for 15 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Primers were designed using Primer Express® (Applied Biosystems), synthesized by Operon Biotechnologies (Huntsville, AL, USA) and tested for amplification efficiency. The genes tested by QPCR were β -*hsd* (hydroxysteroid dehydrogenase), *11* β -*hsd*, *20* β -*hsd*, aquaporin 8 (*aqp8*), cytochrom P450 17 (*cyp17*), aromatase (*cyp19*), estrogen receptor α (*esr1*), estrogen receptor β 1 (*esr2a*), follistatin (*foll*), follicle-stimulating hormone (*fsh*), follicle-stimulating hormone receptor (*fshr*), insulin-like growth factor 1 (*igf1*), insulin-like growth factor 1 receptor (*igf1r*), inhibin (*inhib*), luteinizing hormone receptor (*lhr*), midkine-related growth factor (*mdka*), Nedd4 family interacting protein (*nedd4fip*), p38 mitogen-activated protein kinase (*p38mapk*), steroidogenic acute regulatory protein (*star*), tissue inhibitor of metalloproteinase 3 (*timp3*), vitellogenin (*vlg*), and zona pellucida 3 (*zp3*). The sequences for all the primers can be found on Table S1. Results were normalized to 18S rRNA and analyzed using the Ct method (Applied Biosystems, Foster City, CA, USA).

3. Results

3.1. GV migration in FHM ovary after P4 treatment

All four control samples had centrally located oocytes post-clearing with no evidence of off-center GV's or GVBD in the ovary subsample. Following P4 treatment, the GV's in the advanced oocytes had migrated 50–75% of the way to the oocyte periphery. Evidence of GVBD was visible in 5–10% of the oocytes after P4 treatment. Photographs representing the three stages of cleared eggs observed in the control and P4 treatments are shown in Figure 1.

3.2. Gene expression profiling in the FHM ovary following P4 treatment

There were a total of 1,702 (NR e-value of $<10^{-04}$) gene probes differentially expressed by P4 with 1,243 transcripts increasing in abundance and 459 decreasing in steady state mRNA abundance ($p < 0.05$). Significantly more genes were increased than decreased in abundance after P4 treatment (d.f.=1, $\chi^2=200.01$, $p < 0.0001$). Interestingly, there was little change in steroid-related transcripts, including nuclear receptors and genes involved in steroidogenesis.

Transcripts with the largest increase in relative abundance (> 2 -fold) included *stabilin 1 precursor (stab1)*, *zinc finger protein 91 (HPF7, HTF10) (znf9; HPF7, HTF10)*, *neurabin-II (neural tissue-specific F-actin binding protein II) (protein phosphatase 1 regulatory subunit 9B) (spinophilin) (p130) (PP1bp134)*, and *calcium channel, voltage-dependent, L type, alpha 1C subunit (cacna1c)* ($p < 0.05$). Transcripts with the largest decrease in relative abundance (> 2 -fold) included *12S ribosomal RNA gene*, *U6 snRNA-associated Sm-like protein LSM6 (Sm protein F) (lsm6)*. A complete list of differentially expressed genes is provided in Table S2.

3.3. Real time PCR

Twenty-two genes were analyzed by QPCR, mostly due to their involvement in steroidogenesis or ovary maturation [61]. Of these 22, four genes were significantly up-regulated (*20b-hsd*, *foll*, *p38mapk* and *zp3*) while *aqp8* was down-regulated (Table S3).

3.4. Functional Enrichment

Functional enrichment analysis provided novel insight into the Gene Ontology molecular function terms affected by P4 (Table 2). Overall, there was a significant induction in genes involved in protein serine/threonine kinase activity (d.f. = 1; $\chi = 26.8$; $p < 0.0001$), ATP binding (d.f. = 1; $\chi = 60.6$; $p < 0.0001$), and calcium channel activity (d.f. = 1; $\chi = 7.0$; $p = 0.008$). In contrast, there was an overwhelming response for genes that coded for proteins that are structural constituents of the ribosome, which were down-regulated after P4 treatment (d.f. = 1; $\chi = 30.9$; $p = 0.008$).

Transcripts involved in the molecular function of protein serine/threonine kinase activity that were increased >2-fold included the TBP-associated factor 1 isoform 1 (*tafl1*) and interleukin-1 receptor-associated kinase 4 (*irak4*). Table 3 shows all the transcripts involved in serine/threonine kinase signaling. Transcripts involved in the molecular function of ATP binding that were increased over two-fold included the interleukin-1 receptor-associated kinase 4 (*irak4*), the novel mcm domain containing protein (*mcm9*), the ATP-binding cassette, sub-family A member 4 (*abca4*), and the TBP-associated factor 1 isoform 1 (*tafl1*) while *nppb* was decreased >2-fold. The calcium channel, voltage-dependent, L type, alpha 1C subunit (*cacna1c*), involved in calcium channel activity, was increased >2-fold.

3.5. Pathway analyses

Human homologues for 202 transcripts (89%) were confidently identified for FHM genes. These were manually obtained from NCBI. The FHM sequences were compared to the human NR database in NCBI and if there was an amino acid identify score >60%, then the protein was considered to have a human homolog. Using the Genbank mapping function in Pathway Studio, an additional 7 transcripts could not be mapped, reducing the final dataset to 195 transcripts. To get an idea of how the regulated genes may fit together in pathways, we used PathwayStudio to identify common regulators (genes upstream of the regulated genes), common targets (genes downstream of the regulated genes), direct interactions among the differentially expressed genes, over-represented signaling pathways, and sub-networks. The number of direct interactions (database based on the literature) among transcripts and the progesterone receptors were few, suggesting most of the interactions among regulated transcripts in the ovary are due to indirect or unknown effects of P4 (Figure 2). Any direct effects between nuclear progesterone receptor and regulated molecules were primarily due to the intermediate protein SRC. Also noteworthy is that the direct effects in the pathway involved transcripts that were increasing in steady state mRNA abundance. Pathway Studio signaling pathways that were over represented by highly regulated transcripts included cell cycle regulation, insulin action, B cell activation, and the hedgehog pathway (Table 4).

We also identified enriched sub-networks using two different approaches. First, using Pathway Studio, we identified significant sub-networks for groups containing more than 5 entities (Table 5). Pathway Studio uses a proprietary algorithm called Sub-Network Enrichment Analysis (SNEA) to identify key regulators, targets, and binding partners based on experimental data. These networks (Fig 3) included transcripts regulated by tumor protein p53 (*tp53*), and E2F transcription factor 1 (*e2f1*). Notably, there are some common genes regulated by these molecules that include the damage-regulated autophagy modulator, TNF receptor-associated factor 4, CD59 molecule, complement regulatory protein, and cyclin-dependent kinase inhibitor 2A. Binding partners of histone deacetylase 1 (*hdac1*), protein modification targets of polo-like kinase 1 (*plk1*), caspase 3 (*cas3*), and mitogen activated kinase protein (*mapk*) were also affected by P4 treatment. In a second approach, we mapped orthologs of the DEGs from the P4 treatment onto networks that maximize their specific connectivity to known functions, pathways, and diseases in the Ingenuity Knowledge Base [4]. These network analyses give insight into relationships that exist between genes in the data of interest and known interactions. The highest scoring network (Figure 4) was enriched in molecules related to RNA post-transcriptional modification, as well as developmental disorder and hematological disease. Interestingly, and consistent with the above-mentioned findings, all ribosomal proteins included in this network were down-regulated.

4. Discussion

4.1 Progesterone effects on steroidogenesis and maturation genes

The major endocrine event associated with the termination of vitellogenesis and oocyte maturation are an acute increase in plasma LH levels and an LH-driven switch in the steroidogenesis pathway from the production of predominantly E2 during vitellogenesis to the production of MIS [27]. This switch is normally associated with decreased transcription of P450 aromatase and increased 20 β -HSD activity and often transcription in granulosa cells [28,48,52]. Our data showed an increase in 20 β -HSD, as confirmed by QPCR, but we did not capture a significant decrease of aromatase expression in the FHMs. This is consistent with the observations from both Villeneuve et al [61], where no changes on aromatase transcription in mature ovaries were detected; and the observations by Nakamura et al [36], where aromatase transcripts increased during vitellogenesis but not after vitellogenesis was complete. The enzyme CYP17 has been shown to be up-regulated during ovary maturation [28,51]. This enzyme is up-regulated in our data, although it could not be confirmed by QPCR. This could be explained by the fact that two different forms of CYP17 encoded by two different genes have been identified in fish, so our primers and probes could be detecting the different forms [66,67]. Nevertheless, perhaps the fact that the FHM gonad used in the current experiments contained all stages of oocytes may have dampened the changes in aromatase and CYP17 expression in the mature oocytes after P4 treatment.

Aquaporins are a family of integral proteins that facilitate transport of water across the cell membrane and have been shown to play a crucial role in oocyte hydration [1,28]. An aquaporin ortholog, similar to *aqp8* characterized in humans, was found to be differentially regulated in FHM ovaries with peak up-regulation in the vitellogenic stage [61]. Our results

show that *aqp8* was down-regulated in P4-treated ovaries, which would be consistent with the previous study, as mature ovaries had less *aqp8* transcripts than vitellogenic. Both studies suggest that *aqp8* might play an important role in oocyte maturation in fish.

4.2 Progesterone effects on claudins

Our data revealed two claudin isoforms to be down-regulated with P4 treatment. Claudins mediate cellular adhesion and migration and are the major tight junction transmembrane proteins. Tight junctions form tight seals between adjacent cells, functioning as a gate that regulates paracellular movement of ions and small molecules and functions as a dynamic barrier between the external and internal environment [12]. The claudin component of tight junctions has been shown to determine their size and specificity [12]. Over twenty claudin family members have been identified in most vertebrate species [22]. During embryogenesis, distinct claudin expression corresponding to sites of interactions required for patterning are observed in different tissues [12]. Some of the claudin isoform transcripts of maternal origin have been shown to be downregulated in the early stages of zebrafish embryogenesis [1,26]. Furthermore, several claudin isoforms were shown to be down-regulated in zebrafish oocytes during maturation and after MIH exposure [7]. Interestingly, claudins are being suggested as potential therapeutic targets for several uses, such as enhancing the delivery of pharmaceutical agents by targeting the endothelial tight junctions so that they become leakier [5]. Future studies should help elucidate the complex functions of this enticing family of proteins, as well as their involvement in oocyte maturation.

4.3 Progesterone effects on kinase activities

Several Gene Ontology categories related to kinase activity were significantly affected by P4. These categories include protein tyrosine phosphatase activity, protein kinase activity, MAPK kinase kinase activity, protein serine/threonine kinase and tyrosine kinase activities. Using dispersed rat ovarian cells incubated in the presence of choriogonadotropin and 3-isobutyl-1-methylxanthine, Azhar and Menon [45] showed a correlation between high P4 production and increase in protein kinase activity ratio. Furthermore, *p38mapk*, which has been shown to be involved in oocyte maturation [16,60], was up-regulated by the P4 treatment. These results suggest the involvement of MAPK in the GVBD process and are consistent with previous findings on the central role of MAPK in the morphological changes during oocyte maturation [1,9].

4.3.1 Serine/threonine kinases—A primary cellular mechanism in the regulation of protein function involves phosphorylation cascades. Protein serine/threonine kinases are enzymes that add phosphate groups to susceptible serines and threonines. The active kinase phosphorylates target proteins, initiating downstream biological responses within the cell that include changes in protein activity or a change in cellular localization of a protein. Signaling via serine/threonine phosphorylation has been implicated in many cell processes and is well known to be involved in the regulation of the cell cycle. For instance, the serine/threonine protein kinase AKT plays a substantial in oocyte maturation in several species [15,19,20,57]. Although we did not measure protein activity, AKT was found up-regulated in our dataset, suggesting that AKT might also have a crucial role in FHM oocyte maturation. The maternal embryonic leucine zipper kinase (*melk*), a member of the Snf1/

AMPK serine/threonine kinase family and down-regulated in our dataset, is conserved in the animal taxa and is relatively high in expression in ovulated mouse eggs [14] and bovine oocytes [53] compared to later stages of embryogenesis. It has been demonstrated that *melk* plays a role in regulating cell cycle progression and that it is an important regulator of proliferation and survival of cancerous cells [37]. However, the function of *melk* in the ovary is less studied and is not as well characterized compared to its function in the central nervous system. It is plausible that *melk* also regulates cell cycle progression during final maturation of the follicle, and this is partially mediated by P4.

Some functions of Nek (NIMA (never in mitosis gene a)-related kinases) kinase serine/threonine family include chromosomal condensation and centrosome assembly during the cell cycle [39]. Two members of this family, *nek2* and *nek4*, were up-regulated after P4 exposure in our dataset. In Nile tilapia (*Oreochromis niloticus*), Matsuoka and colleagues [29] demonstrated using in situ hybridization that *nek2* is expressed most abundantly in meiotic cells within oocytes. Interestingly, *nek* proteins are also known to be involved in the G2/M phase transition of the cell cycle in co-operation with cyclin-dependent kinase 2 (*cdc2*). Cdc2 and cyclin B (*Cyc-b*) comprise the maturation-promoting factor (MPF) heterodimer and direct actions of P4 on *nek* mRNA steady state abundance may be one mechanism by which P4 regulates the cell cycle during final maturation. Despite the complex underlying mechanisms, our data support the involvement of the serine/threonine kinases family in the FHM ovary during GVBD.

4.4 Progesterone effects on ribosomal proteins

The accumulation of specific maternal information and molecules, such as RNAs and proteins, is crucial for the ability of an oocyte to develop into a viable embryo. These maternal factors can be stored for a significant amount of time and their use will sustain embryonic development to the stage where zygotic gene activation (ZGA) takes over [1,46]. This ZGA requires at least three events for the progression of development: destruction of oocyte-specific transcripts, replacement of maternal transcripts with zygotic transcripts, and reprogramming in the pattern of gene expression coupled with the generation of novel transcripts that are not expressed in the oocyte [46]. During oocyte maturation, the recruitment, translation, and degradation of maternal mRNAs are extremely important processes [46]. Oocyte maturation initiates the degradation of maternal RNA that continues through the 2-cell stage. At that point >90% of maternal RNAs have been degraded. This degradation process might be one mechanism that drives the developmental switch from oocyte to embryo [46].

Transcriptional regulation in maturing oocytes is complex and highly conserved between species, and is characterized by differential degradation, storage and recruitment of distinct maternal RNAs [9]. In the cap-dependent translational initiation, typical for eukaryotic cells and in particular for maturing oocytes, the cap structure attracts the eukaryotic initiation factor eIF4F complex, an heterotrimeric complex that contains a cap-binding protein, an RNA-dependent helicase and a protein that serves as adapter, eIF4G [9]. This protein, up-regulated in our dataset, has substantial translational stimulatory activity and is essential for initiation of translation and resumption of maturation in *Xenopus* oocytes [62].

All this activity in maturing oocytes is consistent with the fact that processes involving RNA polymerase and transcriptional activators are highly enriched in our dataset (Table S4). Furthermore, a large number of genes that code for structural constituent of ribosomes and genes that have a role in transcription initiation factor activity were either up or down-regulated by P4 during GVBD. Interestingly, all ribosomal proteins included in the inferred network shown in Figure 4 were down-regulated, which suggests turn off of translation.

4.5 Progesterone effects on IGF and hedgehog signaling

Transcriptional response to P4 exposure resulted in some over represented signaling pathways including cell cycle regulation, insulin action, B cell activation, and the hedgehog pathway. These data are consistent with other reports showing that these signaling pathways are key pathways mediating oocyte maturation. For example, IGF1 induces GVBD in common carp *Cyprinus carpio* follicles [32] and relatively low concentrations of IGF (5 nM) can increase GVBD by 25–30%. Insulin-like growth factor has also been shown to induce final oocyte maturation in red seabream (*Pagrus major*) [17], and induce GVBD in white perch (*Morone americana*) [64]. In addition, final oocyte maturation in teleost fish includes transcripts involved in the immune system and the overall process has been described as an inflammatory immune response at the transcriptome level [3]. Consistent with this notion, genes involved in B cell activation were preferentially affected with P4 treatment.

Perhaps most interesting was that genes involved in the hedgehog-signaling pathway were significantly altered by P4 in the ovary. This pathway is involved in developmental patterning of organs and most studies investigating this pathway in teleost fish have focused on its role in neural tissue development. Recent studies in mammals have provided data on the possible role of hedgehog signaling in the ovary. For example, sonic hedgehog stimulated basal androstenedione production in theca cells of bovine follicles [50]. Of note, P4 production by bovine theca cells did not appear to be affected by sonic hedgehog. In addition, the study demonstrated that sonic hedgehog treatment stimulated proliferation (number) of both small and large bovine theca cells along with IGF1 treatment. Effects of hedgehog proteins on steroidogenesis have also been found in fetal Leydig cell precursors through the up-regulation of P450 side-chain cleavage enzyme (*CYP11A1*) expression [65]. Russel et al. [44] showed in the mouse ovary that hedgehog signaling also targets the granulosa layer based on gene expression changes of hedgehog ligands in this tissue. All this information suggests that both the sonic hedgehog pathway and the IGF1 signaling pathway may be activated in concert to regulate final stages of maturation.

Conclusions

Using a microarray-based approach, this study begins to characterize molecular signaling cascades in the teleost ovary that are sensitive to P4. The most intriguing finding is that there is an induction of genes involved in kinase activity and calcium signaling, suggesting that there is an overwhelming genomic response for active cell signaling and protein activation during GVBD. P4-responsive pathways in the ovary are important to characterize because studies have demonstrated that aquatic pollutants such as organochlorine pesticides are capable of interacting with both fish and human PRs [8,25].

It should be pointed out that a limitation to this study is that the thecal and granulosa cells could not be separated, and molecular signaling cascades within each cell are likely unique. We believe the responses are probably more dramatic, but have been blunted by the presence of mixed oocytes. These molecular signals may reflect the compartmentalization of steroid metabolism in the oocyte as well as GVBD. Despite this limitation, this study identifies novel signaling cascades that may have predominant roles in oocyte maturation. Future studies should continue to investigate specific responses within the oocyte and utilize specific agonist/antagonists for Pgrm and Pgr in order to distinguish signaling cascades mediated by each type of receptor in the teleost ovary.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Babin PJ, Cerdà J, Lubzens E. The fish oocyte: from basic studies to biotechnological applications. 2007
2. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society Series B*. 1995
3. Bobe J, Nguyen T, Fostier A. Ovarian function of the trout preovulatory ovary: new insights from recent gene expression studies. *Comparative Biochemistry and Physiology - Part a: Molecular & Integrative Physiology*. 2009; 153:63–68.
4. Calvano SE, Xiao W, Richards DR, Felciano RM, Baker HV, Cho RJ, et al. A network-based analysis of systemic inflammation in humans. *Nature*. 2005; 437:1032–1037. [PubMed: 16136080]
5. Campbell M, Kiang A-S, Kenna PF, Kerskens C, Blau C, O'Dwyer L, et al. RNAi-mediated reversible opening of the blood-brain barrier. *J Gene Med*. 2008; 10:930–947. [PubMed: 18509865]
6. Chen SX, Bogerd J, García-López A, de Jonge H, de Waal PP, Hong WS, et al. Molecular cloning and functional characterization of a zebrafish nuclear progesterone receptor. *Biology of Reproduction*. 2010; 82:171–181. [PubMed: 19741208]
7. Clelland ES, Kelly SP. Tight junction proteins in zebrafish ovarian follicles: stage specific mRNA abundance and response to 17beta-estradiol, human chorionic gonadotropin, and maturation inducing hormone. *General and Comparative Endocrinology*. 2010; 168:388–400. [PubMed: 20553723]
8. Das S, Thomas P. Pesticides interfere with the nongenomic action of a progestogen on meiotic maturation by binding to its plasma membrane receptor on fish oocytes. *Endocrinology*. 1999; 140:1953–1956. [PubMed: 10098537]
9. Eichenlaub-Ritter U, Peschke M. Expression in in-vivo and in-vitro growing and maturing oocytes: focus on regulation of expression at the translational level. *Hum. Reprod. Update*. 2002; 8:21–41. [PubMed: 11866238]

10. Franczak A, Kurowicka B, Kowalik M. The effect of oxytocin on progesterone secretion, phosphoinositide hydrolysis and intracellular mobilisation of Ca²⁺ in porcine luteal cells. *Acta Veterinaria*. 2009
11. Greeley MS, Calder DR, Taylor MH, Hols H. Oocyte maturation in the mummichog (*Fundulus heteroclitus*): Effects of steroids on germinal vesicle breakdown of intact follicles in vitro. *General and*. 1986
12. Gupta IR, Ryan AK. Claudins: unlocking the code to tight junction function during embryogenesis and in disease. *Clinical Genetics*. 2010; 77:314–325. [PubMed: 20447145]
13. Hanna RN, Daly SCJ, Pang Y, Anglade I, Kah O, Thomas P, et al. Characterization and expression of the nuclear progesterin receptor in zebrafish gonads and brain. *Biology of Reproduction*. 2010; 82:112–122. [PubMed: 19741205]
14. Heyer BS, Kochanowski H, Solter D. Expression of Melk, a new protein kinase, during early mouse development. *Dev. Dyn*. 1999; 215:344–351. [PubMed: 10417823]
15. Hoshino Y, Yokoo M, Yoshida N, Sasada H, Matsumoto H, Sato E. Phosphatidylinositol 3-kinase and Akt participate in the FSH-induced meiotic maturation of mouse oocytes. *Mol. Reprod. Dev*. 2004; 69:77–86. [PubMed: 15278907]
16. Hsieh M, Thao K, Conti M. Genetic Dissection of Epidermal Growth Factor Receptor Signaling during Luteinizing Hormone-Induced Oocyte Maturation. *PLoS ONE*. 2011; 6:e21574. [PubMed: 21738714]
17. Kagawa H, Kobayashi M, Hasegawa Y, Aida K. Insulin and insulin-like growth factors I and II induce final maturation of oocytes of red seabream, *Pagrus major*, in vitro. *General and Comparative Endocrinology*. 1994; 95:293–300. [PubMed: 7958758]
18. Kagawa H, Takano K, Nagahama Y. Correlation of plasma estradiol-17 beta and progesterone levels with ultrastructure and histochemistry of ovarian follicles in the white-spotted char, *Salvelinus leucomaenis*. *Cell Tissue Res*. 1981; 218:315–329. [PubMed: 6942931]
19. Kalous J, Kubelka M, Solc P, Susor A, Motlík J. AKT (protein kinase B) is implicated in meiotic maturation of porcine oocytes. *Reproduction*. 2009; 138:645–654. [PubMed: 19633130]
20. Kalous J, Solc P, Baran V, Kubelka M, Schultz RM, Motlík J. PKB/AKT is involved in resumption of meiosis in mouse oocytes. *Biology of the Cell*. 2012; 98:111–123. [PubMed: 15842198]
21. Kazeto Y, Goto-Kazeto R, Trant JM. Membrane-bound progesterin receptors in channel catfish and zebrafish ovary: changes in gene expression associated with the reproductive cycles and hormonal reagents. *General and Comparative Endocrinology*. 2005; 142:204–211. [PubMed: 15862564]
22. Kollmar R, Nakamura SK, Kappler JA, Hudspeth AJ. Expression and phylogeny of claudins in vertebrate primordia. *Proc. Natl. Acad. Sci. U.S.A*. 2001; 98:10196–10201. [PubMed: 11517306]
23. Lahnsteiner F, Berger B, Kletzl M, Weismann T. Effect of 17beta-estradiol on gamete quality and maturation in two salmonid species. *Aquat Toxicol*. 2006; 79:124–131. [PubMed: 16837076]
24. Lessman CA. Oocyte maturation: converting the zebrafish oocyte to the fertilizable egg. *General and Comparative Endocrinology*. 2009; 161:53–57. [PubMed: 19027744]
25. Li J, Li N, Ma M, Giesy JP, Wang Z. In vitro profiling of the endocrine disrupting potency of organochlorine pesticides. *Toxicology Letters*. 2008; 183:65–71. [PubMed: 18992306]
26. Lo J, Lee S, Xu M, Liu F, Ruan H, Eun A, et al. 15,000 unique zebrafish EST clusters and their future use in microarray for profiling gene expression patterns during embryogenesis. *Genome*. 2003
27. Lubzens E, Young G, Bobe J, Cerdà J. Oogenesis in teleosts: How fish eggs are formed. *General and Comparative Endocrinology*. 2010; 165:367–389. [PubMed: 19505465]
28. Lubzens E, Young G, Bobe J, Cerdà J. Oogenesis in teleosts: How fish eggs are formed. *General and Comparative Endocrinology*. 2010; 165:367–389. [PubMed: 19505465]
29. Matsuoka Y, Kobayashi T, Kihara K, Nagahama Y. Molecular cloning of Plk1 and Nek2 and their expression in mature gonads of the teleost fish Nile tilapia (*Oreochromis niloticus*). *Mol. Reprod. Dev*. 2008; 75:989–1001. [PubMed: 18095313]
30. Miura C, Higashino T, Miura T. A progesterin and an estrogen regulate early stages of oogenesis in fish. *Biology of Reproduction*. 2007; 77:822–828. [PubMed: 17687117]

31. Monod G, Rime H, Bobe J, Jalabert B. Agonistic effect of imidazole and triazole fungicides on in vitro oocyte maturation in rainbow trout (*Oncorhynchus mykiss*). *Mar. Environ. Res.* 2004
32. Mukherjee D, Mukherjee D, Sen U, Paul S, Bhattacharyya SP. In vitro effects of insulin-like growth factors and insulin on oocyte maturation and maturation-inducing steroid production in ovarian follicles of common carp, *Cyprinus carpio*. *Comparative Biochemistry and Physiology - Part a: Molecular & Integrative Physiology.* 2006; 144:63–77.
33. Nagahama Y. 17 alpha,20 beta-dihydroxy-4-pregnen-3-one, a maturation-inducing hormone in fish oocytes: mechanisms of synthesis and action. *Steroids.* 1997; 62:190–196. [PubMed: 9029736]
34. Nagahama Y, Adachi S. Identification of maturation-inducing steroid in a teleost, the amago salmon (*Oncorhynchus rhodurus*). *Developmental Biology.* 1985
35. Nagahama Y, Yamashita M. Regulation of oocyte maturation in fish. *Development.* 2008
36. Nakamura I, Evans JC, Kusakabe M, Nagahama Y, Young G. Changes in steroidogenic enzyme and steroidogenic acute regulatory protein messenger RNAs in ovarian follicles during ovarian development of rainbow trout (*Oncorhynchus mykiss*). *General and Comparative Endocrinology.* 2005; 144:224–231. [PubMed: 16102755]
37. Nakano I, Smith MM. Maternal embryonic leucine zipper kinase is a key regulator of the proliferation of malignant brain tumors, including brain tumor stem cells. *Journal of.* 2007
38. Nishimura R, Shibaya M, Skarzynski DJ, Okuda K. Progesterone stimulation by LH involves the phospholipase-C pathway in bovine luteal cells. *J. Reprod. Dev.* 2004; 50:257–261. [PubMed: 15118253]
39. O'regan L, Blot J, Fry AM. Mitotic regulation by NIMA-related kinases. *Cell Div.* 2007; 2:25. [PubMed: 17727698]
40. Ohta K, Yamaguchi S, Yamaguchi A, Gen K, Okuzawa K, Kagawa H, et al. Biosynthesis of steroids in ovarian follicles of red seabream, *Pagrus major* (Sparidae, Teleostei) during final oocyte maturation and the relative effectiveness of steroid metabolites for germinal vesicle breakdown in vitro. *Comp. Biochem. Physiol. B, Biochem. Mol. Biol.* 2002; 133:45–54. [PubMed: 12223211]
41. Paulos P, Runnalls TJ, Nallani G, La Point T, Scott AP, Sumpter JP, et al. Reproductive responses in fathead minnow and Japanese medaka following exposure to a synthetic progestin. Norethindrone, *Aquat Toxicol.* 2010; 99:256–262.
42. Peluso JJ, Pappalardo A. Progesterone regulates granulosa cell viability through a protein kinase G-dependent mechanism that may involve 14-3-3sigma. *Biology of Reproduction.* 2004; 71:1870–1878. [PubMed: 15286034]
43. Pickford DB, Morris ID. Effects of endocrine-disrupting contaminants on amphibian oogenesis: methoxychlor inhibits progesterone-induced maturation of *Xenopus laevis* oocytes in vitro. *Environ Health Perspect.* 1999; 107:285–292. [PubMed: 10090707]
44. Russell MC, Cowan RG, Harman RM, Walker AL, Quirk SM. The hedgehog signaling pathway in the mouse ovary. 2007
45. Salman Azhar KMJM. Receptor-mediated gonadotropin action in the ovary. Regulatory role of cyclic nucleotide phosphodiesterase(s) in intracellular adenosine 3':5'-cyclic monophosphate turnover and gonadotropin-stimulated progesterone production by rat ovarian cells. *Biochemical Journal.* 1979; 180:201. [PubMed: 226066]
46. Schultz RM. The molecular foundations of the maternal to zygotic transition in the preimplantation embryo. *Hum. Reprod. Update.* 2002; 8:323–331. [PubMed: 12206467]
47. Scippo M-L, Argiris C, Van De Weerd C, Muller M, Willemsen P, Martial J, et al. Recombinant human estrogen, androgen and progesterone receptors for detection of potential endocrine disruptors. *Analytical and Bioanalytical Chemistry.* 2004; 378:664–669. [PubMed: 14579009]
48. Senthilkumaran B, Yoshikuni M, Nagahama Y. A shift in steroidogenesis occurring in ovarian follicles prior to oocyte maturation. *Molecular and Cellular Endocrinology.* 2004; 215:11–18. [PubMed: 15026170]
49. Sorensen PW, Murphy CA, Loomis K, Maniak P, Thomas P. Evidence that 4-pregnen-17, 20β, 21-triol-3-one functions as a maturation-inducing hormone and pheromonal precursor in the percid fish, *Gymnocephalus cernuus*. *General and Comparative Endocrinology.* 2004; 139:1–11. [PubMed: 15474530]

50. Spicer LJ, Sudo S, Aad PY, Wang LS, Chun S-Y, Ben-Shlomo I, et al. The hedgehog-patched signaling pathway and function in the mammalian ovary: a novel role for hedgehog proteins in stimulating proliferation and steroidogenesis of theca cells. *Reproduction*. 2009; 138:329–339. [PubMed: 19433502]
51. Sreenivasulu G, Senthilkumaran B. A role for cytochrome P450 17 α -hydroxylase/c17-20 lyase during shift in steroidogenesis occurring in ovarian follicles prior to oocyte maturation. *The Journal of Steroid Biochemistry and Molecular Biology*. 2009; 115:77–85. [PubMed: 19500725]
52. Tanaka M, Nakajin S, Kobayashi D, Fukada S, Guan G, Todo T, et al. Teleost ovarian carbonyl reductase-like 20 β -hydroxysteroid dehydrogenase: potential role in the production of maturation-inducing hormone during final oocyte maturation. *Biology of Reproduction*. 2002; 66:1498–1504. [PubMed: 11967216]
53. Thlie A, Papillier P, Penner S, Perreau C, Traverso JM, Uzbekova S, et al. Differential regulation of abundance and deadenylation of maternal transcripts during bovine oocyte maturation in vitro and in vivo. *BMC Dev. Biol.* 2007; 7:125. [PubMed: 17988387]
54. Thomas P. Rapid steroid hormone actions initiated at the cell surface and the receptors that mediate them with an emphasis on recent progress in fish models. *General and Comparative Endocrinology*. 2012; 175:367–383. [PubMed: 22154643]
55. Thomas P, Sweatman J. Interference by atrazine and bisphenol-A with progesterin binding to the ovarian progesterin membrane receptor and induction of oocyte maturation in Atlantic croaker. *Mar. Environ. Res.* 2008; 66:1–2. [PubMed: 18378294]
56. Tokumoto T, Tokumoto M, Nagahama Y. Induction and inhibition of oocyte maturation by EDCs in zebrafish. *Reprod Biol*. 2005
57. Tomek W. Activation of Akt (protein kinase B) stimulates metaphase I to metaphase II transition in bovine oocytes. *Reproduction*. 2005; 130:423–430. [PubMed: 16183860]
58. Tubbs C, Pace M, Thomas P. Expression and gonadotropin regulation of membrane progesterin receptor alpha in Atlantic croaker (*Micropogonias undulatus*) gonads: role in gamete maturation. *General and Comparative Endocrinology*. 2010; 165:144–154. [PubMed: 19539624]
59. Upadhyaya N, Haider S. Germinal vesicle breakdown in oocytes of catfish, *Mystus vittatus* (Bloch): relative in vitro effectiveness of estradiol-17 β , androgens, corticosteroids, progesterone, and other pregnane derivatives. *General and Comparative Endocrinology*. 1986; 63:70–76. [PubMed: 3770447]
60. Villa-Diaz LG, Miyano T. Activation of p38 MAPK During Porcine Oocyte Maturation. 2004
61. Villeneuve DL, Garcia-Reyero N, Martinovi D, Cavallin JE, Mueller ND, Wehmas LC, et al. Influence of ovarian stage on transcript profiles in fathead minnow (*Pimephales promelas*) ovary tissue. *Aquat Toxicol*. 2010; 98:354–366. [PubMed: 20363515]
62. Wakiyama M, Imataka H, Sonenberg N. Interaction of eIF4G with poly(A)-binding protein stimulates translation and is critical for *Xenopus* oocyte maturation. *Curr. Biol*. 2000; 10:1147–1150. [PubMed: 10996799]
63. Webb M, Van Eenennaam JP. Effects of steroid hormones on in vitro oocyte maturation in white sturgeon (*Acipenser transmontanus*). *Fish Physiology and*. 2000
64. Weber GM, Moore AB, Sullivan CV. In vitro actions of insulin-like growth factor-I on ovarian follicle maturation in white perch (*Morone americana*). *General and Comparative Endocrinology*. 2007; 151:180–187. [PubMed: 17320084]
65. Yao HH-C, Whoriskey W, Capel B. Desert Hedgehog/Patched 1 signaling specifies fetal Leydig cell fate in testis organogenesis. *Genes Dev*. 2002; 16:1433–1440. [PubMed: 12050120]
66. Zhou L-Y, Wang D-S, Kobayashi T, Yano A, Paul-Prasanth B, Suzuki A, et al. A novel type of P450c17 lacking the lyase activity is responsible for C21-steroid biosynthesis in the fish ovary and head kidney. *Endocrinology*. 2007; 148:4282–4291. [PubMed: 17569754]
67. Zhou L-Y, Wang D-S, Shibata Y, Paul-Prasanth B, Suzuki A, Nagahama Y. Characterization, expression and transcriptional regulation of P450c17-I and -II in the medaka, *Oryzias latipes*. *Biochem. Biophys. Res. Commun.* 2007; 362:619–625. [PubMed: 17723227]

Figure 1.

Photographs of chemically cleared fathead minnow eggs in control and P4 treatments. A) Example of control ovary tissue showing centrally located GV's in vitellogenic and oocytes ready for maturation; B) Close-up of cleared oocyte in "pre-maturation" stage with central GV; C) Oocyte after P4 treatment with an off-center GV; D) Oocyte that has undergone GVBD (absence of GV) after P4 exposure.

Figure 2.

Pathway analyses of direct interactions among transcripts mediated by P4 in the ovary. Also included in the figure are both the membrane-bound and the nuclear progesterone receptors to better elucidate the effects of P4 on gene expression. Red is indicative of an increase in mRNA abundance and blue is indicative of a decrease in mRNA abundance. CACNA1C, calcium channel, voltage-dependent, L type, alpha 1C subunit; CD59, CD59 molecule, complement regulatory protein; CDKN2A, cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4); FOXA1, forkhead box A1; IQGAP1, IQ motif containing GTPase activating protein 1; MYH9, myosin, heavy chain 9, non-muscle; PAK1, p21 protein (Cdc42/Rac)-activated kinase 1; PGR, progesterone receptor; PGRMC1, progesterone receptor membrane component 1; PKM2, pyruvate kinase, muscle; SNX9, sorting nexin 9; UNC45A, unc-45 homolog A (C. elegans); SPEN, spen homolog, transcriptional regulator (Drosophila); TRAF4, TNF receptor-associated factor 4; SRC, v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian).

Figure 3.

Pathway analyses of A) tumor protein p53 (TP53) B) E2F transcription factor 1 (E2F1), and C) miRNA 124-1. These sub-networks were identified within the group of highly regulated transcripts. Red is indicative of an increase in mRNA abundance and blue is indicative of a decrease in mRNA abundance. 1200002N14Rik, damage-regulated autophagy modulator; CD59, CD59 molecule, complement regulatory protein; CDKN2A, cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4); E2F1, E2F transcription factor 1; FEN1, flap structure-specific endonuclease 1; IFRD1, interferon-related developmental regulator 1; MCM5, minichromosome maintenance complex component 5; MYBL2, v-myb myeloblastosis viral oncogene homolog (avian)-like 2; PKD1, polycystic kidney disease 1 (autosomal dominant); POLI, polymerase (DNA directed) iota; PPP1R13B, protein phosphatase 1, regulatory (inhibitor) subunit 13B; TACSTD1, epithelial cell adhesion molecule; TOP2A, topoisomerase (DNA) II alpha 170kDa; TRAF4, TNF receptor-associated factor 4.

developmentally down-regulated 8; RAD21: double-strand-break repair protein rad21 homolog; Rnr: RNase R; RPL11: 60S ribosomal protein L11; RPL13A: 60S ribosomal protein L13a; RPL24: 60S ribosomal protein L24; RPL26: 60S ribosomal protein L26; RPL32: 60S ribosomal protein L32; RPL35A: 60S ribosomal protein L35a; RPL36A: 60S ribosomal protein L36a; RPL39: 60S ribosomal protein L39; RPL5: 60S ribosomal protein L5; RPL7: 60S ribosomal protein L7; RPS10: 40S ribosomal protein S10; RPS11: 40S ribosomal protein S11; RPS15: 40S ribosomal protein S15; RPS2: 40S ribosomal protein S2; RPS3A: 40S ribosomal protein S3, A isoform; RPS4X: 40S ribosomal protein S4, X isoform; RPS5: 40S ribosomal protein S5; VEGF: vascular endothelial growth factor.

Table 1
Chemicals that have been found to alter maturation response in several egg-laying vertebrates

Chemical	Type	Maturation effect	Exposure	Species	Reference
Tamoxifen	drug	▲	In vitro	zebrafish	Tokumoto et al [56]
4-hydroxytamoxifen	drug	▲	In vitro	zebrafish	Tokumoto et al [56]
DES	drug	▲	In vitro	zebrafish	Tokumoto et al [56]
Pentachlorophenol	pesticide	▼	In vitro	zebrafish	Tokumoto et al [56]
17 α ,20 β DHP	hormone	▲	In vitro	zebrafish	Tokumoto et al [56]
Prochloraz	fungicide	▲	In vitro	rainbow trout	Monod et al [31]
Imazalil	fungicide	▲	In vitro	rainbow trout	Monod et al [31]
Epoxiconazole	fungicide	▲	In vitro	rainbow trout	Monod et al [31]
17 β - estradiol	hormone	▼	In vivo	salmon	Lahnsteiner et al [23]
Testosterone	hormone	▲	In vitro	w sturgeon	Webb et al [63]
Progesterone	hormone	▲	In vitro	w sturgeon	Webb et al [63]
Cortisol	hormone	▲	In vitro	w sturgeon	Webb et al [63]
17 α ,20 β DHP	hormone	▲	In vitro	w sturgeon	Webb et al [63]
Kepone	pesticide	▲	Receptor	seatrout	Das & Thomas [8]
DDD-o,p	pesticide	▼	Receptor	seatrout	Das & Thomas [8]
Methoxychlor	pesticide	▼	In vitro	xenopus	Pickford & Morris [43]
Atrazine	herbicide	▼	In vitro	Atlantic croaker	Thomas & Sweatman [55]

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Chemical	Type	Maturation effect	Exposure	Species	Reference
Bisphenol A	plasticizer	inhibitor ▼	In vitro	Atlantic croaker	Thomas & Sweatman [55]

Table 2

Functional enrichment for genes involved in molecular function (Gene Ontology). GO classes were significantly over-represented (FDR-adjusted $p < 0.05$). The complete list of molecular functions that were significant at $p < 0.05$ (prior to FDR-adjustment) is provided in Appendix 2.

GO Category	GO Term	p-value	p-value (FDR adjusted)
GO:0003735	structural constituent of ribosome	4.92E-15	7.73E-12
GO:0004329	formate-tetrahydrofolate ligase activity	1.61E-07	0.00013
GO:0016986	transcription initiation factor activity	1.75E-06	0.00092
GO:0005524	ATP binding	2.90E-06	0.0011
GO:0003980	UDP-glucose:glycoprotein glucosyltransferase activity	0.00010	0.028
GO:0005262	calcium channel activity	0.00012	0.028
GO:0005215	transporter activity	0.00013	0.028
GO:0004674	protein serine/threonine kinase activity	0.00028	0.056 ^a
GO:0003723	RNA binding	0.00034	0.059 ^a

^(a) These Gene Ontologies were close to $p < 0.05$ and are also shown in the table for comparison.

Table 3

Differentially expressed transcripts in the FHM ovary ($p < 0.05$) that were involved in the molecular function of serine/threonine kinase activity. Shown are the NCBI accession (protein) for the transcript, gene identity, fold change (relative to control), and p-value. Two probes for *PREDICTED: similar to TBP-associated factor 1 isoform 1* both indicated a significant increase in expression and the probe indicating the lower fold change relative to control was removed from the table and subsequent analyses.

NCBI Accession	Gene Identity	Fold Change	p-value
AAH50520	maternal embryonic leucine zipper kinase	-1.6	0.001
NP_001007415	protein kinase-like protein SgK196	-1.5	0.021
NP_031916	dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1a	-1.42	0.02
XP_691570	<i>PREDICTED: similar to Traf2 and NCK interacting kinase</i>	-1.41	0.046
AAI09421	mitogen-activated protein kinase 10	-1.4	0.05
XP_687276	<i>PREDICTED: similar to type I serin/threonine kinase receptor</i>	-1.24	0.05
AAH75930	mitogen-activated protein kinase-activated protein kinase 5	1.19	0.043
BAA13204	mitogen-activated protein kinase kinase kinase 4 isoform b	1.22	0.023
XP_689681	<i>PREDICTED: similar to tyrosine-protein kinase transmembrane receptor ROR2 precursor</i>	1.25	0.025
XP_685053	<i>PREDICTED: similar to serine/threonine-protein kinase 17A</i>	1.26	0.022
XP_696856	<i>PREDICTED: hypothetical protein XP_691764</i>	1.26	0.025
CAF92350	<i>PREDICTED: citron Rho-interacting kinase-like</i>	1.26	0.042
AAH46888	ribosomal protein S6 kinase, polypeptide 1	1.23	0.046
AAH44129	mitogen-activated protein kinase kinase 3	1.37	0.016
CAF96367	<i>PREDICTED: similar to protein kinase PRK2</i>	1.38	0.041
AAH65990	eph-like receptor tyrosine kinase 6	1.39	0.012
AAH44485	similar to serine/threonine kinase 38 like	1.41	0.021
CAI11833	novel protein similar to vertebrate mitogen-activated protein kinase kinase kinase 7 (MAP3K7)	1.44	0.025
XP_695199	<i>PREDICTED: similar to SNF-1 related kinase</i>	1.42	0.041
NP_071861	serine/threonine kinase 32B	1.43	0.007
CAF06181	protein-tyrosine kinase	1.44	0.008
CAF97108	5'-AMP-activated protein kinase catalytic subunit alpha-1	1.45	0.034
XP_687040	<i>PREDICTED: similar to tubulin tyrosine ligase-like protein 4</i>	1.43	0.038
CAG05180	<i>PREDICTED: PTK7 protein tyrosine kinase 7</i>	1.48	0.003
XP_590006	<i>PREDICTED: similar to tyrosine kinase 2 isoform 1</i>	1.49	0.047
AAH65603	P21/Cdc42/Rac1-activated kinase 1	1.49	0.004
XP_692754	<i>PREDICTED: similar to ribosomal protein S6 kinase, 52kDa, polypeptide 1</i>	1.53	0.037
AAH37268	Protein kinase C, eta	1.53	0.005
XP_699612	<i>PREDICTED: similar to BMP2 inducible kinase</i>	1.54	0.022
XP_695441	<i>PREDICTED: similar to Serine/arginine-rich protein specific kinase 1</i>	1.55	0.026
XP_698765	<i>PREDICTED: similar to Protein kinase N2 (Protein kinase C-like 2)</i>	1.55	0.008
AAH43764	<i>PREDICTED: similar to serine/threonine kinase TAO1</i>	1.55	0.014
NP_001025232	transient receptor potential cation channel, subfamily M, member 7	1.55	0.045
CAI12053	<i>PREDICTED: similar to Serine/threonine-protein kinase Nek1</i>	1.55	0.008
XP_690225	<i>PREDICTED: similar to thymoma viral proto-oncogene 3 isoform 1</i>	1.57	0.025

NCBI Accession	Gene Identity	Fold Change	p-value
AAH54633	<i>similar to NIMA (never in mitosis gene a)-related expressed kinase 4</i>	1.59	0.015
NP_001003609	<i>microtubule associated serine/threonine kinase-like (mastl)</i>	1.63	0.013
XP_416804	<i>PREDICTED: similar to ribosomal protein S6 kinase 2</i>	1.64	0.025
NP_808430	<i>AP2 associated kinase 1</i>	1.66	0.027
NP_998243	<i>protein kinase PKK</i>	1.69	0.022
CAI21056	<i>similar to NIMA (never in mitosis gene a)-related kinase 2</i>	1.77	0.004
XP_684787	<i>PREDICTED: similar to protein-tyrosine kinase fyn isoform c</i>	1.83	0.04
AAH65959	<i>pdpk1b 3-phosphoinositide dependent protein kinase-1b</i>	1.88	0.011
XP_683044	<i>PREDICTED: similar to TBP-associated factor 1 isoform 1</i>	2.16	0.046

Table 4

Adriane signal pathways that are over represented by highly regulated transcripts ($p < 0.01$) after P4 treatment. The total number of entities indicates the number of entities shown in the group or pathway, the expanded number of entities also includes the number of entities of the pathway and contained in complexes and functional classes.

Signaling Pathways	Total Entities	Expanded # of Entities	p-value
Cell Cycle Regulation	135	2164	0.00002
Insulin Action	50	905	0.00003
B Cell Activation	65	1178	0.00011
Hedgehog Pathway	17	624	0.00132
Translation Control	86	984	0.01127

Table 5

Sub-networks for expression targets, binding partners, and protein modification targets involving highly regulated transcripts ($p < 0.01$) in the ovary. Total number of neighbors refers to all entities within a network and overlap refers to the common entities between the sub-network and P4-mediated transcripts.

Sub-network ^a	Total # of Neighbors	Overlap	p-value
Expression Targets of TP53	522	11	0.008
Expression Targets of E2F1	178	5	0.024
Expression Targets of MIRN124-1	189	5	0.030
Binding Partners of HDAC1	192	5	0.039
Protein Modification Targets of PLK1	62	5	0.001
Protein Modification Targets of CASP3	233	8	0.005
Protein Modification Targets of MAPK	260	8	0.010
Protein Modification Targets of CDC2	250	7	0.026
Protein Modification Targets of MAPK1	275	7	0.041
Protein Modification Targets of casein kinase II, catalyst	283	7	0.046

^aNomenclature follows that for mammalian proteins