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Alterations in gene expression during fasting-induced atresia of early secondary ovarian follicles of coho salmon, *Oncorhynchus kisutch*

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ABSTRACT

Molecular processes that either regulate ovarian atresia or are consequences of atresia are poorly understood in teleost fishes. We hypothesized that feed restriction that perturbs normal ovarian growth and induces follicular atresia would alter ovarian gene expression patterns. Previtellogenic, two-year old coho salmon (*Oncorhynchus kisutch*) were subjected to prolonged fasting to induce atresia or maintained on a normal feeding schedule that would promote continued ovarian development. To identify genes that were specifically up- or down-regulated during oocyte growth in healthy, growing fish compared to fasted fish, reciprocal suppression subtractive hybridization (SSH) cDNA libraries were generated using ovaries from fed and fasted animals. Differential expression of genes identified by SSH was confirmed with quantitative PCR. The SSH library representing genes elevated in ovaries of fed fish relative to those of fasted fish contained steroidogenesis-related genes (e.g., *hydroxy-delta-5-steroid dehydrogenase*), Tgf-beta superfamily members (e.g., *anti-Mullerian hormone*) and cytoskeletal intermediate filament proteins (e.g., *type I keratin s8*). Overall, these genes were associated with steroid production, cell proliferation and differentiation, and ovarian epithelialization. The library representing genes elevated in ovaries of fasted fish relative to fed fish contained genes associated with apoptosis (e.g., *programmed cell death protein 4*), cortical alveoli (e.g., *alveolin*), the zona pellucida (e.g., *zona pellucida protein c*), and microtubules (e.g., *microtubule associated protein tau*). Elevated expression of this suite of genes was likely associated with the initiation of atresia and/or a reduced rate of follicle development in response to fasting. This study revealed ovarian genes involved in normal early secondary oocyte growth and potential early markers of atresia.

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1. Introduction

Numerous studies in mammals have shown that ovarian follicular atresia is a hormonally-controlled apoptotic process (Hughes and Gorospe, 1991; Hsueh et al., 1994). However, the cellular and molecular mechanisms regulating follicular atresia in fishes are much less clear. In fishes, atresia is most commonly observed during the process of resorption of either postovulatory follicles or vitellogenic follicles that fail to mature and ovulate (Drummond et al., 2000; Wood and Van Der Kraak, 2001; Luckenbach et al., 2008a; Santos et al., 2008). In addition to normal atresia that occurs during ovarian development to adjust follicle number to maintain ovarian homeostasis (and ultimately determine fecundity), environmental stressors such as hypoxia, extreme water temperatures, and reduced food availability may disrupt brain-pituitary-gonad (BPG) axis signaling and induce atresia (Guraya, 1986). For example, a recent study we conducted with previtellogenic to early vitellogenic coho salmon (*Oncorhynchus kisutch*) revealed that prolonged fasting impaired endocrine function (reduced levels of follicle-stimulating hormone (Fsh) in the pituitary, as well as plasma estradiol-17β (E2) and insulin-like growth factor 1 (Igf1)), and increased follicular atresia relative to normally fed fish (Yamamoto et al., 2011a). In addition, we found that ovarian expression of steroidogenesis-related genes (e.g., *steroidogenic acute regulatory protein*; *star*) was reduced, while expression of apoptosis-related genes (e.g., *caspase 3*, *caspase 8* and *fas-associated death domain*) was elevated in fasted fish relative to normally fed fish (Yamamoto et al., 2011a). Hence, this previous study showed that feed restriction significantly increased the appearance of atretic follicles and ovarian expression of apoptosis-related genes, while reducing ovarian expression of steroidogenesis-related genes.

Still, atresia is an extremely complex process and little is known about other factors/processes in the ovary that either regulate atresia

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or are consequences of atresia. Our overarching hypothesis is that the pattern of ovarian gene expression will reflect the status of gonadal development. Furthermore, we hypothesize that environmental stressors that perturb normal ovarian growth would alter the ovarian gene expression pattern, finally leading to atresia. There are a variety of conditions that may alter normal gonadal growth, each of which may induce a signature gene expression pattern or 'fingerprint.' Thus, identification of the suite of genes up- or down- regulated during healthy follicular growth compared to follicular atresia is important to establishing tools to assess reproductive health in fishes and ultimately determining the factors that regulate atresia.

The primary aim of this study was to identify new gene transcripts that are regulated in the salmon ovary when ovarian growth and development are impaired and the rate of atresia is increased by prolonged feed restriction. A second goal of the study was to identify genes that might serve as molecular markers of the initiation of atresia. We used coho salmon in this study because it is a semelparous species (spawning only once in its life and then dying) that exhibits synchronous follicle development. This unique reproductive life history allows for stage-specific analysis of a relatively homogenous clutch of ovarian follicles, which is not possible with iteroparous species.

2. Materials and methods

2.1. Animals and sampling

Details regarding the animals used in this study and sampling procedures were previously reported (Yamamoto et al., 2011a). Fish were reared and handled according to the policies and guidelines of the University of Washington Institutional Animal Care and Use Committee (IACUC Protocol #2313-09). In brief, two-year old coho salmon with ovarian follicles at the late cortical alveolus (LCA) stage were either fasted (no feed provided) or normally fed (1.5% body wt./day) over a 17-week period beginning in March, approximately 10 months before the normal time of spawning. Fish were sub-sampled 0, 3, 9, 14, and 17 weeks after fasting was initiated. A piece of ovary from each fish was fixed for staging with paraffin histology and the remaining tissue was snap frozen for later RNA isolation and suppression subtractive hybridization (SSH).

2.2. RNA isolation and cDNA synthesis

Ovarian tissues were individually homogenized using a TissueLyser II (QIAGEN, Germantown, MD) and total RNA was isolated with Tri-Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. For SSH, 2 total RNA pools were generated from 14-week fed and fasted fish ovaries ($n = 8$ fish contributing to each pool) and mRNA was further isolated via the MicroPoly(A) Purist kit (Thermo Fisher Scientific, Waltham, MA). For the across-stage comparisons of gene expression by qPCR, mRNA was also isolated from individual total RNA samples ($n = 8$ fish per group at each time point) to mitigate issues associated with comparing ovarian follicles during different stages of oogenesis, which may be dramatically different in size and RNA composition (Luckenbach et al., 2008b). For the 14-week time point, the same ovary samples were used for qPCR as were used for SSH. For each sample, 50 ng of mRNA was reverse transcribed using SuperScript II (Thermo Fisher Scientific) with random primers (Promega, Madison, WI) in 10 μl reactions.

2.3. Subtractive hybridizations and analyses

Reciprocal SSH cDNA libraries were generated using ovarian mRNA from fed and fasted animals collected at week 14. This time point was selected for SSH, because as previously reported (Yamamoto et al., 2011a), it was the earliest time point when fasted salmon showed significantly reduced plasma E2 levels and pituitary Fsh content, and a higher proportion of follicles either delayed in development or initiating atresia relative to the main cohort of follicles in the ovary. Importantly, the stage of the main cohort of follicles appeared histologically the same (lipid droplet (LD)-stage) for both the fed and fasted fish at this time (Fig. 1). Therefore, we predicted that differences in ovarian gene expression at this time would best reflect the effects of disruption of the BPG axis and atresia onset rather than simply reflecting differential gene expression across follicle stages, as follicles of fed and fasted fish were at different stages by week 17 (Fig. 1).

SSH was performed with 1 μg of pooled mRNA per treatment group using the PCR-Select cDNA Subtraction and Advantage cDNA PCR kits (Takara Bio, Shiga, Japan) as previously described (Luckenbach et al., 2008b). Two subtractions were performed: the original intended subtraction (forward subtraction), and a reverse subtraction in which the tester serves as the driver and driver as the tester. Subtracted cDNA samples were cloned in TOPO pCR 2.1 (Thermo Fisher Scientific) and plated on LB agar containing 100 μg/ml kanamycin. A total of 480 clones per library were randomly selected for sequencing with Big Dye Terminator (Thermo Fisher Scientific) on an ABI 3730 sequencer using the M13 reverse primer. Sequence chromatogram files were trimmed for quality (Phred), vector-screened (cross match), and analyzed in the following manner to determine transcript identities: sequences ≤50 bp were removed and the remaining sequences were analyzed for redundancy with CAP3 (Huang and Madan, 1999), analyzed locally using blastx against the NCBI nonredundant (nr) protein database, and further analyzed using blastn against the NCBI nucleotide (nt) database if the sequence corresponded primarily or completely to the untranslated region (Ashburner et al., 2000). Genomic database analyses were conducted July 2015. The minimum significant e-value was 10^{-5} for blast searches.

2.4. Quantitative PCR

Partial cDNAs obtained from SSH that showed high-redundancy in the libraries were selected for further validation and analyses with qPCR. Since our previous study revealed that prolonged fasting induced ovarian atresia, gene transcripts were also selected that showed connections (e.g., GO results and/or literature searches) to atresia/apoptosis. In total, ~50 PCR primer sets were designed from sequences obtained by SSH and undigested SSH cDNA was used as template to screen for differentially expressed genes with semi-quantitative PCR as described by Goetz et al. (2004). Genes that appeared to be differentially expressed in the initial screen were then assessed with qPCR (Table. 1). Primers for qPCR analyses are listed in Table 2. The primers for several genes were the same as previously reported (Luckenbach et al., 2008b, 2013; Yamamoto et al., 2011a).

All qPCR assays were run on an ABI Prism 7700 Sequence Detector in 96-well plates as described previously (Yamamoto et al., 2011a). Data for target genes were normalized to *elongation factor-1 alpha* (*eef1a*), because previous studies in coho salmon revealed that use of mRNA as template and normalization of qPCR data to *eef1a* generated results that best reflected transcript abundance on a per follicle basis when comparing across different stages of primary and early secondary oocyte growth (e.g., Luckenbach et al., 2008b; Yamamoto et al., 2011b). Primers for *eef1a* were the same as previously reported (Luckenbach et al., 2008b). Levels of ovarian *eef1a* mRNA were not significantly different between fed and fasted fish at any time point (Supplementary Fig. 1).

2.5. Statistical analyses

Ovarian transcript levels of fed and fasted fish at each time point were compared by unpaired *t*-tests, or Welch's *t*-test in the absence of homogeneity of variance (Prism 6, GraphPad Software, San Diego, CA). Time course data within a treatment were subjected to one-way analysis of variance (ANOVA) followed by Tukey's multiple mean comparison