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Characterizing ovarian gene expression during oocyte growth in Atlantic cod (*Gadus morhua*)

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ABSTRACT

Vertebrate oocytes undergo dramatic changes during development as they accumulate many RNA transcripts, glycoproteins, and yolk proteins, necessary to ensure proper fertilization and embryogenesis. Oogenesis in teleosts often requires several years for completion, but very little is known about the early developmental stages. Recently, two-stage gene expression comparisons were made during oocyte growth in coho salmon (*Oncorhynchus kisutch*) and Atlantic cod (*Gadus morhua*), but more broad-scale, comprehensive assessments have not been conducted. The objectives of the present study were to characterize the gene expression patterns throughout oocyte growth in cod and compare them to changes previously identified in coho salmon. A quantitative PCR survey was conducted using 50 genes at six ovarian stages, ranging from the onset of primary growth (oocyte differentiation) to late vitellogenesis. Most expression patterns could be grouped into three major clusters, consisting of oocyte-derived (cluster 1) and likely follicle cell (clusters 2 and 3) genes. Oocyte genes were elevated during primary growth, while many follicle cell transcripts were abundant during oocyte differentiation and vitellogenesis. Few expression changes identified in coho salmon were evident in cod, which is likely due to differences in reproductive strategies. These results demonstrate that dynamic changes in gene expression occur during oocyte growth in teleost fish.

1. Introduction

Oogenesis in vertebrates is a highly conserved, dynamic process, during which diploid oogonia develop into fertilizable ova (Patiño and Sullivan, 2002). Oogenesis in oviparous teleosts, in particular, has received considerable research attention because of their commercial importance in aquaculture and fisheries. Among the more than 20,000 species, teleosts exhibit vast differences in their reproductive strategies, and eggs differ considerably in size, composition/density, and developmental rates. Despite these differences, however, the basic stages of oogenesis appear to be universal among teleosts and have been categorized as: 1) transformation of oogonia into oocytes (onset of meiosis), 2) primary oocyte growth, 3) cortical alveoli stage, 4) vitellogenesis, 5) maturation, and 6) ovulation. Descriptions of these stages have been detailed in several review papers (Wallace and Selman, 1981; Tyler and Sumpter, 1996; Patiño and Sullivan, 2002; Lubzens et al., 2010).

In the early stages of oogenesis, oogonia are transformed into primary growth oocytes following the initiation of meiosis. Primary oocytes subsequently arrest in meiotic prophase I and become surrounded by steroidogenic follicle cells (theca and granulosa), forming the ovarian follicle structure. In teleost fish, primary growth is often prolonged for years, during which oocytes transcribe and accumulate enormous quantities of RNA transcripts for use in later stages of development (Song and Wessel, 2005). Secondary growth is marked by the appearance of cortical alveoli that develop in the periphery of the oocyte. Cortical alveoli are variable-sized, membrane-bound vesicles comprised of glycoproteins and lectins that increase in number, eventually filling the oocyte cytoplasm (Wessel et al., 2001). These vesicles are stored and released into the perivitelline space upon fertilization ("cortical reaction"), causing a restructuring of the egg envelope proteins comprising the chorion (Selman et al., 1993; Lubzens et al., 2010).

The later stages of secondary development are characterized by enormous growth due to the incorporation of hepatically-synthesized vitellogenin. Estrogens produced by follicle cells stimulate synthesis of vitellogenins that are secreted in the systemic circulation and enter oocytes by receptor-mediated endocytosis (Carnevali et al., 2006). In the oocyte, vitellogenins are proteolytically cleaved into yolk proteins and free amino acids that serve as an osmotic gradient during oocyte maturation and as a nutritional reservoir during embryonic development (Patiño and Sullivan, 2002). Following vitellogenesis, the follicle attains competency to respond to a gonadotropin surge and undergoes oocyte maturation and ovulation, releasing a mature egg cell (ovum) (Bobe et al., 2004, 2006).

Our understanding of the processes occurring during fish oogenesis has increased greatly in recent years (Lubzens et al., 2010), aided by whole transcriptome studies that have identified thousands of ovarian transcripts (Cerdá et al., 2008; Kleppe et al., 2012; Reading et al., 2012). Despite these advances, many gene functions remain poorly understood, especially during the early stages of oogenesis. Studies such as

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those examining gene expression in developing ovaries (Baron et al., 2005) and during precocious puberty (von Schalburg et al., 2005) have been conducted, but the majority of research has primarily focused on later developmental stages (i.e. vitellogenesis, oocyte maturation, and ovulation) (Patiño and Sullivan, 2002). Recently, two-stage gene expression comparisons were made during primary and early secondary growth and mid and late vitellogenesis in coho salmon (*Oncorhynchus kisutch*) and Atlantic cod (*Gadus morhua*), respectively (Luckenbach et al., 2008; Breton et al., 2012). While informative, these studies do not convey gene expression profiles across broad developmental scales. Further, the two species have very different life histories and reproductive strategies. Coho salmon are an anadromous, semelparous species, and spawn before dying, after undertaking long migrations from marine to riverine environments. Consequently, all of their oocytes develop and ovulate in unison (synchronous ovarian development; Wallace and Selman, 1981). Cod, in contrast, are a long-lived, highly fecund marine species that spawn annually (iteroparous) after attaining sexual maturity at 2–4 years of age (O'Brien, 1998). As they spawn multiple times over a protracted spawning season, their ovaries contain heterogeneous oocyte populations that develop at different rates (group synchronous ovarian development; Wallace and Selman, 1981) and ovulate at 2–4 day intervals (Kjesbu, 1989). Atlantic cod reproduction has been well studied due to its importance to commercial fisheries and aquaculture, but little is known about gene expression changes during oocyte growth. The objectives of the present study were to provide a comprehensive assessment of the gene expression patterns throughout oocyte growth in cod and compare them to changes previously identified in synchronous coho salmon (Luckenbach et al., 2008). A real time quantitative PCR survey was conducted using 50 genes at six ovarian stages, ranging from the onset of primary growth (oocyte differentiation) to late vitellogenesis.

2. Material and methods

2.1. Animals and sampling

Juvenile Atlantic cod (*G. morhua*, Gadidae) were hatchery-reared from captive broodstock (2008–2009) at Great Bay Aquaculture, LLC (Portsmouth, NH, USA) and transported to the University of New Hampshire (UNH) recirculating systems (Durham, NH, USA). Fish were raised under UNH Institutional Animal Care and Use committee guidelines, fed a commercial diet (Skretting, Stavanger, Norway), and held at 8–12 °C with a simulated natural photoperiod until entering their first reproductive season. Cod were sampled throughout this period to collect ovaries at six developmental stages: oocyte differentiation (8 months old, ~240 days post hatch (DPH), n = 3), 1.0 year old primary growth (~360 DPH, n = 6), 1.5 year old primary growth (~540 DPH, n = 6), early secondary growth (1.8 years old, ~650 DPH; n = 3), mid vitellogenesis (2.0 years old, ~720 DPH, n = 3) and late vitellogenesis (2.0 years old, ~720 DPH, n = 3) (Fig. 1). All cod were euthanized with 200 mg/L tricaine methanesulfonate (Argent Chemical Laboratories, Redmond, WA, USA), and ovaries were dissected and held on ice.

Ovarian fragments from all individuals were collected for histological analyses and RNA extractions. Histological samples were preserved in 10% formalin for 24–48 h, processed for routine hematoxylin and eosin staining (Humason, 1972), and examined using a compound microscope. Ovarian fragments for RNA analyses were immediately stored in RNALater (Ambion, Austin, TX, USA) and kept at −70 °C until RNA extractions were performed.

To accurately identify mid and late vitellogenic growth stages, ovarian fragments from vitellogenic ovaries were preserved in Ringer's solution and identified as described previously (Breton et al., 2012). Briefly, ovarian samples were photographed in duplicate using a dissecting microscope (20–30× magnification), and oocyte diameters from the 10 largest oocytes/image were measured using Image J software (Abramoff et al., 2004). Diameters were assessed for both mid (474 ± 5, 475 ± 9, 490 ± 10 μm) and late (736 ± 21, 736 ± 20, 815 ± 17 μm) vitellogenic stages. In addition, ovarian samples from late vitellogenic tissues were examined to ensure they hadn't entered the maturation stage by clearing with ethanol: formalin:acetic acid (6:3:1) and observing the (central) position of the germinal vesicle (King et al., 1994).

2.2. RNA extractions and cDNA synthesis

Ovarian fragments for RNA extractions were removed from RNALater, blotted on Whatman #1 filter paper (Whatman, Inc., Sanford, ME, USA), sectioned using a razor blade, weighed (4.0–41.2 mg tissue, depending on stage), and added to 500 μL Tri Reagent. Tissues were homogenized using microtubes and pestles (Fisher Scientific, Pittsburgh, PA, USA) and brought to 1.0 mL using Tri reagent. Extractions were performed using standard phenol/chloroform procedures (Molecular Research Center, Cincinnati, OH, USA), and mRNA was further isolated (15 μg total RNA) using the MicroPoly(A) Purist kit (Ambion, Austin, TX, USA). mRNA was quantified using an ND 1000 NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and cDNA synthesis was performed using 500 ng mRNA, 2.5 μM oligo dT primer (20mer), and 200 units of SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA).

2.3. Primer design

Primers were designed for genes (n = 25) identified in cod vitellogenic growth stage libraries by Breton et al. (2012) and genes (n = 19) identified during early oocyte growth in coho salmon (*O. kisutch*; Luckenbach et al., 2008). All genes identified in those studies were used when possible, including those corresponding to genes with unknown functions (unknown transcripts 1 and 2, *wu:fb21f05*, *zgc:112095*; Breton et al., 2012). Additionally, previously developed cod primers for 20β-hydroxysteroid dehydrogenase (carbonyl reductase 1-like), aromatase, eukaryotic elongation factor 1α (Mittelholzer et al., 2007), follicle stimulating hormone receptor, luteinizing hormone receptor (Mittelholzer et al., 2009), and cytochrome P450 side chain cleavage (Breton et al., 2012) were used, since they were also included in prior oocyte growth studies (Luckenbach et al., 2008; Breton et al., 2012). The 50 genes assayed are presented in Table 1.

To develop Atlantic cod-specific primers for *O. kisutch* genes, putative cod orthologous sequences were first identified using a reciprocal BLAST (NCBI, Bethesda, MD, USA) strategy. *O. kisutch* sequences were compared to the *G. morhua* EST database, and cod ESTs with significant similarity (e value $< 1.0^{-5}$) to salmon genes were further compared to the general nucleotide database to confirm identification. For most cod ESTs (15/19 genes), significant similarity to orthologous genes was detected in multiple fish species. In addition, the Atlantic cod genome assembly (Star et al., 2011, gadMor1, Ensembl software, European Bioinformatics Institute, Cambridge, UK) was also used to confirm cod ESTs as present in protein coding regions that matched orthologous genes (based on the UniGene database). Two other *O. kisutch* sequences (egg envelope glycoprotein and cyclin E) exhibited significant similarity to cod ESTs but could not be identified as orthologs in the general nucleotide database. Using the cod genome assembly, however, these ESTs were identified as a zona pellucida gene (*zpax*) and a likely 3′ untranslated region (UTR) fragment of cyclin E. Other cod ESTs with significant similarity to *O. kisutch* alveolin and cathepsin D could not be confirmed as orthologs, and previously identified cod sequences (GenBank acc. no. CO542059.1 and Wang et al., 2007) were used instead for primer design. Lack of similar sequences in cod databases precluded inclusion of serum lectin isoform 2 from subsequent primer design, while somatic lipoprotein receptor was also excluded due to similarity with the vitellogenin receptor sequence in this species. Primer sets for all putative orthologs were designed to