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Research paper

Transcriptomic signatures for ovulation in vertebrates

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ABSTRACT

The central roles of luteinizing hormone (LH), progestin and their receptors for initiating ovulation have been well established. However, signaling pathways and downstream targets such as proteases that are essential for the rupture of follicular cells are still unclear. Recently, we found anovulation in nuclear progestin receptor (Pgr) knockout (Pgr-KO) zebrafish, which offers a new model for examining genes and pathways that are important for ovulation and fertility. In this study, we examined expression of all transcripts using RNA-Seq in preovulatory follicular cells collected following the final oocyte maturation, but prior to ovulation, from wild-type (WT) or Pgr-KO fish. Differential expression analysis revealed 3567 genes significantly differentially expressed between WT and Pgr-KO fish (fold change $\geqslant 2$, $p < 0.05$). Among those, 1543 gene transcripts were significantly more expressed, while 2024 genes were significantly less expressed, in WT than those in Pgr-KO. We then retrieved and compared transcriptional data from online databases and further identified 661 conserved genes in fish, mice, and humans that showed similar levels of high (283 genes) or low (387) expression in animals that were ovulating compared to those with no ovulation. For the first time, ovulatory genes and their involved biological processes and pathways were also visualized using Enrichment Map and Cytoscape. Intriguingly, enrichment analysis indicated that the genes with higher expression were involved in multiple ovulatory pathways and processes such as inflammatory response, angiogenesis, cytokine production, cell migration, chemotaxis, MAPK, focal adhesion, and cytoskeleton reorganization. In contrast, the genes with lower expression were mainly involved in DNA replication, DNA repair, DNA methylation, RNA processing, telomere maintenance, spindle assembling, nuclear acid transport, catabolic processes, and nuclear and cell division. Our results indicate that a large set of genes (>3000) is differentially regulated in the follicular cells in zebrafish prior to ovulation, terminating programs such as growth and proliferation, and beginning processes including the inflammatory response and apoptosis. Further studies are required to establish relationships among these genes and an ovulatory circuit in the zebrafish model.

1. Introduction

Ovulation is a physiological process that releases a fertilizable oocyte from follicular cells and is an essential reproductive event for the preservation of a species. It is well established that luteinizing hormone (LH) initiates a cascade of signaling, including up-regulation of progestin and its nuclear progestin receptor (PGR) which activates various downstream targets and signaling pathways, eventually leading to follicular rupture. However, our understanding of the molecular mechanisms that control ovulation is far from complete. For example, there is limited evidence of downstream targets and signaling pathways that PGR regulates. A few genome-wide transcriptome analyses of differentially expressed genes in the follicular cells of preovulatory oocytes suggest conserved mechanisms in the regulation of gene expression in humans, macaques, and mice (Hernandez-Gonzalez et al., 2006; Wissing et al., 2014; Xu et al., 2011). It is still unknown whether these "ovulatory genes and pathways" are also presented and conserved in other vertebrates. To our knowledge, there are no published transcriptomic analyses of gene expression focusing on the follicular cells of preovulatory oocytes in non-mammalian vertebrates. All studies conducted so far have not separated follicular cells from the oocytes or used mixed stages of oocytes that preclude detailed comparisons between different species (Chapman et al., 2014; Gohin et al., 2010; Reading et al., 2012).

Zebrafish is an alternative vertebrate model for studying gene function, signaling pathways in development, and various physiological processes because of their low cost, rapid development, and

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relative simplicity. Unlike mammalian models, zebrafish release and fertilize mature oocytes outside the body, developing their embryos externally. Because embryos develop externally, females do not undergo cumulus-oocytes complex (COC) expansion, luteinization, or implantation processes that happen concurrently or subsequently with ovulation, making it relatively easy to distinguish genes exclusively involved in ovulation (Hagiwara et al., 2014). In addition, follicular cell layers can be collected separately from preovulatory oocytes, which are relative large (>650 μm), for biochemical and molecular analyses (Hanna and Zhu, 2011). Our recent study has also shown that Pgr is an important transcription factor induced by luteinizing hormone (LH) and is essential for ovulation in zebrafish (Zhu et al., 2015). In Pgr knockout (Pgr-KO) female zebrafish mature oocytes were trapped within follicular cells unable to ovulate, leading to infertility. Our results are consistent with the complete anovulatory and infertile phenotype reported in PGR-KO mice and rats (Lydon et al., 1995; Kubota et al., 2016). These results prompted us to hypothesize that ovulation is controlled by conserved genes and signaling pathways in vertebrates.

In this study, we first conducted a genome-wide differential gene expression analysis in the follicular cells of preovulatory oocytes from wildtype (WT) in comparison to Pgr-KO zebrafish using RNA-Seq and bioinformatics tools. We hypothesize that changes in gene expression in WT would be important for ovulation, while lack of changes in gene expression in Pgr-KO due to anovulation could serve as reference. We then conducted a comparison analysis of genome-wide differentially regulated genes in the follicular cells of preovulatory oocytes of three key vertebrate species, i.e., zebrafish, mouse, and human. We found that Pgr regulates a network of conserved signaling pathways, biological processes, and genes that control ovulation. Dramatic differences in the expression between WT and Pgr-KO of various genes include *ptgs2* (prostaglandin-endoperoxide synthase 2a, 2b), *runx1* (runt-related transcription factor 1), *ptger4b* (prostaglandin E receptor 4b), *rgs2* (regulator of G-protein signaling 2), and *adamts9* (a disintegrin-like and metalloproteinase with thrombospondin motifs). This varied expression across species provides a list of candidate genes to study the molecular mechanisms underlying ovulation.

2. Materials and Methods

2.1. Zebrafish husbandry

Generation and characterization of Pgr mutant lines have been described previously (Zhu et al., 2015). The WT zebrafish used in this study was a Tübingen strain initially obtained from the Zebrafish International Resource Center and propagated in our lab according to the following procedure. Fish were kept at constant water temperature (28 °C), a photoperiod of 14 h of light with 10 h of dark (lights on 9:00, lights off at 23:00), pH at 7.2, and salinity conductivity from 500 to 1200 μS in automatically controlled zebrafish rearing systems (Aquatic Habitats Z-Hab Duo systems, Florida, USA). Fish were fed three times daily to satiation with a commercial food (Otohime B2, Reed Mariculture, CA, USA) containing high protein content and supplemented with newly hatched artemia (Brine Shrimp Direct, Utah, USA). The Institutional Animal Care and Use Committee (IACUC) at East Carolina University approved experimental protocols.

2.2. Collection of preovulatory follicular cell layers

Follicular cells of preovulatory oocytes were collected from three WT or three Pgr-KO female zebrafish at the same developmental stage, i.e., immediately following oocyte maturation but prior to ovulation (See Fig.1 for detail). We limited our sample size to n = 3 for each group, to balance the high cost of RNA-seq and minimum requirement of statistical analyses. Follicular cells are two thin layers of cells (~20 μm in thickness, panels D1 and D2 in Fig.1) containing theca and granulosa cells, surrounding a gigantic oocyte in zebrafish (Ø > 650 μm for preovulatory oocyte, Fig.1). Follicular cells could be physically separated from oocytes (Hanna and Zhu, 2011), which typically have an approximate 1000 fold higher amount of total RNAs than those in surrounding follicular cells. However, physical separation of granulosa cells from theca cells is impractical due to small cell size and no distinguishable physical properties of these two cell types.

All the fish used in the experiment were approximately four months old. Individual, well-fed, mature, and healthy female zebrafish were housed separately from male fish by a middle divider in a spawning tank the night before sampling. Oocyte maturation and ovulation were synchronized between different individuals, and spawning typically happened within 30 min once the lights switched on and the middle divider was removed from these well-fed and individually housed fish. To obtain preovulatory oocytes, ovaries were removed within an hour before the lights turned on in the morning following an appropriate anesthetic overdose (MS-222: 300 mg/L in buffered solution). Each excised individual ovary was then placed in a zebrafish Ringer's solution (116 mM NaCl, 2.9 mM KCl, 1.8 mM CaCl2, 5 mM HEPES, pH 7.2) and examined under a dissecting microscope. We selected majority of ovaries containing healthy preovulatory follicles with a translucent appearance, indicating the occurrence of oocyte maturation and proximal to ovulation (panel F2 in Fig.1). A few ovaries had no preovulatory oocytes, incomplete absorption of previous left-over matured oocytes, or unhealthy oocytes were discarded. Individual, follicle-enclosed, healthy mature oocytes were teased away from immature oocytes by gently pipetting in and out several times using a Pasteur pipette. To determine cell specific changes of transcripts regulated by Pgr in the follicular cells, we manually peeled follicular cells off preovulatory oocytes in zebrafish Ringers' solution under a dissecting microscope using a pair of fine, clean glass needles as described previously (Hanna and Zhu, 2011). Follicular cells were collected into a 1.7 ml microcentrifuge tube and homogenized immediately in a 300 μl TRIzol solution by a sonicator (Sonic Dismembrator, Fisher Scientific, Pittsburgh, PA, USA). Samples were stored in a −80 °C freezer until RNA extraction. Each sample contained follicular cell layers separated carefully from 45 to 130 preovulatory oocytes of one fish. The process for collecting one sample was limited to less than an hour, avoiding significant degradation or changes of the transcripts (unpublished data). We collected six samples in six different days, in order to sample at the same time point and same developmental stages.

2.3. RNA isolation

Total RNA was extracted using TRIzol and a Qiagen RNeasy kit according to a modified protocol. An equal volume of cold 100% ethanol was added into the aqueous phase of the solution following phase separation of TRIzol. Samples were then loaded onto an RNeasy spin column, centrifuged (8000*g*, 30 s), washed once with 700 μl RW1, twice with 500 μl RPE, and eluted in 25 μl of RNase free water according to Qiagen's instructions. The approximate concentration and purity of samples were examined using a Nanodrop 2000 Spectrophotometer. An aliquot of RNA sample with OD 260/280 > 1.8 and OD 260/230 > 1.6 was used for RNA-Seq analysis and the remainder was used for quantitative real-time PCR (qPCR) assay.

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