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Gene expression profiling of bovine ovarian follicular and luteal cells provides insight into cellular identities and functions

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ABSTRACT

After ovulation, somatic cells of the ovarian follicle (theca and granulosa cells) become the small and large luteal cells of the corpus luteum. Aside from known cell type-specific receptors and steroidogenic enzymes, little is known about the differences in the gene expression profiles of these four cell types. Analysis of the RNA present in each bovine cell type using Affymetrix microarrays yielded new cell-specific genetic markers, functional insight into the behavior of each cell type via Gene Ontology Annotations and Ingenuity Pathway Analysis, and evidence of small and large luteal cell lineages using Principle Component Analysis. Enriched expression of select genes for each cell type was validated by qPCR. This expression analysis offers insight into cell-specific behaviors and the differentiation process that transforms somatic follicular cells into luteal cells.

1. Introduction

1.1. Mammalian ovarian follicle and corpus luteum structure and function

A key feature of the mammalian female reproductive cycle is the ovarian follicle, which contains an oocyte, granulosa cells (GCs), and theca cells (TCs). The somatic GCs and TCs create a microenvironment that determines oocyte quality and maturation by synthesizing steroid and peptide hormones, secreting extracellular matrix, and signaling to control the development and health of the follicle/oocyte (Albertini et al., 2001; Hennet and Combelles, 2012). The TCs are primarily responsible for the synthesis of androgens within the ovary via the enzyme cytochrome P450 17A1 (CYP17A1) (Young and McNeilly, 2010). The mural GCs are positioned against the basement membrane on the periphery of the antrum while the cumulus GCs surround and can physically interact with the oocyte. Both of these GCs convert androgens to estrogens with the cytochrome P450 enzyme aromatase (CYP19A1) (Erickson and Hsueh, 1978). When ovulation occurs in response to a surge of luteinizing hormone (LH), the following series of events occurs: the follicle ruptures, the cumulus-oocyte-complex is released, and the remaining GCs and TCs differentiate into luteal cells as the ovulated follicle transforms into the corpus luteum (Stouffer and Hennebold, 2015). The morphology of the corpus luteum consists of large luteal cells (LLCs, ≥25 μm) and small luteal cells (SLCs, 12–25 μm) intermixed and accompanied by other cells that migrate into the tissue (Donaldson and Hansel, 1965; Fitz et al., 1982; Heath et al., 1983). Both LLCs and SLCs secrete progesterone, a steroid hormone that is required for the maintenance of pregnancy in most

Abbreviations: GC, granulosa cell; IPA, Ingenuity® Pathway Analysis; LLC, large luteal cell; PC, principle component; PCA, principle component analysis; SLC, small luteal cell; TC, theca cell.

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species including humans and cattle. However, in cows and sheep the SLCs contain the majority of the luteinizing hormone receptors (LHCGR) and the LLCs express the bulk of the prostaglandin F2 alpha (PGF2α) receptors (PTGFR) (Fitz et al., 1982; Mamluk et al., 1998; Wiltbank et al., 2012). The corpus luteum becomes highly vascularized in order to distribute progesterone, which inhibits the secretion of LH and thus prevents ovulation. For subsequent ovulation to occur the corpus luteum must regress, and this luteolysis can be triggered by PGF2α (Stouffer and Hennebold, 2015). Alternatively, when fertilization of the oocyte and implantation are successful, maternal recognition of pregnancy results in the maintenance of the corpus luteum which, in turn, plays a key role supporting the developing embryo. Anti-luteolytic mechanisms such as secretion of signaling molecules from the conceptus result in gene expression changes in the LLCs and SLCs (Romero et al., 2013). For example, both luteal cell types in ruminant species respond to the conceptus secretion of IFNT by increasing expression of ISG15 (interferon-stimulated gene, 15 kDa) (Romero et al., 2013). Thus, ovarian somatic cells play essential roles in oocyte and embryo fates.

1.2. Gene expression profiles of ovarian somatic cells

The physiological roles of GCs and TCs in the follicle are well studied in a variety of mammalian species including humans, non-human primates, rodents, sheep, and cattle (Edson et al., 2009). While there are some species-specific differences, many aspects of ovarian physiology are well conserved. A wide variety of microarray-based investigations have been performed in various species as well, often with the goal of understanding the changes in a single cell type in response to time, external stimuli, or disease conditions (Coskun et al., 2013; Kezele, 2005; McKenzie et al., 2004; Owens et al., 2002; Skinner et al., 2008; Tsubota et al., 2011; Uyar et al., 2013; Wood et al., 2003; Xu et al., 2011). There are far fewer direct comparisons of the transcriptomes of specific cell types with the goal of identifying cell type markers and functional differences, but some have been performed including a recent direct comparison of the bovine GC and TC transcriptomes which identified cellular markers unique to GC and TC in addition to the traditional markers (steroidogenic enzymes and receptors) (Hatzirodos et al., 2015). Other studies have assessed the shifts in transcription patterns that occur in ovine and bovine GCs and TCs during follicular development (Bonnet et al., 2011; Hatzirodos et al., 2014a, b; Khan et al., 2016) or the gene expression changes in GCs and TCs during the attainment of follicle dominance and preovulatory status in the cow (Zielak et al., 2008) and the horse (Donadeu et al., 2014). However, characterization and comparison of the transcriptomes of GC and TC with LLC and SLC cell types is not currently available. Therefore, there is a gap in knowledge regarding how the follicular cells' gene expression profiles relate to the luteal phase of the reproductive cycle.

The transition from follicle to corpus luteum has also not been fully addressed by microarray analyses, though there are publications covering the short-term changes that happen in bovine GCs and TCs in response to the luteinizing hormone (LH) surge and intrafollicular prostanoids (Christenson et al., 2013; Li et al., 2009). A study of the GCs before and after human chorionic gonadotropin (hCG) administration in women undergoing controlled ovarian stimulation identified many of the same differentially expressed genes (Wissing et al., 2014). Other research conducted on the transcriptome of the corpus luteum has focused on the mechanisms of luteal regression in cattle and non-human primates (Bogan et al., 2009; Casey et al., 2005; Goravanahally et al., 2009) or on changes at progressive stages in the luteal life cycle (early, mid, mid-late, late, and very-late) (Bogan et al., 2008). However, these luteal microarrays did not distinguish between SLCs and LLCs.

1.3. Luteal cell type distinctions and lineages

There are currently no published microarray assessments of LLC and SLC gene expression profiles. What is known of the disparate functions of these cell types in sheep and cows comes from immunohistochemistry, small-scale transcriptional analysis, and cell culture-based experiments. The major known functional differences are that the basal progesterone secretion of LLCs is about 6–20× greater than that of SLCs, but SLCs are able to robustly respond to LH to amplify their progesterone production while LLCs have a modest steroidogenic response to LH (Alila et al., 1988; Fitz et al., 1982; Harrison et al., 1987). Importantly, in addition to the lack of a comprehensive transcriptome for LLCs and SLCs, the question of their cellular origin and lineage has not been addressed with the latest technologies. The prevailing understanding is that in cows LLCs originate from the GCs that remain in the follicle after ovulation while the TCs give rise to SLCs (Donaldson and Hansel, 1965; Hansel et al., 1991). With new technology and a comprehensive assessment of the transcriptomes of the GC, TC, SLC, and LLC populations, possible lineage markers for future investigation can be identified in addition to attaining an improved understanding of the relative functions of each cell type. Thus the objective of this study was to comparatively analyze RNA microarrays of these four ovarian somatic cells in order to corroborate existing GC and TC transcriptomes, provide novel transcriptome data for LLCs and SLCs, perform bioinformatic analyses to expand on the functional roles of these cells in ovarian physiology, and determine whether the existing luteal cell lineage model is supported by transcriptome analysis.

2. Methods

2.1. Follicular cell isolation

Follicular granulosa (n = 4 cows) and theca cells (n = 3 cows) were isolated from estrogen-active dominant follicles in ovaries of beef cows (75% Red Angus, 25% MARC III) from the physiology herd located at the University of Nebraska Agricultural Research and Development Center. The University of Nebraska-Lincoln Institutional Animal Care and Use Committee approved all procedures and facilities used in this experiment. Estrous cycles of cows were synchronized with a modified Co-Synch protocol using gonadotropin releasing hormone (GnRH) and a controlled internal drug release device (CIDR; 1.38 g progesterone, Zoetis) for 7 days with a PGF2α (25 mg/mL; Lutalyse, Pfizer Animal Health) injection at CIDR removal (Summers et al., 2014). Ovariectomy was performed approximately 36 h after CIDR removal (Youngquist et al., 1995). Upon ovariectomy, the largest (>10 mm diameter) antral follicle from each cow's ovaries was aspirated/dissected and the granulosa cells (≥94% purity), theca cells (≥82% purity), and follicular fluid were isolated as described previously (Summers et al., 2014). The purity of the follicular cell types using the same isolation method was determined by culturing 1 K cells per chamber on a 4-chamber glass slide, performing immunofluorescence detection of aromatase and smooth muscle actin, and manually counting the cells of six randomly selected regions. For the microarray, both granulosa and theca cells were homogenized in Tri-reagent (Sigma-Aldrich) for RNA isolation. It is important to note that follicles and RNA samples were collected from a large number of cows for use in various experiments, and those used for the microarray analyses were selected based on RNA quality and evidence of cell population enrichment. Thus, the GC and TC samples discussed in this article are not pairs from the same cows.