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Global gene expression analysis indicates that small luteal cells are involved in extracellular matrix modulation and immune cell recruitment in the bovine corpus luteum

Vijay Simha Baddela[a], Dirk Koczan[b], Torsten Viergutz[a], Andreas Vernunft[a], Jens Vanselow[a,*]

[a] Reproductive Biology, Leibniz Institute for Farm Animal Biology (FBN), 18196, Dummerstorf, Germany
[b] Institute for Immunology, University of Rostock, 18055, Rostock, Germany

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ABSTRACT

Genome wide mRNA expression analysis of small and large luteal cells, isolated from the mature staged corpora lutea (CL), was not performed in any species. In the current study, we have isolated bovine small and large luteal cells from mid-cycle (day 10–11) animals and characterized their transcriptomes using “GeneChip™ Bovine Gene 1.0 ST Arrays”. A total of 1276 genes were identified to be differentially expressed between small and large luteal cells. Data evaluation revealed that novel functions, extracellular matrix synthesis and immune cell recruitment, were enriched in small luteal cells. On contrary, functions regarding the regulation of folliculogenesis, luteal regression, fatty acid and branched chain amino acid metabolism were differentially enriched in large luteal cells. Overall, the current data offer a first and detailed insight into the functional roles of small and large luteal cells in the mature bovine corpus luteum.

1. Introduction

Corpus luteum (CL) is a heterogeneous, ephemeral endocrine structure that develops from differentiated somatic cells of ovulated follicles. As in many other species, the CL of cows contains luteal cells of two different types. Large luteal cells (LLC) originate from the avascular granulosa cell layer and have a diameter of 25–50 µm, and small luteal cells (SLC) with an approximate diameter of 10–25 µm, which however are derived from the vascular thecal cell layers (Spitschak and Vanselow, 2012; Weber et al., 1987; Hansel et al., 1987). The life cycle of the CL contains a rapid growth phase, during which differentiation of follicular cells, vascularization and tissue remodeling events take place. The growth phase is followed by a mature phase in which remodeling events are ceased, but the CL is involved in unparalleled production of progesterone. During a non-fertile reproductive cycle, the mature CL is demolished by uterine prostaglandin F2α (PGF2α) induced processes. Alternatively, maternal recognition of pregnancy occurs after fertilization due to signals from the embryo, thus leading to the survival of a mature CL throughout pregnancy. Progesterone produced by the CL is essential for blastocyst implantation and thereafter maintenance of pregnancy. Higher progesterone concentrations in the blood will further inhibit the release of gonadotropins from the pituitary gland and thus prevents the successive development of ovarian follicles (Lesoon and Mahesh, 1992; Girmus and Wise, 1992).

It has been apparent from numerous studies that follicular granulosa and theca cells perform different functions as dictated by their respective gene expression profiles (Nimz et al., 2009; Christenson et al., 2013; Orisaka et al., 2006; Romereim et al., 2017). Hence, it is also likely that their descendants, LLC and SLC have different gene expression patterns and functions in the CL. As evident from a hand full of in vitro studies performed in ruminants, mostly in the late 20th century, small and large luteal cells of the CL display distinctive features. For example, LLC are readily characterized by a bigger size (30–50 µm), numerous mitochondria and dense clusters of secretory granules in the cytoplasm (Fields et al., 1992). SLC display greater concentrations of LH receptors on their plasma membrane and fuel progesterone production even at very low doses of LH in vitro. On the contrary, LLC exhibit a nearly 3 fold higher expression of prostaglandin F receptor than SLC (Mamluk et al., 1998). Furthermore, PGF2α could increase LH induced progesterone production in SLC compared to its negative effects in LLC. In sheep and cows, LLC have also been responsible for the CL's production of oxytocin (Rodgers et al., 1983a, 1983b), which was suggested to regulate luteal regression.

Genome wide gene expression studies have been extensively performed to understand the functional genomics of cells, tissues, and organs in different species. Most of the global expression studies in CL

* Corresponding author. Reproductive Biology, Leibniz Institute for Farm Animal Biology (FBN), Wilhelm-Stahl-Allee 2, 18196, Dummerstorf, Germany.
E-mail address: vanselow@fbn-dummerstorf.de (J. Vanselow).

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have considered whole CL as a sample rather than sorted LLC and SLC populations. Interpretations drawn from such studies could not be specifically applied to luteal cells as CL is a heterogeneous tissue. Technical difficulties associated with the separation of large and small luteal cells render these cells to be difficult to study separately to a similar extent as follicular cells. Although earlier studies on SLC and LLC intuitively established a few functional differences between them (Fields et al., 1992; Mamluk et al., 1998; Rodgers et al., 1983a, 1983b; Farin et al., 1989), detailed analyses are yet to be performed. Recently, mRNA profiling of follicular and luteal cells has been reported in beef cattle, in which luteal cells were separated via elutriation of disaggregated CL of ovaries, obtained from an abattoir (Romereim et al., 2017). As abattoir samples are likely to have unknown genetics and physiological backgrounds, data drawn from such samples could potentially differ from the samples of healthy cycling animals. Apart from that, comparative genome wide gene expression profiles of small and large luteal cells of CL are not currently available in any other species. Thus, the current study is planned to meticulously isolate LLC and SLC population from mature CL, obtained from diestrus staged animals (day 9–11), and characterize corresponding transcriptomes using bovine mRNA microarrays.

2. Materials and methods

2.1. Animal's approval, maintenance and CL collection

Corpora lutea, obtained from six healthy lactating Holstein cows, were considered as discovery samples to identify differences on the transcriptome level between small and large luteal cells. A supra-institutional animal ethics committee approved these animals for CL collection under the project No M-V/TSD/7221.3-1.1-038/12. All cows were maintained under the same conditions on a regular diet. During the period of investigation, the heat was synchronized by an injection of PGF2α analog (0.5 mg Cloprostenol/animal, PGF Veyx forte®) for luteolysis. The follicular development was monitored daily by trans-rectal ultrasound examination with a 10-5 MHz linear ultrasound probe (L52, MicroMaxx®, SonoSite Inc., USA) until the ovulation was noticed. The day of noticed ovulation was considered as day 1 of the ovarian cycle. To obtain mature CL (Day 10–11), all animals were fixed followed by a peridural anesthesia with 6 ml of a 2% Procain hydrochlorid solution (Isocain ad. us. vet.®, Selectavet Dr. Otto Fischer GmbH, Germany) and an additional sedation with one ml of a 2% Xylazine solution (Xylariem®, Pharma.Partner-GmbH, Germany). After the perineal area was cleaned, the wall at the craniodorsal end of the vagina (Fornix vaginae) was opened by a stitch incision towards the direction of the CL carrying ovary. The entry to the abdominal cavum was bluntly extended by hand so that the surgeon's fingers could enter the abdomen and grasp the CL carrying ovary in order to retract it into the vaginal cavum. The CL was manually enucleated from the ovarian parenchyma and the ovary was subsequently released into the abdomen. The corpus luteum was immediately stored in ice cold PBS and rapidly (< 15 min) taken to the laboratory for luteal cell preparation.

2.2. CL digestion and separation of luteal cells

All chemicals for tissue disaggregation were purchased from Biochrom AG (Berlin, Germany) if not indicated otherwise. CL were weighed and washed in PBS (without Ca^{2+} and Mg^{2+}) and, finely sliced. CL slices were transferred to 30 ml 1 x Hank's solution (with Mg^{2+}/Ca^{2+} and Phenolred) with HEPES (25 mM) and 0.1% collagenase (Serva, Heidelberg, Germany), and incubated for 45 min at 37 °C under continues stirring. The resulting cell and tissue suspension was filtered through a stainless steel screen (100 meshes per inch, Sigma). The filtrates were transferred into 50 ml tubes and centrifuged at 400x*g* for 5 min (Allegra x-12R centrifuge, Beckman-Coulter, Krefeld, Germany) to sediment the cells, which were re-suspended in MEM with 10% FCS. Undigested tissue chunks were removed from the screen, transferred to fresh collagenase solution, and digested by repeating the above steps for two more times. Dissociated cells were incubated with DNase1 (0.01 mg/ml, Roche, Mannheim, Germany) in MEM at room temperature followed by a centrifugation at 400x*g* for 5 min. Cell pellets were then resuspended in MEM and transferred on top of a discontinuous percoll gradient, made with 1.02, 1.03, 1.04 and 1.05 g/ml in 1 x Hank's solution w/o Ca^{2+} and Mg^{2+} and phenol red/HEPES (25 mM) with 2% Ficoll 400 (Sigma) and 2% bovine serum albumin (Sigma) as described earlier (Spitschak and Vanselow, 2012; Polei et al., 2014). Gradients were centrifuged for 20 min at 1700x*g* without brake. All interfaces were manually collected in different tubes and visualized using a light microscope to identify the luteal cell fractions. Finally, cells of two different interfaces (1.04 and 1.05) were selected and resuspended 1:1 in MEM w/o FCS and pooled together to get mixed luteal cell populations.

Mixed luteal cell populations were stained with the DNA fluorochrome, Hoechst 33342 (8.9 µM, Sigma, Germany) for 30 min at 37 °C to stain all nucleated cells. The cells were directly used for flow cytometric sorting using a MoFlo XDP high-speed flow cytometry sorter (Beckman Coulter, USA) equipped with an air cooled Coherent Sapphire laser (Coherent Laser Group, USA) set at 488 nm, 100 mW, and a Uniphase Xcyte laser (JDSU, USA) operating at 355 nm, 100 mW. The optimal settings to sort the luteal cells were a 100-µm flow tip at 30 psi (207 kPa) with a one-droplet envelope in the purification mode. The flow-rate was 3000 cells per second and the sorting efficiency was 94 ± 2%. The signal used for determining the cell size was based on the forward scattering (BP 488 ± 5 nm). The threshold was set so that not more than 10 events per second were detected when running only sheath fluid. The cells with lower forward scattering intensities (corresponding to cell sizes) were considered as small luteal cells whereas, the cells with higher forward scattering intensities were considered as large luteal cells. The purified luteal cell populations were collected in PBS and re-analyzed via a flow cytometry analyzer (Gallios, Beckman Coulter, USA). The resultant fractions of small and large luteal cells had an average diameter of 15 ± 2 and 31 ± 3 µm, respectively, and were enriched to 98% SLC and 93% LLC (Fig. 1). The cells were centrifuged (300x*g*, RT, 5 min) and the dry cell pellets were stored at −80 °C. Microscopic examination of enriched SLC and LLC fractions indicated that the SLC fractions contained a small percent of similarly sized, but non yellowish (possibly non steroidogenic) cells, which could be endothelial, fibroblast or immune cells that are generally closely associated with steroidogenic luteal cells in the mature CL. The viability of the separated SLC and LLC fractions could not be determined because Hoechst 33342 indistinguishably labels living and dead cells. However, the corpora lutea were processed very rapidly in order to minimize cell death.

2.3. RNA isolation, cDNA synthesis and qRT-PCR analysis

RNA was isolated from small and large luteal cells using the RNeasy mini kit (Qiagen, Hilden, Germany) with an integrated DNase1 digestion according to the manufacturer's recommendation. All the samples were initially passed through QIA-shredder columns to completely homogenize the lysate. One fraction of RNA was stored for qPCR validation purpose while the other fraction was used for microarray library preparation.

For qPCR analysis, 15 µl of total RNA was taken for cDNA synthesis using the SensiFAST cDNA synthesis kit (Bioline, Luckenwalde, Germany). Briefly, the RNA was mixed with 4 µl of 5 x trans amplification buffer and 1 µl of reverse transcriptase enzyme in a 20 µl reaction. The reaction was incubated at 25 °C for 10 min, 42 °C for 15 min and 85 °C for 5 min in a Biometra PCR instrument.

The relative abundance of selected transcripts was quantified by qPCR amplification using the SensiFast SYBR green NO-ROX kit (Bioline, Luckenwalde, Germany) and gene specific primers

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