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Occludin expression and regulation in small follicles of the layer and broiler breeder hen

C.S. Stephens *, P.A. Johnson

Department of Animal Science, Cornell University, Ithaca, NY 14853, USA

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ABSTRACT

Synchronized yolk accumulation and follicle development are essential for egg production in oviparous species. In birds, yolk is incorporated into the oocyte by an avian specific yolk receptor (LR8), and it has been suggested that occludin (OCLN), a tight junction protein, mediates transfer of yolk material to the oocyte surface. OCLN may be a key regulator of yolk accumulation and follicle growth, however, the expression and regulation of OCLN in granulosa cells during various stages of follicle development is unknown. In the first experiment, we found that *LR8* and *OCLN* mRNA were highest in small follicles within the ovary. In addition, *OCLN* decreased with increasing follicle size. *OCLN* mRNA was more abundant in the germinal disc region of the granulosa cell layer than the non-germinal disc region. In addition, we found epidermal growth factor (EGF) and activin B, decreased *OCLN* mRNA, while activin A increased *OCLN*. In the second experiment, restricted fed (RF) broiler breeder hens were randomly divided into two groups and one group remained on RF and the other was fed *ad libitum* (FF). *OCLN* expression in granulosa cells of 3–5 mm follicles of FF hens was lower compared to RF hens and yolk weights were higher in the FF group, however, *LR8* mRNA in small whole follicles (<3 mm) did not differ between groups. In conclusion, the level of feed intake is related to or may directly regulate *OCLN* mRNA expression or may have an indirect effect through paracrine or autocrine factors in the ovary.

1. Introduction

Yolk accumulation into ovarian follicles is directly related to reproductive efficiency and egg production in avian species. In the domestic hen, yolk is produced in the liver and subsequently travels to the ovary, where it passes between granulosa cells, and is incorporated into the oocyte by receptor mediated endocytosis (Barber et al., 1991; George et al., 1987; Stifani et al., 1990). The yolk receptor (LR8) is a chicken homologue of the mammalian low density lipoprotein receptor (LDLR) with eight ligand binding domain repeats (Bujo et al., 1994). The importance of LR8 for yolk accumulation is evident in laying hens that carry a point mutation in the gene, as the mutation results in reduced yolk accumulation and very limited egg production (Barber et al., 1991; Nimpf et al., 1989). Protein expression for LR8 has been well characterized in laying hens (Shen et al., 1993). Interestingly, LR8 is present on the oocyte surface before yolk accumulation occurs, and LR8 is found in small oocytes of immature birds that do not actively accumulate yolk (Recheis et al., 2005). Morphological or structural changes within the granulosa cells that surround the oocyte likely modulate yolk access to the receptor and could explain differences in yolk accumulation when there are no differences or changes in LR8 expression.

Ovarian follicles in the domestic laying hen are arranged in several different stages, distinguished by the amount of yolk material present. There is a large pool of small, slow-growing follicles, 1 to 8 mm in diameter, and a smaller number of fast-growing pre-ovulatory follicles (approximately 5–7) arranged in a size hierarchy (F5-F1). Once the largest follicle (F1) ovulates, the second largest will subsequently ovulate the next day. The arrangement of follicles in the pre-ovulatory hierarchy ensures a regular and constant supply of yolk-filled oocytes for efficient egg production. As follicles leave the hierarchy through ovulation, a single follicle 6–8 mm in diameter is selected and becomes the smallest in the hierarchy (F5) and begins to rapidly accumulate yolk. Relatively little is known about how a newly selected follicle undergoes morphological and physiological changes in order to accumulate approximately 17 grams of yolk material (Gilbert, 1971) and transform from 6 to 8 mm to 40 mm in diameter (Wyburn et al., 1965) in 5–7 days. A better understanding of yolk uptake could potentially lead to improved ovulation rate in some birds.

* Corresponding author at: Department of Animal Science, Cornell University, Morrison Hall, Ithaca, NY 14853, USA.
E-mail addresses: ces277@cornell.edu (C.S. Stephens), paj1@cornell.edu (P.A. Johnson).

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Avian ovarian follicles consist of a single oocyte surrounded by layers of somatic cells. Granulosa cells encircle the growing oocyte. The germinal disc contains the nuclear material of the oocyte and the structure and function of the overlying granulosa cell layer is different around the germinal disc, compared to granulosa cells distal to the germinal disc (GD) (Perry et al., 1978). A basement membrane separates the avascular granulosa cell layer from the vascularized theca layer which forms the outermost somatic cell layer of the follicle. Yolk is synthesized in the liver and delivered to the follicle via the vascular system and then travels between granulosa cells before reaching LR8 on the oocyte surface. Increased yolk accumulation is associated with increased permeability of the granulosa cell layer (Perry and Gilbert, 1979). Altered tight junctions between granulosa cells surrounding the oocyte may facilitate the rapid uptake of yolk by allowing increased access of yolk material to LR8 on the oocyte surface (Schuster et al., 2004). The abundance of the tight junction protein occludin (OCLN) located on the periphery of granulosa cells may modulate yolk passage between the granulosa cells and therefore oocyte yolk accumulation (Schuster et al., 2004).

OCLN is involved in both adhesion (Furuse et al., 1996; Van Itallie and Anderson, 1997) and sealing of the paracellular space for selective passage of nutrients (Huber et al., 2000). OCLN protein expression is most abundant in small follicles in the hen and is low in pre-ovulatory follicles (Schuster et al., 2004). The expression pattern of OCLN in the hen ovary suggests that it could function by sealing the paracellular space between granulosa cells, preventing or permitting the transport of yolk material. Schuster et al. (2004) were the first to document the expression of OCLN protein in follicles but they studied OCLN in whole small follicles and in the granulosa cells from large follicles, so the results from the two tissues are not directly comparable (Schuster et al., 2004).

In the first experiment of this study, we verified the localization of the mRNA for LR8. Additionally, we determined the expression and regulation of OCLN mRNA in whole small follicles and in the granulosa cells of follicles from laying hens around the time of follicle selection, when the rate of yolk accumulation begins to increase. In the second experiment in this study, *LR8* and *OCLN* mRNA expression were investigated in small follicles and the granulosa cells of broiler breeder hens, which exhibit excessive yolk accumulation when allowed to feed *ad libitum*.

2. Materials and methods

2.1. Animals

In the first experiment, single-comb white leghorn (Babcock B300 strain) laying hens, in their first year of lay, were housed in individual cages and given *ad libitum* access to feed and water throughout post-hatch life. Hens were maintained on a light schedule of 15 h of light and 9 h of darkness. Egg production from individual hens was recorded daily. Hens with regular laying patterns were selected and ovaries were collected within 2 h of ovulation for tissue collection and granulosa cell culture experiments.

In the second experiment, Cobb 700 broiler breeder hens were reared in floor pens and maintained on a commercial restricted feeding (RF) program according to primary breeder guidelines (Breeder Management Guide, 2008) from 2 weeks of age. During their peak egg production, determined by the highest percentage of laying hens, 22 hens were selected and randomly divided into two groups. One pen of hens was given *ad libitum* access to feed (FF; n = 11), and one pen remained on RF (146 g/bird/day; n = 11) for 6 weeks prior to euthanasia. Two hens in the RF group and 1 hen from the FF group were excluded from the analysis because their ovaries were regressed at the termination of the experiment. The Institutional Animal Care and Use Committee of Cornell University approved all animal procedures and techniques.

2.2. Tissue collection

All tissue collection occurred immediately after birds were euthanized by CO_2 asphyxiation. In the first experiment, the ooplasm and surrounding granulosa cells were isolated from 3 mm follicles and pooled separately from individual laying hens (n = 4 laying hens) according to the procedure described by Wang et al. (Wang et al., 2007). In a separate experiment, individual (1, 2, 3, 4, 5, and 6 mm) whole follicles (n = 4–6 laying hens) and granulosa cell layers from 3–5, 6–8, 9–16 mm and F1 follicles (separate hens; n = 4–6 laying hens) were collected for RNA extraction. The granulosa cell layers were isolated by gently shaking the follicle wall in 4 °C Krebs-Ringer bicarbonate buffer. The granulosa cell layers were subsequently pooled from each hen according to follicle size (3–5, 6–8, 9–16 mm) and RNA was immediately extracted.

To determine expression of *OCLN* mRNA in the germinal disc and non-germinal disc regions of the granulosa cell layer, F1 and F4 follicles were split in half (n = 4–6 hens). One half contained the germinal disc and the associated granulosa cells (GD region; GDR) (Johnson et al., 2008) and the other half contained granulosa cells that did not contain the germinal disc. RNA was then extracted, following granulosa cell isolation.

In the second experiment, whole 3 mm follicles (3 from each hen) were collected from FF and RF broiler breeder hens for LR8 mRNA expression analysis (n = 8–10 broiler hens in each group). Granulosa cells from 3 to 5 mm (n = 6–8 broiler hens in each group) and 6 to 8 mm (n = 3–5 broiler hens in each group) follicles were collected from broiler breeder hens for RNA extraction. Eggs were collected daily, over the 6 week period from all FF and RF hens. The eggs were cracked and yolk was separated from albumin to record yolk weight as an indication of yolk accumulation.

2.3. RNA isolation and cDNA synthesis

RNA extraction was conducted using an RNAeasy Mini Kit (Qiagen Inc., Valencia CA) after tissue collection. All extractions were conducted with an on-column ribonuclease-free deoxyribonuclease treatment (Qiagen Inc., Valencia CA). Reverse transcriptase reactions were performed using 1 μg of total mRNA in a 20 μl volume using the high capacity cDNA RT kit (Applied Biosystems, Foster City, CA). The primer pair for *LR8* was previously described (Seol et al., 2007): F 5′-GCCAGGATCGTAGACTTGTGCTC 3′ and R 5′-CAC ATGAAGTAGCCAGCCAATGC 3′. The *OCLN* primers were: F 5′-GTCT GTGGGTTCCTCATCGT-3′ and R 5′- GTTCTTCACCCACTCCTCCA- 3′. These primers were designed to span introns using Primer3 software for SYBR green assays (Rozen and Skaletsky, 1998).

2.4. PCR analysis

Quantitative real time PCR reactions (ABI StepOnePlus Real-Time PCR System) were set up in a 25 μl reaction volume. Standard curve and samples were run in duplicate with a final concentration of 1X Power SYBR Green (Applied Biosystems, Foster City, CA, USA) and 300 nM of primer pairs. Control reactions containing no template and reactions lacking reverse transcriptase were also run in duplicate. The unknown sample expression was determined from the standard curve and normalized to *18S* mRNA (Applied Biosystems, Foster City, CA).

2.5. Granulosa cell culture

Granulosa cells were isolated from 3 to 5 and 6 to 8 mm follicles from 2 hens for each replicate experiment (n = 2–7 replicates),

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