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The avian ovary and follicle development: some comparative and practical insights

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Abstract: This review focuses on recent research related to the avian ovary and, in particular, cellular mechanisms mediating embryonic growth of the ovary, postembryonic follicular development, and the process of follicular selection. Attention is drawn to a variety of fundamental, yet unresolved, aspects of ovarian function, several of which are uniquely related to avian reproductive strategies. Some notable differences in ovarian function between birds and the most closely related extant group, the crocodilians, are illustrated. Moreover, because the sauropsid (reptilian and avian) and synapsid (mammalian) lineages diverged shortly after the emergence of amniotes some 340 million years ago, consideration is given to both derived and convergent characteristics of ovarian function in avian species versus those in eutherian mammals. Two examples of divergent characteristics are the differences in sex-determining mechanisms between birds and mammals and the requirement for progesterone synthesized within the follicular granulosa as a requirement for ovulation in virtually all species of birds, compared to the site of synthesis and role for estradiol in mammals. As an example of a convergent process, both birds and select mammals (e.g., humans, cattle, and horses) typically ovulate a single egg per reproductive cycle, yet the processes associated with follicular selection occur via unique mechanisms. Finally, reference is made to several practical outcomes from studies of the avian ovary, including applications within the poultry industry and use of the domestic hen ovary as a model for human ovarian cancer.

Key words: Ovary, embryo, granulosa, oocyte, theca, follicle selection

1. Introduction

Evolutionarily, the avian lineage descended directly from ancestral archosaurs and, in particular, theropod dinosaurs. Birds and crocodilians represent the 2 groups of extant archosaurs, and, as such, their ovaries share a number of common, derived traits. For instance, at any one time during a reproductive season the ovary contains growing follicles at all stages of maturation (slow-growing previtellogenic to more rapidly growing preovulatory), and ovarian follicles in both lineages maintain a monolayer of granulosal cells (a characteristic that facilitates yolk transport to the oocyte). Nevertheless, some reproductive characteristics of extant crocodilians and birds demonstrate some notable differences, particularly with regards to clutch size. At the organ level, crocodilians possess 2 ovaries, although 1 ovary is typically larger and produces a greater number of mature follicles. Within a breeding cycle a considerably greater number of preovulatory follicles grow simultaneously and mature over a period of months, not days. Compared to virtually all birds, crocodilians produce proportionately smaller preovulatory follicles but a significantly larger clutch size (range of 12 to 48 among extant crocodilians). By comparison, birds maintain a strict follicular hierarchy consisting of 2 to approximately 6 preovulatory follicles, and ovulate at most a single follicle per day. Such differences in clutch size are no doubt related to the minimal parental care of the eggs during incubation and immediately after hatch in crocodilians compared to the significant parental energy by most birds.

The derived characteristic of a single, left avian reproductive tract (ovary plus oviduct) dates back to enantiornithine birds from the Early Cretaceous period. The characteristics of ovarian asymmetry and preovulatory follicle hierarchy are generally believed to be at least in part reflections of weight reduction for flight (1). Nevertheless, an alternative and not mutually exclusive proposal is that the presence of a single ovary/oviduct limits calcification to a single egg at a time, thus minimizing physical contact between eggs that could lead to malformations. Presumably, this adaptation maximized embryo survival (2). Interestingly, the flightless kiwi has 2 functional ovaries (yet only 1 oviduct!), and in a 2-egg clutch, temporally separated, can alternate ovulations from the dominant left ovary to the right ovary. Given

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that the time from ovulation to oviposition in the kiwi is approximately 8 to 9 days, the interval between ovulations in a clutch can range from 12 to 30 days (T. Jensen, San Diego Zoo Institute for Conservation Research, personal communication). At this time, the mechanism that enables the coordination of alternating ovulations between the 2 ovaries is not known. Although a number of other species, including several falcons, develop a smaller right ovary during the breeding season, apparently no documented evidence demonstrates that this ovary is capable of developing and ovulating viable eggs.

2. Organization of the avian embryonic ovary and germ cell development

In all birds, the female is the heterogametic sex (ZW), while the male is homogametic (ZZ). Although a number of studies have reported that the primary sex ratio of birds can be modified at or shortly after mating by environmental factors (e.g., diet, quality of mate, stress), the mechanism mediating primary sex ratio bias remains unknown (for review, see 3). Sex determination and differentiation of the male testes during early embryonic development is attributed to a dosage effect related to the Z-linked transcription factor gene, doublesex and mab-3-related transcription factor 1 (*DMRT1*) (4), perhaps supplemented by another Z-linked gene, *HEMGN*, encoding the hemogen protein (5). At this time, specific mechanisms downstream of DMRT1 responsible for directing differentiation of the testes are not well characterized. Conversely, in the absence of this gene-dosage effect, the default sex is female.

In the domestic hen, both ovaries initially begin to develop after approximately 72 h of incubation. The outer cortex of the left ovary becomes noticeably larger after the sixth day, and the right ovary begins to regress. Asymmetric ovarian development has been attributed to pituitary homeobox 2 (PITX2) expression within the left gonad that suppresses estrogen receptor-□ (EP□) in the right gonad (6). Initiation of aromatase expression (CYP19A1) is activated by the forkhead box L2 (FOXL2) transcription factor, and the synthesis of estrogen is a prerequisite for subsequent growth and organization of the left ovary (7). In particular, estrogen promotes expression of R-spondin 1 (*RSPO1*), a regulator of the WNT/□-catenin signaling pathway. Expression of *RSPO1* is restricted to the female embryo and appears to be critical for somatic cell organization within the cortical layer around the time of germ cell meiosis (8).While both embryonic ovaries express anti-Mullerian hormone (AMH), elevated concentrations of estrogen from the larger left ovary repress the AMH-specific receptor, AMHRII, thus protecting the left müllerian duct from AMH-induced regression via apoptosis. Conversely, treatment of female embryos with an estrogen synthesis inhibitor increases AMHRII expression that results in the masculinization of the reproductive tract (9). Moreover, ectopically expressed aromatase in a 10-day-old male embryo is sufficient to suppress expression of testes determining genes (e.g., *DMRT*, *AMH*) and promote functional ovarian development (10). This requirement for estrogen to promote ovarian development is apparently conserved from its crocodilian ancestors. Finally, results from a recent study (11) have identified several microRNAs (MIR) that exhibit sexually dimorphic expression during chick embryo development, yet the specific gene targets and role in the organization of the ovary remain to be elucidated.

Primordial germ cells (PGCs) originally derived from the embryo germinal crescent invade the female gonadal anlagen by day 3 to 4, yet a greater number of PGCs expressing the germ cell marker *VASA* (also called *DDX4*; encodes an ATP-dependent RNA helicase from the DEAD-box protein family) invade the left ovary compared to the right (12). Significantly, chickens develop 2 distinct stem cell lines, one committed to the development of germ cells and a second embryonic stem cell line that contributes to somatic tissues. Both cell lines have been demonstrated to proliferate, in vitro, and can be genetically modified (13–15). These cell characteristics have enabled the recent development of fully transgenic chickens (e.g., 16).

Expression of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) receptors occurs by day 5 of development, and circulating concentrations of FSH in the plasma of female chicken embryos appear by day 8. Thereafter, FSH treatment of the female embryo, in vivo and in vitro, induces expansion of both the ovarian surface epithelium and somatic cells within the medullary cords (17,18). It has been estimated that the number of germ cells, some 450 that initially invade the ovary via the circulatory system, increases to 28,000 on the ninth day of incubation and to an approximate 680,000 on day 17. Initiation of meiosis can be detected by day 15.5 of incubation. In ovaries cultured in vitro, FSH stimulates germ cell expansion and the formation of clusters (or cysts; a result of incomplete cytokinesis) via the actions of estrogen. Together with basic fibroblast growth factor, FSH also delays germ cell meiosis during the early stages of ovarian development, while epidermal growth factor (EGF) promotes proliferation and cell survival. For instance, FSH induces expression of *MIR181A*, which acts to downregulate the meiosis-inducing factor, *NR6A1* (19,20). Conversely, LH treatment was found to reduce *MIR181A*, and is proposed to increase the nuclear receptor gene *NR6A1*. Treatment with LH also initiates 3□HSD expression and increases progesterone production, which together with *NR6A1*, serves to eventually initiate meiosis (20). One additional factor implicated in initiating meiosis is the increased expression of retinaldehyde

dehydrogenase 2 (*RALDH2*) and the synthesis of retinoic acid (21). Finally, the initiation of meiosis among different populations of ovarian germ cells is asynchronous; thus it is evident that the mechanisms that trigger the onset of meiosis are complex.

Between day 17 and hatch, a dramatic reduction occurs in the total number of germ cells via the process of apoptosis. Although neither the mechanism nor reasons for this reduction are clear, this observation is consistent with prenatal reduction in germ cells in mammals (e.g., 22). At the time of hatch, germ cells remain mostly in the diplotene stage of meiotic prophase I (now called oocytes) and have begun to organize into primordial follicles. Currently, no evidence exists in birds for the continued proliferation of avian germ cells after hatch. Overall, a clearer understanding of the cellular and molecular mechanisms (including MIRs) that regulate PGC and embryonic gonadal development will ultimately enable practical use of genetic modifications and routine production of transgenic avian species.

3. Posthatch organization and development of follicles

During the late embryonic and early posthatch period, germ cell cysts break down to form individual oocytes. Somatic (presumptive granulosa) cells begin to contact and eventually encapsulate oocytes. This process represents the assembly of a primordial follicle (an oocyte surrounded by a few, flattened granulosal cells). As in mammals, those oocytes that fail to become enclosed by a granulosal layer will undergo a second wave of apoptosis. An inner, perivitelline layer (consisting of the zona pellucida glycoproteins ZP1, ZP2, ZP3, and ZPD) forms between granulosal cells and the oocyte, and this will subsequently support fertilization and acrosome exocytosis (23) as well as serve as a barrier against microbial contamination (see Section 6). Most primordial follicles (approximately 0.05 mm in diameter) remain loosely associated in clusters in a quiescent state until sexual maturation (24).

The activation of primordial follicles to the primary follicle stage represents a change in the morphology of granulosal cells from flat and elongated to cuboidal in nature. Those primary follicles that appropriately organize remain viable and can continue to remain quiescent within the ovarian cortex layer for months or years. The transition to an active growing primary follicle is accompanied by the formation of the thecal layer derived from mesenchymal cells. Unlike mammalian follicles, the granulosa layer remains as a single cell layer throughout the remainder of follicular development. The factors involved in activating the transition from quiescent to slow growing follicles have yet to be adequately investigated in avian species. Potential factors previously implicated in mammalian follicular activation include AMH and KIT ligand (KITL). These factors are reportedly expressed at highest levels in undifferentiated (≤1 mm) follicles from the hen, and KITL is regulated by factors produced within the avian oocyte (25,26). In addition, several bone morphogenetic proteins (BMPs) known to be expressed within the avian ovary (including BMP4, BMP7) promote this transition in mammals, while AMH as well as BMP antagonists (e.g., Gremlin1 and Gremlin2) treatment can block this transition (27).

Compared to mammals, few studies have investigated endocrine/paracrine/autocrine factors and associated receptors expressed specifically by ovarian follicles during the extended period after hatch through sexual maturation. Nevertheless, a variety of factors reportedly are expressed immediately prior to puberty within the whole avian ovary, including the βA and βB subunits of inhibin/activin, GnRH-I, together with receptors encoding the FSH receptor (FSHR), LH receptor (LHR), estrogen receptor-β (ERβ), gonadotropin-inhibitory hormone receptor (GnIHR), plus several different neuroendocrine cell types (28). As yet, a specific function for most of these ovarian factors relative to the onset of sexual maturation is not established.

4. Follicular development at sexual maturation

The number of viable follicles within the hen ovary remaining at the time of sexual maturation is reduced to some 12,000, yet even many of these will eventually be lost to atresia on an on-going basis. Under an appropriate stimulatory photoperiod, the initiation of follicular growth begins at puberty, and in wild birds this event will reoccur with each successive breeding season. To the greatest extent, photo refractoriness and reproductive seasonality have been eliminated in the domestic hen; consequently, continuous new follicular growth can be maintained for a year or longer. Nevertheless, in such birds a decline in egg production eventually occurs with advancing age (around 80+ weeks), and this is accompanied by a period of molt. The poultry industry may sometimes "force molt" hens in an effort to fully regress the ovary and subsequently initiate new follicular growth and an additional period of egg production.

Increasing photoperiod provided at sexual maturation or at the beginning of the breeding season acts upon a gonadotropin-releasing hormone (GnRH) pulse generator within the hypothalamus to initiate pulsatile GnRH-I secretion into portal vasculature and stimulate pituitary gonadotropin secretion. In turn, GnRH secretion is negatively modulated by ovarian steroids that act within a population of neurons within the arcuate nucleus to modulate kisspeptin, neurokinin B, and dynorphin expression (KNDy neurons). Peptide secretion from the KNDy neurons is proposed to act as a pacemaker

for pulsatile GnRH secretion (reviewed by 29); however, the neural circuitry that directly integrates stimulatory photoperiodic signals with the GnRH pulse generating neurons remains unidentified.

No fewer than 93 MIRs (of as yet unknown function) are differentially expressed within the ovary of immature pullets compared to sexually mature hens, and a subpopulation of these continue to be expressed at different levels during subsequent follicular growth and development (30). The initial, slow growth of follicles measuring 60–100 μm in diameter occurs over several months via the uptake of a lipoprotein-rich yolk by the oocyte, concurrent with the expansion of the single layer of granulosal cells. As in mammals, this early follicular growth occurs independently of gonadotropins but is regulated by various autocrine and paracrine factors. During a second phase of growth, a subpopulation of follicles increases to a diameter of 3–8 mm. This development, and in particular *FSHR* expression within the granulosa, is regulated by a variety of growth factors in a positive (BMP4 and BMP6) or negative (epidermal growth factor receptor ligands; EGFRLs) fashion (31,32). In mammals, AMH reportedly reduces "FSH responsiveness" in small growing follicles. In both mammalian and hen granulosal cells, *AMH* mRNA decreases as follicle size increases, and eventually AMH expression becomes almost nondetectable by the time ovarian follicles become FSH-dependent. Clearly, it is possible that AMH plays some role in regulating avian follicle development and perhaps even the process of follicle selection. Unfortunately, due to species differences in AMH protein sequences between birds and mammals, the mammalian AMH protein fails to demonstrate biological activity, in vitro, in cultured chicken cells.

Although both the avian granulosal and thecal layers from slow growing, prehierarchal (<9 mm diameter) follicles express *FSHR* mRNA, only the theca expresses LH receptor (*LHR*) mRNA prior to follicular selection. As in mammals, LHR expression within the granulosal layer of birds is promoted in large part by FSH-induced cyclic adenosine monophosphate (cAMP) production but only subsequent to follicular selection. An important difference between birds and mammals is that prior to follicular selection the granulosal layer from hen prehierarchal follicles does not respond to FSH with increased cAMP formation (33). The granulosa also expresses vasoactive intestinal peptide (VIP) receptors (VPAC1 and VPAC2) throughout follicular development, yet similar to FSH, VIP fails to induce cAMP formation prior to follicular selection (34). Each of these receptors represents a member of the G protein-coupled receptor (GPCR) family, in which the adaptor proteins, βARRESTINs, are known to modulate receptor signaling. In the laying hen, the absence of FSHR and VPAC signaling via cAMP has recently been attributed to βARRESTIN-mediated desensitization (34). In the absence of such receptor-mediated cell signaling, several critical cAMP-dependent genes encoding steroidogenic enzymes (e.g., *CYP11A* and CYP17) as well as steroidogenic acute regulatory protein (*STAR*) are not expressed at this stage of follicle development, and the granulosa remains incapable of producing steroids until the time of follicular selection (33). Consequently, all steroid production during this period of early follicular growth is necessarily limited to the adjacent thecal layer. Some follicles within the prehierarchal stage of development will ultimately undergo atresia mediated via the process of apoptosis, yet unlike in mammals this cannot be attributed to a loss/withdrawal of FSH support.

5. Follicular selection

An unresolved question in both birds and monovulatory mammals (e.g., woman, cow, mare) pertains to the identity of the most proximal factor that mediates follicular selection. In addition, the mechanism by which this process is generally restricted to ovulation of 1 follicle per reproductive cycle, together with why is it "that one," remains elusive. Clearly, oviparous birds and viviparous mammals have derived very different cellular mechanisms associated with follicular selection. Several of these differences are described here.

Much like in mammals, the follicle selected each ovulatory cycle into the preovulatory hierarchy of birds emerges from a relatively small number of growing follicles, and growth during the period immediately following selection is dependent on support by FSH. In the laying hen, the prehierarchal cohort typically numbers 6 to 12 follicles each measuring approximately 6–8 mm in diameter. Unlike mammals, however, all follicles within this cohort do not undergo atresia after a follicle is selected for final maturation and ovulation. Specifically, subdominant follicles in mammals undergo atresia due to the loss of FSH support in the face of declining levels of circulating FSH. In cattle, the emergence of the dominant follicle is attributed to the FSH-induced insulin-like growth factor binding protein protease, pregnancy-associated plasma protein-A. The activity of this protease is predicted to increase free IGF within the follicular fluid of the dominant follicle, which in turn synergizes with FSH to both enhance estradiol production and ensure continued viability of the emerging dominant follicle (reviewed in 35). By comparison, unselected follicles within the avian prehiearchal cohort must be maintained in a viable but undifferentiated state to provide for selection of a subsequent follicle some 24 h later. Moreover, there is no evidence in birds that FSH plays a role in the maintenance or growth of follicles prior to selection (see section 4). Instead, published data from the laying hen

support the proposal that both the continued viability of prehierarchal follicles and the undifferentiated status of the granulosa are maintained by mitogen-activated protein kinase (MAPK) signaling (33). In part, inhibitory actions of MAPK signaling may occur via transcriptional regulatory actions of the inhibitor of differentiation/DNA binding (ID) protein isoforms ID1, ID3, and ID4 (36). The transcriptional targets of these ID proteins are currently the subject of ongoing studies.

A current, but as yet incomplete, working model for follicular selection in the hen is presented in Figure 1. A key concept proposed is that the acquisition of receptor-mediated signaling via cAMP within the granulosal layer of the most recently selected follicle occurs as a result of an "escape" from inhibitory MAPK signaling. Subsequent to follicular selection and the initiation of GPCR signaling, increased ID2 expression promoted by cAMP signaling was determined to promote the progression of granulosal cell differentiation. With regards to this model, perhaps the most important concept remaining to be resolved is the mechanism by which this proposed inhibitory signaling is reduced or terminated, together with how "that one" follicle attains a selective advantage during that ovulatory cycle.

According to this model presented for birds, the acquisition of GPCR-mediated signaling via cAMP within the granulosal cell layer is both sufficient and necessary to initiate follicular growth and differentiation of the granulosa immediately after selection. As a result, each of the following processes related to the subsequent growth and maturation of the most recently selected follicle is a direct consequence of this cAMP/protein kinase A signaling: 1) initial expression within the granulosa of steroidogenic enzymes encoding CYP11A (37) and CYP17 (38), plus initiation of STAR expression and the capacity for progesterone production (39); 2) increased vascularization mediated, at least in part, via enhanced vascular endothelial growth factor to accommodate rapid yolk transport (34); 3) enhanced expression of antiapoptotic proteins (e.g., BCLXL, MCL1, inhibitor of apoptosis proteins) specifically within the granulosa layer to ensure maintenance of follicular viability (40); 4) enhanced BMAL expression and the initiation of clock protein rhythmicity within the granulosa (41); and 5)

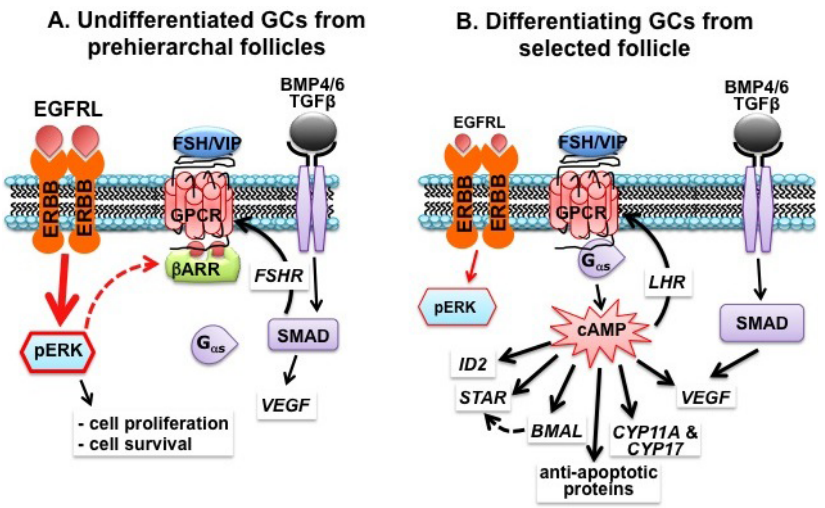

Figure 1. Current model for cellular processes related to follicle selection in the avian ovary. The function of the theca (not shown) does not change appreciably during the transition from prehierarchal to preovulatory follicle; this compared to a dramatic change within the granulosa. **A.** Granulosal cells (GCs) from prehierarchal (unselected) follicles express G-protein coupled receptors (GPCR), including follicle stimulating hormone receptor (FSHR) and vasoactive intestinal peptide receptors (VPAC1 and VPAC2). Amounts of *FSHR* mRNA and FSHR protein together with vascular endothelial growth factor (VEGF; promotes blood vessel formation within the adjacent theca layer) within undifferentiated GCs are maintained by SMAD signaling induced by transforming growth factor b and bone morphogenetic proteins (BMP4, BMP6). Nevertheless, FSH and VIP fail to promote GPCR signaling via cAMP due to □-arrestin that prevents stimulatory G-proteins (G□s) from binding to the receptor. The absence of signaling via cAMP is mediated, at least in part, via epidermal growth factor receptor ligand/extracellular-signal-regulated kinase/ERBB receptors (62). **B.** It is proposed that at follicle selection, an attenuation of ERBB signaling (potentially via MAPK-selective phosphatases; 63) initiates GPCR-mediated cAMP formation, which in turn promotes: expression of mRNA-encoding inhibitor of differentiation protein 2, CYP11A, and CYP17; the initiation of STAR expression and the capacity for progesterone production; dramatically increased vascularization mediated, at least in part, via enhanced VEGF; enhanced expression of antiapoptotic proteins; enhanced BMAL expression; and the subsequent expression of LHR (see text for additional details and references).

the subsequent expression of LHR within the granulosa layer, which maintains steroid production during final maturation and eventually mediates the ovulatory process (42). Apparently, a common feature after follicular selection between birds and monovulatory mammals initially pertains to the capacity for FSH signaling followed by the transition to LH-dependence as a mechanism to promote expression of requisite factors for granulosal cell differentiation and survival of the preovulatory follicle.

Finally, the observation that the number of follicles within the avian preovulatory hierarchy remains essentially constant from one laying sequence to the next implies that there occurs no selection of a new follicle into the preovulatory hierarchy on the day when there is no ovulation (e.g., the "pause day" at the end of a laying sequence). At this time the mechanism by which such coordination between the processes of follicular selection and ovulation occur remains obscure, yet it likely involves circadian rhythmicity (see Section 7).

6. Preovulatory follicles and ovulation

A number of comprehensive reviews describe the process of follicular growth and steroidogenesis specifically in preovulatory follicles, together with the proposed role of growth factors and gonadotropins in the final stages of follicular development (see, for instance: 43,44). Perhaps this focus on preovulatory follicles reflects the relative ease in which granulosa and theca from domesticated fowl (chickens, turkeys, quail) can be collected from these large follicles and cultured in vitro. Moreover, there appears to be the perception that the rapid growth of preovulatory follicles consists of dramatic developmental processes. In fact, compared to the complex and varied cellular events that occur at the time of follicle selection, much of postselection follicular development represents growth due to yolk incorporation and increasingly greater amounts of progesterone production by the granulosa. Presumably, increased number and size of blood vessels maintain the ordered hierarchy of preovulatory follicles as each follicle progresses through final development, which in turn accommodates delivery of progressively greater amounts of yolk. Only a few notable processes that occur during the final stages of preovulatory follicle growth are noted here.

As occurs in mammals, steroidogenesis in preovulatory follicles occurs within multiple layers of the theca. Specifically, cells expressing aromatase and synthesizing estrogens from androgen precursor are localized to the externa while pregnenolone, progesterone, and androgen precursors are produced almost exclusively within the theca interna (45). The adjacent granulosa layer produces progesterone, de novo, from cholesterol and pregnenolone and has the capacity to further process progesterone to testosterone but not to estrogen. Ovulation in birds, as in many fish, amphibians and reptiles, is induced by the stimulatory action of ovarian progesterone (in birds, derived predominantly from the granulosa layer of the largest preovulatory follicle) and pituitary LH.

In nondomesticated birds, follicular selection, preovulatory follicle growth, and ovulations occur on a near-daily basis to generate a species-specific number of eggs within a clutch. Even within a species the number of preovulatory follicles developed and eventually ovulated is dependent on a number of environmental factors, including food availability, ambient temperature, and social factors. Unlike mammals, no corpus luteum develops following ovulation. Instead, the postovulatory follicle rapidly regresses over the course of a few days via the process of apoptosis combined with the activity of several matrix metalloproteinases. Nonetheless, the most recent postovulatory follicle retains some function, as its removal is reported to delay the timing of the next oviposition (46).

A prerequisite for avian oviparity is the capacity to rapidly invest large amounts of yolk components (e.g., very low-density lipoprotein, vitellogenin, □2-macroglobulin, riboflavin binding protein, complement factor-3) within the oocyte during the last few days prior to ovulation. Specifically, rapid uptake of very low-density lipoprotein and vitellogenin uptake is accomplished at the time of follicle selection first by increasing paracellular transport of yolk precursors through the granulosa to reach the oocyte membrane and subsequently by uptake via the avian oocyte-specific receptor (LR8) localized within the oocyte membrane (47).

Along with the incorporation of yolk is the development of the germinal disc, a region visible as a small white plaque that sequesters 99% of the oocyte organelles. This region also concentrates proteins and messenger (m) RNA that are required for fertilization and directs early embryonic divisions prior to activation of the embryonic genome (estimated to occur at the 30,000 to 50,000 cell stage). The critical importance of this region to preovulatory follicular viability is evidenced by the fact that destruction of the germinal disc results in a rapid progression of follicle atresia via apoptosis. Compared to the outer layer, granulosal cells surrounding the germinal disc remain largely undifferentiated (e.g., steroidogenically incompetent and mitotically active). Granulosal cells within the germinal disc express greater amounts of EGF and EGF receptor compared to very low LHR (48). This is of significance in light of the proposed role of EGFRLs in the maintenance of GCs from prehierarchal follicles in an undifferentiated state, as discussed above. Moreover, it is possible that, as previously described in mammals, EGFRLs may eventually play a role in the resumption of

oocyte meiosis in response to the LH surge at ovulation. Unfortunately, the cellular events and mechanisms that result in meiotic maturation in birds have not been elucidated.

There is now considerable information regarding immune functions within the avian ovary. For instance, immunocompetent cells (e.g., dendritic cells, macrophages, plus B and T cells) are within the theca (but not granulosal layer) of ovarian follicles. The active induction of immunoglobulin Y (IgY; the functional equivalent to mammalian IgG) by B cells results in its accumulation within the egg as a mechanism to passively immunize the developing embryo. Yet a variety of innate ("nonspecific") immune factors also exist within the ovary and throughout the reproductive tract. These include Toll-like receptors (TLRs) that engage pathogen-associated molecular patterns (commonly expressed by microbes), proinflammatory cytokines and chemokines, and avian β-defensins (AvBDs, which have both chemotactic effects and wide-spectra antimicrobial actions). At least 8 different TLRs are expressed within the theca and/or granulosa of developing follicles (reviewed by 49). Moreover, no fewer than 11 AvBDs are expressed within ovarian tissues; in particular, AvBD12 is localized to the perivitelline layer of the egg. While the activation of TLRs has been implicated in the induction of AVBDs, there is still much to learn regarding the regulation of AvBD transcription.

Finally, it is worth cautioning that not all factors ascribed a function within the mammalian ovary should be assumed to play a physiological role in the function of avian follicles. As one example, treatment of avian ovarian tissues with the metabolically active mammalian hormone leptin has been implicated in a variety of functions in the hen ovary, including cell proliferation, cell viability/apoptosis, ovarian regression, and puberty. Nevertheless, an avian homolog to the mammalian leptin gene has yet to be identified within the avian genome, and the existence of a functional homolog to this factor in birds has been questioned (50). Thus, the various biological effects within the avian ovary reported following treatment with leptin of mammalian origin should currently be viewed with skepticism.

7. Mechanisms that regulate circadian rhythmicity in avian ovarian follicles

To date, how photoperiodic signals are communicated from both retinal photoreceptors and deep encephalic photoreceptors within the mediobasal hypothalamus to the avian ovary is unclear (51,52). Seasonal and daily photoperiodic signals serve to phase rhythms of ovarian activity. Virtually all tissues and cells within the body, including ovarian granulosal cells (e.g., 53), exhibit circadian rhythms that are phased by photoperiod, and this rhythmicity is coordinated by a dominant, central circadian pacemaker located in the suprachiasmatic nuclei (SCN) within the hypothalamus. Within SCN neurons, circadian rhythmicity is mediated via transcriptional/posttranslational feedback loops driven by protein dimers consisting of "clock" genes (e.g., *BMAL1-CLOCK* and *PERIOD [PER]-CRYPTOCHROME* [*CRY*]). Clear anatomical evidence from mammals shows that SCN cells communicate circadian time via VIP-expressing neurons that connect both directly and indirectly to peripheral endocrine targets (54). Significantly, clock protein loops in several peripheral tissues are sustained and synchronized by VPAC2-mediated AMP synthesis (55). Moreover, mutations of VIPergic signaling compromise the SCN molecular pacemaker by diminishing the amplitude and intercellular synchrony of circadian gene expression.

Not unexpectedly, differentiated granulosal cells from preovulatory follicles of birds (quail, chicken: 41,56) demonstrate a circadian rhythmicity that is entrained by signals originating in the SCN and phased relative to the external photoperiod. Nakao et al. (41) demonstrated that the rhythmic expression of STAR mRNA within granulosal cells from chicken preovulatory follicles is driven by BMAL1-CLOCK binding to the E-box of the STAR promoter region. These investigators proposed that timing of the preovulatory surge of progesterone and ovulation is dependent upon an LH/LHR/cAMP-responsive clock within the largest preovulatory follicle. Importantly, a second rhythm entailing follicular growth and maturation of the preovulatory (F1) follicle is apparently not controlled by a circadian clock and displays a periodicity something in excess of 24 h. As a result, ovulation of the largest F1 follicle can occur only when the appropriate phase of 2 rhythms coincide (represented by a 6–10 h "open period" each day). To date, the cellular mechanisms regulating and phasing the 24+ h rhythm of follicle maturation have not been identified.

By comparison, undifferentiated granulosal cells from both the rat (57) and quail (41) ovarian follicles fail to demonstrate any endogenous rhythmicity of clock proteins until after initiating the process of differentiation (e.g., subsequent to follicular selection in birds). In early differentiating granulosal cells from the mouse ovary, FSH has been implicated in regulating clock gene expression (57). There is also evidence for the regulation of circadian rhythms by VIP in the SCN as well as several peripheral tissues, and that VIP is indispensable for endogenous encoding of seasonal information (58). Accordingly, it is possible that in birds, photoperiod-driven circadian rhythmicity of cAMP-dependent, differentiation-related genes (e.g., *STAR*) subsequent to follicular selection is phased either by circulating FSH, or alternatively, by VIP that reaches the ovary via VIPergic nerve terminals innervating the theca layer (59).

8. Summary and practical implications

With regards to domesticated chickens, it is unlikely that further significant gains in egg production within the egg-laying industry will be gained based upon conventional breeding and management strategies. On the other hand, much could be gained from implementing new technologies, including the use of molecular and transgenic techniques. This would include the development of rapid, mass production techniques for sex identification, ideally at day 1 of incubation. The development of methods to efficiently sex chicks, in ovo, compared to current manual sexing at 1 day of age, would significantly decrease costs and reduce the stress of chick handling after hatch. Perhaps more importantly, for the egg industry to be able to efficiently manipulate the primary sex ratio of an ovulated follicle as a means to produce all female offspring has significant advantages. Such capabilities would have significant economical, practical, and ethical implications for the egg industry, such as eliminating the need to kill 1-day-old male chicks. Other segments of the poultry industry separate offspring according to sex for various reasons; for example, the broiler and turkey industries prefer to segregate sexes to improve feed efficiency and weight uniformity. Finally, early sex manipulation and determination would also provide important tools to avian conservation biologists and breeders for the purpose of preserving endangered species.

In broiler breeder hens, genetic selection for rapid growth has resulted in poor reproductive efficiency, due in large part to the inadvertent development of a hyperphagic condition, particularly when a strict feeding regimen is not implemented. Yet even with controlled feed intake, a "double" preovulatory hierarchy (resulting from more than 1 follicle selected into the preovulatory hierarchy each day) can still develop that inevitably results in more than 1 ovulation per day (double-yolk eggs) and represents an infertile condition. Currently, reasons proposed to explain 2 or more selected follicles per day in broiler breeders include elevated FSHR expression within prehierarchal follicles, excessive production of metabolic hormones such as leptin (but, see discussion above), IGF-I, IGF-II, glucagon, and/or elevated circulating AMH (for review, see 60). Based upon a current working hypothesis (Figure 1), a reasonable prediction is that multiple follicles selected per day result from the initiation of FSH and/or VIP signaling via cAMP in more than 1 prehierarchal follicle. Whether altered production of metabolic hormones may directly or indirectly influence such receptor sensitization is worth investigating.

Finally, the ovary of the aging domestic hen has been utilized as a model for human reproductive cancers. This is based upon observations that the hen develops spontaneous ovarian/oviductal tumors with high incidence (estimated at 30%–35% of hens by 3.5 years of age); the tumors are associated with the accumulation of ascites fluid, plus they biochemically and histologically resemble human tumors of epithelial origin (61). While these authors discuss significant advantages of exploiting this model system, they also note that the absence of routine methodologies available in chickens for targeted gene expression/deletion represents a disadvantage to fully exploiting this model system. In this regard, the gene-targeting technology using avian primordial germ cells as recently described by Schusser et al. (16) presents significant potential for advancing biomedical research of this type.

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