Anim. Reprod., v.10, n.2, p.79-87, Apr./Jun. 2013

Feed restriction inhibits early follicular development in young broiler-breeder hens

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Abstract

Ad libitum feeding causes excessive follicular development and is associated with extensive metabolic changes in broiler-breeder hens. Restricting feed intake reduces excessive follicular development, but the mechanisms mediating this response are unknown. In the present study, the effects of feeding on follicular development in immature broiler-breeder hens were examined. There was an increase in the proportion of follicles 100-300, 300-500 and >500 μm in diameter and a decrease in follicles <100 μm in full-fed (FF) compared to restricted-fed (RF) hens. Increased follicular development in FF hens was associated with a greater expression of steroidogenic transcripts (*STAR, CYP11A1, HSD3B,* and *CYP19*) within the ovarian cortex of FF hens. These transcripts represent markers of more advanced follicular development. However, increased follicular development in FF hens was not associated with changes in the expression of other factors previously implicated in follicular development, including those encoding TGF-beta ligands (*AMH, BMP6, BMP15,* or *GDF9*) or their signaling proteins (SMAD2/3 or SMAD1/5/9). Changes in histone modifications associated with proliferation, including trimethylated histone H3K4, trimethylated histone H3K27, and acetylated histone H3K9, were also not different between treatment groups. However, feed restriction caused serine phosphorylation to localize strongly to the ovarian stroma of FF hens compared to RF hens. In contrast, phosphorylation of tyrosine residues localized more prominently to the surface of granulosa cells from RF hens. Thus, restricted feeding may enhance the efficiency of reproduction by suppressing early follicular development which is associated with changes in granulosa cell protein phosphorylation status.

Keywords: chicken, domestic fowl, follicle, ovary.

Introduction

Avian follicular development is a continuous process from the activation of small cortical follicles (<0.1 mm) to the ovulation of hierarchical follicles. The largest 5-6 follicles (F1-F6, approximately 9-45 mm) comprise the preovulatory hierarchy. The largest follicle ovulates on a nearly daily basis. To replace hierarchical follicles as they ovulate, one prehierarchical follicle 6-8 mm in diameter is selected to enter the hierarchy each day. Granulosa cells from the smallest of the preovulatory follicles have the highest proliferative activity, and this proliferative capacity decreases dramatically as the follicle progresses through the hierarchy (F5 to F1; Tilly *et al.*, 1992; Tischkau and Bahr, 1996; Yao and Bahr, 2001; Wang *et al.*, 2007). The mechanisms responsible for follicle selection are proposed to involve an increase in FSH receptor mRNA expression and a release from the inhibitory effects of MAPK signaling (Woods *et al.*, 2005, 2007). Prior to selection, a number of slow growing follicles undergo atresia (Tilly *et al.*, 1991b; Johnson *et al.*, 1996). Prehierarchical follicles between 1 and 5 mm in diameter have not begun to accumulate large amounts of yolk and therefore appear white in color. The mechanisms controlling the growth of these small (1-6 mm) prehierarchal follicles is not well understood, but is associated with specific changes in gene and protein expression (Diaz *et al*., 2011). Follicles less than 1 mm in diameter represent cortical follicles which are completely embedded within the ovarian cortex. The smallest cortical follicles measuring ~0.05 mm in diameter are presumably recruited at regular intervals into the actively growing population, but the signals initiating follicle activation and early growth in birds are not fully characterized.

Genetic selection for growth and development in poultry lines used for meat production (broilers) has been extremely successful. However, for breeding stock (broiler-breeders) a consequence of increased growth rate is a severe decrease in reproductive efficiency, including fewer eggs produced and lower fertility (Yu *et al.*, 1992a, b; Hocking and McCormack, 1995). Paradoxically, the decrease in egg laying capacity in broiler-breeder hens is related to increased follicular development and an increased incidence of more than one follicle selected per day. Full-fed broiler-breeder hens may have up to 12-14 hierarchical follicles and up to 2 follicles ovulating on a given day. Ovulation of two oocytes can lead to one oocyte entering the body cavity, which can cause infection or result in two oocytes in a single shell which results in non-viable embryos. Currently, the only effective way to control ovarian hyperactivation in broiler-breeder hens is through

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Received: September 5, 2012
Accepted: March 25, 2013

dietary calorie restriction (Yu *et al.*, 1992a, b; Hocking and McCormack, 1995). Thus, the broiler hen is a useful model to study the effects of level of nutrition on ovarian function. In the present study, we used a full-fed *versus* restricted fed model to establish changes within the ovary in young broiler-breeder hens.

Materials and Methods

Animals

Female white Leghorn chicks were purchased from Hy-line International. Animals were raised according to the management protocols established by Hy-line International (Hy-line International, 2007). At 17 weeks of age, hens were photostimulated by increasing light duration to 15 h per day to induce ovarian development. Ovaries from actively laying leghorn hens were collected at 35-45 weeks for determination of follicle number (Table 1). Broiler-breeder hens (Cobb, 2000) were generously donated by a local hatchery (Longneckers Hatchery, Harrisburg, PA). Day old chicks were raised in floor pens for 2 weeks and were then allocated to separate pens (9 animals/pen). One group was fed *ad libitum* (full-fed) and another group was restricted-fed according to commercial grower's guidelines (Cobb, 2008). At 16 weeks, animals were weighed and ovaries were collected for analysis as described below. Some animals were photostimulated (12:12 h light dark cycle) and ovaries were collected at 22-24 weeks to characterize follicular development at the beginning of the laying period (Table 1). All procedures described herein were reviewed and approved by the Pennsylvania State University Institutional Animal Care and Use Committees, and were performed in accordance with The Guiding Principles for the Care and Use of Laboratory Animals.

Table 1. Number of preovulatory and prehierarchal follicles (6-8 mm).

Broiler-Breeder (BB)	Preovulatory follicles (Mean ± SEM)	Prehierarchal cohort (Mean ± SEM)
Full-fed (n = 8)	11.6 ± 1.0^{b}	11.0 ± 1.9^{b}
Restricted-fed (n = 8)	6.8 ± 0.3^{a}	5.0 ± 0.8^{a}
Laying hen (n = 3)	5.4 ± 0.12^{a}	10.7 ± 1.5^{b}

[ab]Significant differences, $P < 0.05$.

Histology and immunofluorescence

Ovaries were collected from 16 week full-fed (FF) and restricted-fed (RF) broiler breeder hens (n = 9/group). Small sections (~1 cm^3) of cortex were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) overnight. Paraffin embedded tissues were sectioned (6 µm) and stained with hematoxylin and eosin. Brightfield images were acquired on a Nikon Te200 compound microscope (Melville, NY) with an attached DP20 Olympus digital color camera and DP software (Center Valley, PA). The diameter of every follicle was measured in three of the largest sections of ovarian cortex from randomly selected FF and RF animals (n = 6), and the proportion of follicles <100, 100-300, 300-500, and >500 µm in diameter was calculated. Sections (6 µm) from randomly selected animals (n = 3) from each group (FF and RF) were used for immunostaining as described previously (Diaz *et al.*, 2011) with the following primary antibodies: anti-histone H3K4me3 (1:800, Cell Signaling, #9751), anti-histone H3K27me3 (1:100, Cell Signaling, #9733), anti-histone H3K9Ac (1:200, Cell Signaling #9671), pSMAD1/5/9 (1:200, Cell Signaling, #9511), pSMAD2 (1:200, Cell Signaling, #3101) pSerine (1:400, Zymed, #61-8100) and pTyrosine (1:50, Cell Signaling, #8954). After washing in PBS with 0.05% tween-20 (PBST), sections were incubated with goat anti-rabbit IgG Alexa Fluor 594 (1:2000, Invitrogen) for 1 h, followed by washing with PBST and mounting with DAPI anti-fade gold (Invitrogen). Epi-fluorescence images were acquired on an AxioScope 2 Plus (Leica, Bannockburn, IL) and a DP70 Olympus digital color camera and DP software (Center Valley, PA).

Isolation of total mRNA and qPCR

Total RNA was isolated from ovarian cortex using the RNAeasy mini kit (Qiagen, Valencia, CA). Equal amounts of total RNA (1 µg) were reverse transcribed into cDNA as described previously (Diaz *et al.*, 2006) using the QuantiTect Reverse Transcription kit (Invitrogen, Carlsbad, CA). Amplification of specific transcripts was conducted using gene specific primers (Table 2). The Ct values for *ACTB* mRNA (actin) were used as the normalizer as described previously (Livak and Schmittgen, 2001; Diaz *et al.*, 2011). For each primer pair, only a single product of the predicted size was identified. All amplification products were sequenced to confirm specificity of the reaction. All transcripts were analyzed in cortex samples of individual animals from FF and RF groups (n = 9). Values shown for transcript abundance are the mean ± SEM.

Data and statistical analysis

Abundance of specific mRNAs was analyzed

by real-time PCR using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Statistical differences in mRNA abundance and proportion of follicles were analyzed by Student's t-test, $P < 0.05$. The data in Table 1 were analyzed by one way ANOVA followed by Fisher's LSD, $P < 0.05$.

Table 2. Primer sequences used for qPCR.

Gene symbol	Forward	Reverse
ACTB	GCGCAAGTACTCTGTCTGGA	TAGAAGCATTTGCGGTGGA
AMH	GAGCAGCATTTTGGGGACT	CTGAGGAGGTGCTGGAAGA
BMP6	TACCAAGTGCTGCAGGAACA	CTGAGGATTCATCACCCACA
BMP15	TTATCCCCAGCAAACAGCA	GGTGTCGTTGAGGAAGAGGA
CYP11A1	CAAGACATGGCGTGACCA	TGAAGAGGATGCCCGTGT
CYP19	CACATGGGAGATTTCTCTGGA	ACGTGAAATACGCTGGAGGA
GDF9	AATCCCAAAGGCCCATAAGA	GAAGAGCAAATCCACCGAGT
HSD3B	CAGCTGCTCTGGGAAGTCA	GGGTCACCCCTGCAGTTT
STAR	CCAGCGTCAAAGAGGTGAA	GAGCACCGAACACTCACAAA

Sequences are 5' to 3'.

Results

Effect of feed restriction on prehierarchal and hierarchical follicles

To demonstrate that the level of feeding employed in this study caused ovarian dysfunction, broiler-breeder hens were raised under full-fed or restricted-fed conditions for 22-24 weeks. The light cycle was changed from 8 to 12 h at 16 weeks to stimulate the development of preovulatory follicles. Ovaries were collected at first lay (22-24 weeks) and the preovulatory (9-45 mm) and prehierarchical (6-8) follicles were counted (Table 1). The full-fed and restricted-fed treatments were maintained for the full duration of the experiments (22-24 weeks). As a comparison, follicles were also counted from actively laying white Leghorn hens that were not subject to ovarian hyperactivation under *ad libitum* feeding conditions. As shown in Table 1, FF broiler hens had an average of 11.6 hierarchical follicles and 11 prehierarchal (6-8 mm) follicles. Feed restriction reduced the number of both groups of follicles by ~50% ($P < 0.05$). In contrast, laying hens had about half as many hierarchical follicles as full-fed broilers (5.4), but a similar number of prehierarchal follicles (10.7; $P < 0.05$).

Effect of feed restriction on ovarian development in young hens

To investigate the effects of feed restriction on ovarian development in immature, 16 week old broiler-breeder hens before photostimulation, chicks (Cobb, 2000) were raised under FF and RF conditions for 16 weeks. Ovaries were then collected for mRNA and protein analysis. Ovarian weight of FF hens was almost three times that of RF birds (Fig. 1A; $P < 0.05$). This is consistent with the larger bodyweight of the FF birds (Fig. 1B, $P < 0.05$). To determine changes in the proportion of follicles at different sizes, sections of ovarian cortex from FF and RF hens ($n = 6$) were stained with H&E and the follicles were counted. FF birds had a higher proportion of follicles 100-300, 300-500, and >500 μm in diameter compared to RF animals, but had fewer follicles <100 μm in diameter than RF hens (Fig. 2A; $P < 0.05$). Greater follicular development in FF hens was associated with increased relative levels (fold change) of steroidogenic transcripts (*STAR*, *CYP11A1*, *HSD3B*, and *CYP19*; Fig. 2B, $P < 0.05$).

Effect of feed restriction on TGF-beta ligands and pSMAD signaling

AMH, BMP6, BMP15, and GDF9 are members of the TGF-beta family that signal through SMAD1/5/9 or SMAD2/3 pathways. The relative levels of *AMH, BMP6,* and *GDF9* mRNA did not differ in the cortex of 16 week old FF and RF hens, but *BMP15* mRNA was moderately decreased in FF compared to RF hens (Fig. 3A; $P < 0.05$). To determine whether signaling through the pSMAD2/3 (GDF9) or pSMAD1/5/9 (AMH, BMP6, BMP15) pathways is altered by feeding level, we examined levels of phosphorylated (activated) pSMAD2 and pSMAD1/5/9 in the cortex of FF and RF hens. Phosphorylated SMAD1/5/9 was similar in FF and RF hens as determined by immunofluorescence (Fig. 3B). Interestingly, pSMAD1/5/9 was much more intense in the granulosa cell layer compared to the ovarian stroma. Levels of pSMAD2 were also similar in FF and RF hens, but unlike pSMAD1/5/9, pSMAD2 was more uniformly distributed in the granulosa and the stroma cells (Fig. 3C).

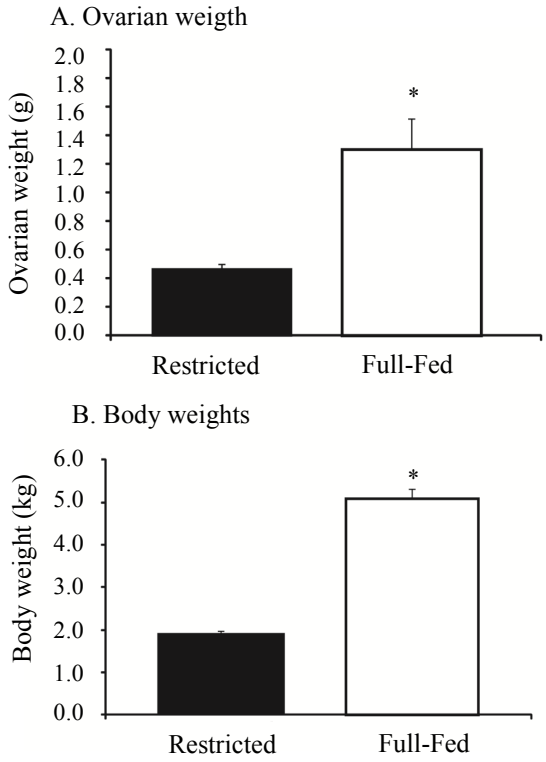

Figure 1. A. Ovarian weights of full-fed and restricted-fed 16 week old hens. B. Body weights of full-fed and restricted-fed hens. *Significant differences by Student's t-test, $P < 0.05$, $n = 9$.

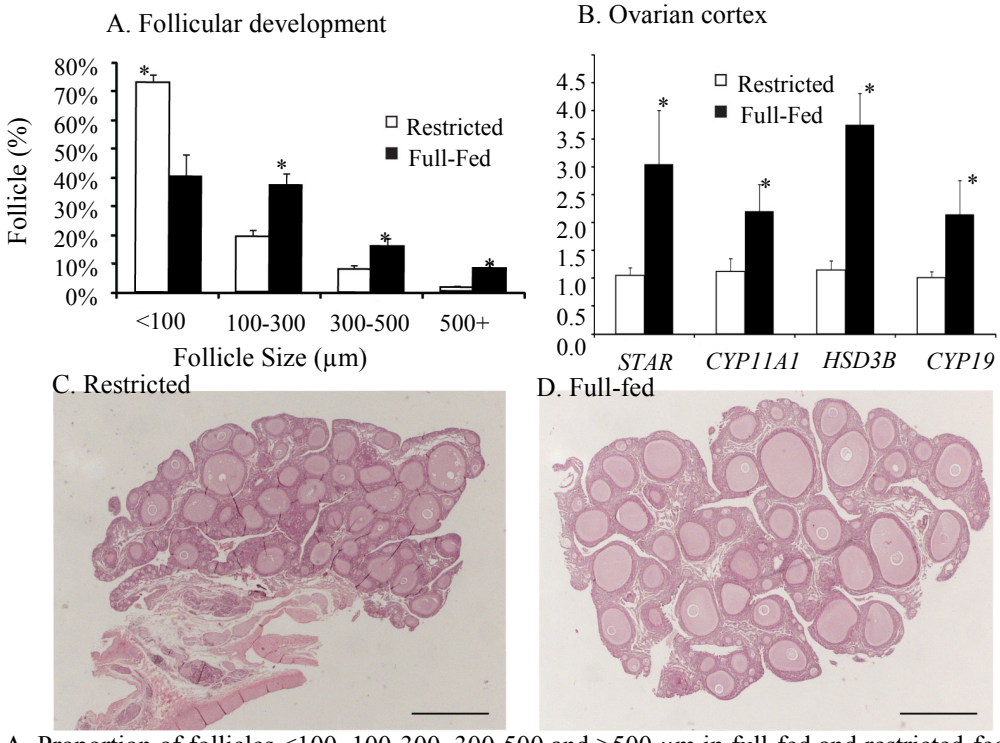

Figure 2. A. Proportion of follicles <100, 100-300, 300-500 and >500 µm in full-fed and restricted-fed 16 week old broiler-breeder hens. B. Fold change of *STAR, CYP11A1, HSD3B,* and *CYP19* mRNA in ovarian cortex from full-fed compared to restricted-fed hens (n = 9). C. Representative H&E section from restricted-fed hens. D. Representative H&E section from full-fed hens. *Significant differences by Student's t-test, $P < 0.05$, $n = 6$-9. Scale bar = 1 mm.

Changes in follicular growth

Ovarian sections from FF and RF hens (n = 3) were immunostained with antibodies recognizing trimethylated histone H3K4, trimethylated histone H3K27, and acetylated H3K9 (Fig. 4sA-C). Immunostaining for trimethylated H4K4 was uniform throughout the ovarian cortex and was similar between FF and RF hens. Trimethylated H3K27 was much more intense in the granulosa compared to the stroma cells, but trimethylated H3K4 was similar in FF and RF hens. Likewise, acetylated H4K9 was also higher in the granulosa compared to the surrounding stroma cells, but was not different between FF and RF hens.

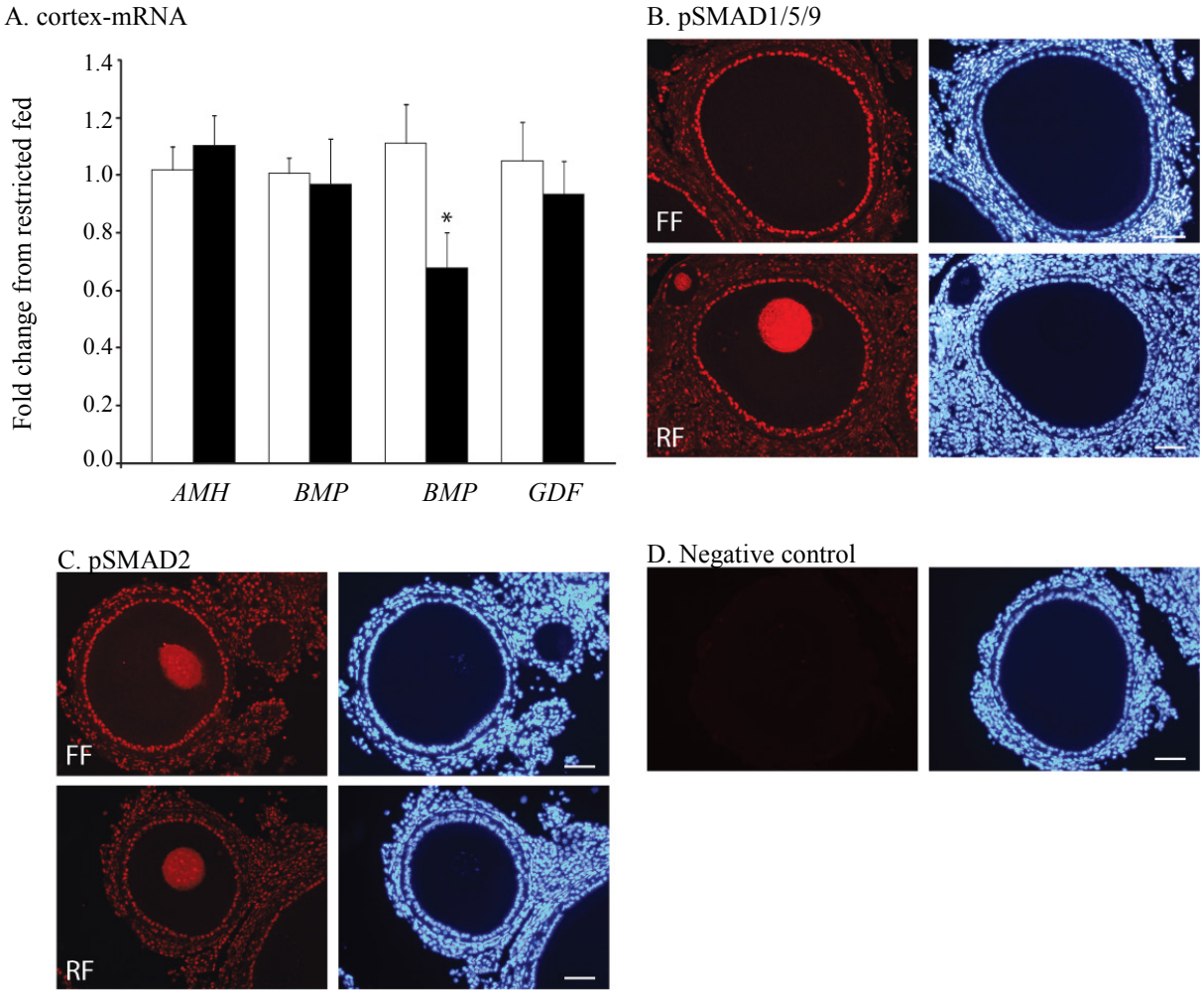

Figure 3. A. Fold change of AMH, BMP6, BMP15 and GDF9 mRNA in ovarian cortex from full-fed compared to restricted-fed hens (n = 9). B. Immunostaining for pSMAD1/5/9 in ovarian cortex from full-fed and restricted-fed hens. C. Immunostaining for pSMAD2 in ovarian cortex from full-fed and restricted-fed hens. D. Negative control incubated with secondary antibody only. Corresponding DAPI images are also shown. Scale bar = 100 µm. Sections from 3 FF and 3 RF hens were analyzed with similar results. *Indicates significant difference by Student's t-test, $P < 0.05$.

Changes in global serine and tyrosine phosphorylation

The overall levels of serine and tyrosine phosphorylation were determined as an indication of kinase activity. Sections of ovarian cortex were incubated with anti-phosphoserine or anti-phosphotyrosine antibodies. The oocyte showed very high and discrete localization of phosphoserine proteins in the cytoplasm in both FF and RF hens (Fig. 5A). The phosphoserine immunostaining signal in the oocyte cytoplasm was associated with vesicle-like structures (Fig. 5B). However, phosphoserine localized more prominently to the ovarian stroma of FF compared to RF hens (Fig. 5A). The oocyte showed little if any phosphotyrosine staining. However, the granulosa cells in particular showed discrete staining on the cell surface (Fig. 5C). Moreover, phosphotyrosine localized more prominently to the granulosa cell plasma membrane in RF than FF hens (Fig. 5C).

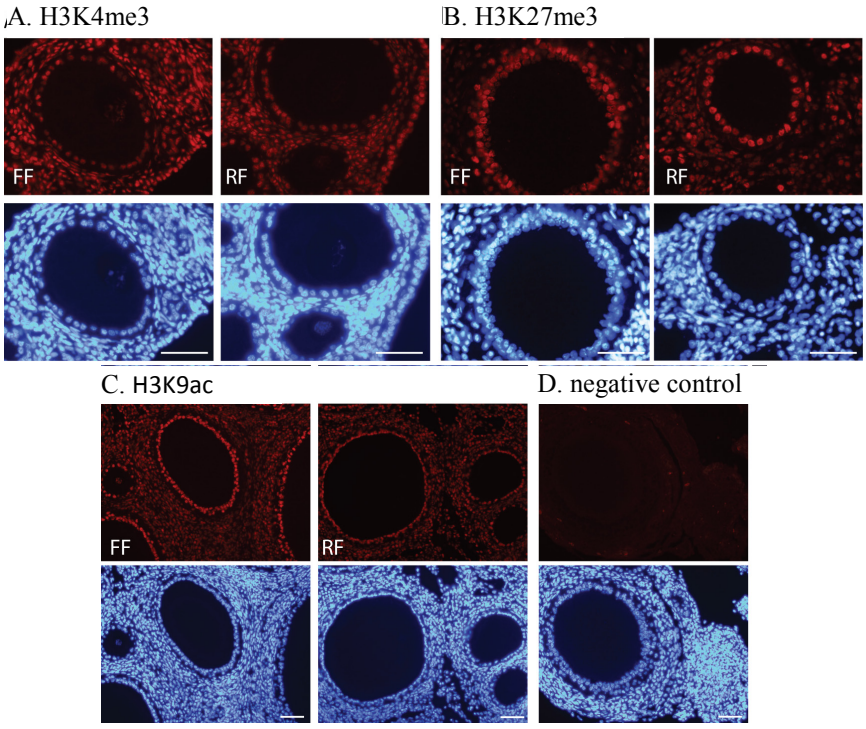

Figure 4. Immunostaining for trimethylated histone H3K4 (A), trimethylated histone H3K27 (B), acetylated histone H3K9 (C), and negative control (D) in ovarian sections from full-fed and restricted-fed hens. Corresponding DAPI images are also shown. Scale bar = 100 µm. Sections from 3 FF and 3 RF hens were analyzed with similar results.

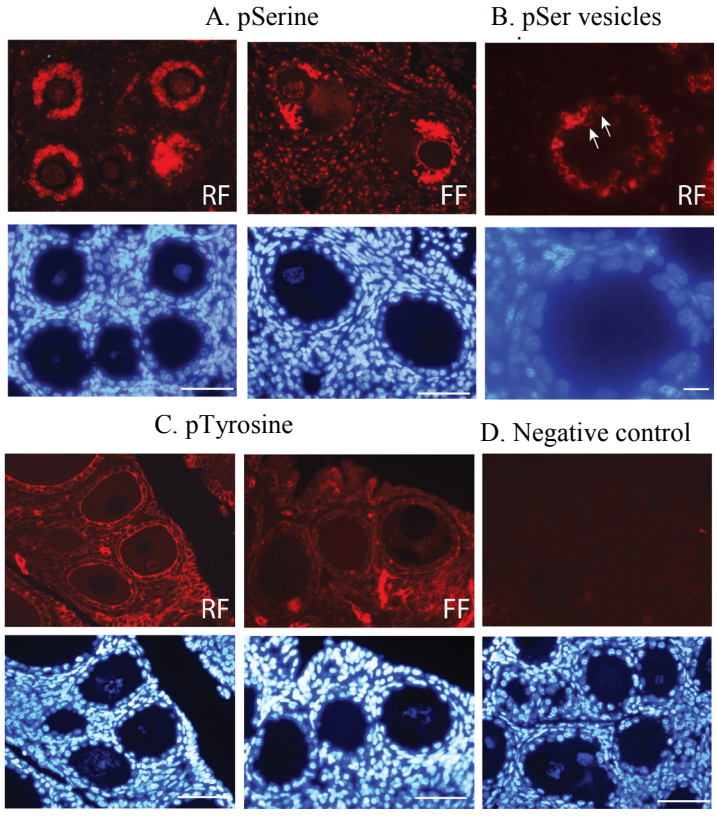

Figure 5. A. Immunostaining for pSerine in ovarian cortex from full-fed and restricted-fed hens. B. Higher magnification of pSerine positive vesicles (white arrows) in the oocyte. C. Immunostaining for pTyrosine in ovarian cortex from full-fed and restricted-fed hens. D. Negative control section incubated with secondary antibody only. Scale bar = 100 µm, except in panel B, scale bar = 10 µm.

Discussion

Dietary factors have profound influences on ovarian function through direct effects on the ovary and indirect endocrine effects on the hypothalamic-pituitary axis. In broiler-breeder hens, free access to feed causes severe ovarian dysfunction. Dietary calorie restriction is the only effective way to normalize ovarian function (Yu *et al.*, 1992a, b; Hocking and McCormack, 1995). However, the physiological changes caused by feed restriction that lead to reduced follicular development are not known. Thus, the broiler-breeder hen is an excellent model to study the effect of increased feed intake and obesity on ovarian function. To begin identifying the direct effect of nutritional status on ovarian function, the present study examined the effect of full or restricted feed intake on follicular development before sexual maturity. Ovarian development before photostimulation is not dependent on gonadotropins and feed restriction does not appear to alter gonadotropin levels in adult animals (Liu *et al.*, 2004; Onagbesan *et al.*, 2006). Thus, by focusing on the immature rearing period, the direct effects of feed restriction on the growth of small follicles can be more easily separated from possible indirect effects on gonadotropin stimulation. The findings show that feed restriction suppresses the growth of small cortical follicles, but these effects are not associated with changes in signaling by the TGF-beta family of proteins or with changes in histone modifications in the granulosa cells. However, feed restriction altered the localization of phosphoserine and phosphotyrosine proteins in small follicles. The findings clearly show that feed restriction decreases early follicular development in young broiler hens. Ovarian weight was 3-4 times greater in full-fed than restricted hens at 16 weeks of age. The increase in ovarian weight parallels a similar increase in body weight, but larger ovaries in FF hens were also the result of more advanced follicular development. FF hens had more actively growing follicles (100-300, 300-500, and >500 µm) than restricted hens. Greater follicular development could be the result of greater follicle activation. Indeed, FF hens had fewer follicles <100 µm in diameter, which includes the quiescent pool of follicles. The depletion on follicles <100 µm in FF hens suggests accelerated activation compared to restricted hens. Consistent with more advanced follicular development, the ovarian cortex of FF hens had higher relative levels of *HSD3B, STAR, CYP11A1,* and *CYP19* mRNA which are known to increase during the development of small cortical follicles (0.5 to 1 mm; Diaz *et al*., 2011). These steroidogenic factors continue to increase throughout the prehierarchical period as follicles produce increasing levels of steroids, such as estradiol (Tilly *et al.*, 1991a; Li and Johnson, 1993; Johnson *et al.*, 2002). To establish that our dietary regimen caused ovarian dysfunction, some animals were exposed to an increasing photoperiod to induce the development of preovulatory follicles (photostimulation). Changes in the number of hierarchical (>9 mm) and prehierarchal follicles (6-8 mm) in FF and RF broiler hens compared to white leghorn hens (Table 1) give some clues as to the cause of ovarian dysfunction. First, both FF broiler and layer hens have ~11 prehierarchal follicles, but FF hens have twice as many hierarchal follicles. This suggests that ad libitum feeding results in broiler-breeder hens having a more permissive follicle selection mechanism that allows more than one follicle to enter the hierarchy each day. Secondly, feed restriction caused a 50% decrease in prehierarchal follicles in RF hens, which reduces the pool of follicles available to enter the hierarchy. Thus, excessive follicular development in full-fed broiler hens is likely caused by both permissive follicle selection combined with increased availability of selectable prehierarchal follicles. Our present findings demonstrate that feed restriction decreases the number of prehierarchical follicles available for selection (6-9 mm) and decreases the development of small cortical follicles, eventually limiting the availability of selectable follicles. Current work is focused on identifying the signaling pathways affected by feed restriction that are responsible for decreased follicular development.

Greater follicular development in FF hens could be caused by greater follicular activation or increased growth or survival of activated follicles. There was no change in specific histone modifications associated with proliferation such as trimethylated histone H3K4 (Schneider *et al*., 2004) and acetylated histone H3K9 (Yang and Seto, 2007) or a decrease in trimethylated histone H3K27, which is associated with repressed chromatin (Trojer and Reinberg, 2007). Thus, changes in histone modifications are not part of the mechanism promoting greater follicular development.

In mammals and other vertebrates, early follicular development is driven by members of the TGF-beta superfamily, such as AMH, BMPs, activin and GDF9. The role of these ligands in avian follicular development is not as well understood. Small follicles (~1 mm) express the highest levels of many TGF-beta proteins, including *GDF9* and *BMP15* (Johnson *et al.*, 2005b, 2008; Elis *et al.*, 2007), suggesting that these ligands are particularly active during the early stages of follicular development. Consistent with this idea, GDF9 from small avian follicles stimulates the proliferation of granulosa cells (Johnson *et al.*, 2005b) and could be a major pathway stimulating follicular development in FF broiler hens. Avian follicles express all the components of the SMAD signaling pathway (Davis *et al.*, 2001; Lovell *et al.*, 2003, 2007; Schmierer *et al.*, 2003; Johnson *et al.*, 2005a, b, 2006, 2008; Al-Musawi *et al.*, 2007; Elis *et al.*, 2007; Diaz *et al.*, 2011). Both activin and GDF9 signal through the SMAD2/3 pathway, while BMPs and AMH signal through a separate SMAD1/5/9 pathway. However, the current findings do not support a

change in the activation of pSMAD proteins as the main cause of increased follicular development in immature broiler-breeder hens. This is in contrast to adult broiler-breeder animals where full-feeding caused an increase in *AMH* mRNA and protein compared to restricted-fed hens (Johnson *et al.*, 2009). This difference could be caused by the different complement of follicles present on the ovary in immature versus adult ovaries.

Protein kinase activity is essential for normal cell homeostasis. Phosphorylation of specific serine and tyrosine amino acid residues in target proteins is a hallmark of many signaling pathways. A surprising finding in the present study was the observed changes in the localization of global serine and tyrosine phosphorylation caused by feed restriction. In full-fed hens, serine phosphorylation was present in both somatic cells and in the oocyte. In RF hens, serine phosphorylation was more apparent in the oocyte than the somatic cells, but confirmation is necessary by more robust quantitative methods. Nevertheless, these observations suggest that feed restriction may regulate overall serine phosphorylation in somatic cells. Whether this has an impact on follicular development and the exact pathways affected awaits further study. Interestingly, we observed robust and discrete pSerine immunostaining in the oocyte cytoplasm that was associated with vesicle-like structures. It is tempting to speculate that these vesicles are transferred to the oocyte plasma membrane where the contents affect granulosa cell function, but this idea needs to be rigorously tested. In contrast to phosphoserine immunostaining, phosphotyrosine was localized most strongly to the surface of granulosa cells from restricted hens. This observation suggests that feed restriction increased the association of one or more phosphotyrosine proteins with the plasma membrane. Many cell surface receptors have tyrosine kinase activity, including those that bind epidermal growth factor (EGF), fibroblast growth factor, vascular endothelial growth factor, and hepatic growth factor. One possibility is that feed restriction increases tyrosine phosphorylation of the EGF receptor (EGFR). Activation of the EGFR and downstream signaling molecules in the granulosa cells is known to suppress follicular growth and differentiation (Woods *et al.*, 2005, Woods and Johnson, 2006). Thus, increased tyrosine phosphorylation could reflect the higher activity of the EGFR pathway that would then suppress follicular growth in restricted-fed hens.

The present findings begin to uncover some direct effects of feed restriction on follicular development. Restricted feeding clearly suppresses the development of small follicles possibly by altering the pathways that activate tyrosine phosphorylation in the membrane compartment of the granulosa cells. The signaling pathways causing this response are not specifically known, but do not appear to include changes in TGF-beta signaling (SMAD signaling) or histone modifications associated with proliferation. However, feed restriction does cause a robust change in kinase signaling. Specifically, increases in granulosa cell tyrosine phosphorylation, which could indicate the activation of a RTK (receptor tyrosine kinase) pathway, may be responsible for suppressing follicular development. Future research will identify the pathways associated with decreased follicular growth and how changes in protein phosphorylation are involved in controlling follicular development in broiler-breeder hens.

Acknowledgments

We thank Ashley Halfhill, Tian Sun, and Dr. Dan Poole for technical assistance during sample collection and Dr. Alan Johnson for sharing the data in Table 1.

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