Bone Morphogenetic Protein 15 May Promote Follicle Selection in the Hen

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Short title: BMP15 and Follicle Selection in the Hen

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18 **Abstract**

19 In the hen, optimal ovulation rate depends on selection of a single follicle into the pre-ovulatory
20 hierarchy. Follicle selection is associated with increased oocyte growth and changes in gene
21 expression in granulosa cells surrounding the oocyte, in preparation for ovulation. This study
22 investigated the expression, function and regulation of bone morphogenetic protein-15 (BMP15)
23 during follicle development in the hen. *BMP15* mRNA expression was analyzed in the ooplasm
24 and granulosa cells of 3 mm follicles and was confirmed to be primarily in the ooplasm. BMP15
25 was detected by immunoblotting in 6 and 8 mm follicles near the time of follicle selection.
26 Expression of mRNA for BMP15 receptors (*BMPR1B* and *BMPR2*) in granulosa cells increased
27 with follicle size, indicating that BMP15 may play an important role around follicle selection.
28 The function of BMP15 was examined by culturing granulosa cells from 3-5 mm and 6-8 mm
29 follicles with recombinant human BMP15 (rhBMP15). BMP15 increased expression of follicle
30 stimulating hormone receptor (*FSHR*) mRNA and decreased anti-Müllerian hormone (*AMH*)
31 mRNA and occludin (*OCN*), factors associated with follicle maturation and growth in the hen.
32 Hormonal regulation of BMP15 was assessed by whole follicle culture with estradiol (E2) which
33 increased *BMP15* mRNA expression. The distinct expression pattern of BMP15 and its
34 receptors, coupled with the effects of BMP15 to increase *FSHR* mRNA and decrease *AMH*
35 mRNA and *OCN* mRNA and protein expression suggest that the oocyte may have a role in
36 follicle selection in the chicken.

37 Keywords: BMP15; hen; ovary; AMH; ovarian follicle

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40 **Introduction**

41 A variety of endocrine, paracrine and autocrine signals in the ovary result in growth,
42 maturation and subsequent ovulation of one or more oocytes. Bone morphogenetic protein-15
43 (BMP15), also termed growth differentiation factor-9B (GDF-9B), along with growth
44 differentiation factor 9 (GDF9), are oocyte-derived members of the transforming growth factor
45 beta superfamily (TGF β) that contribute to the network of signals within the mammalian follicle
46 (Shimasaki *et al.*, 2004). In the oviparous hen, there is little known about the early stages of
47 follicle maturation prior to selection. Comparative biology would suggest that oocyte factors,
48 including BMP15, could play a vital role in follicle maturation. In oviparous species, oocyte
49 competence is especially important as proper accumulation of yolk by the oocyte is closely
50 coordinated with steroidogenesis and follicle maturation. The aim of this study was to determine
51 the expression, function and regulation of BMP15 in the hen ovary around the time of follicle
52 selection.

53 In the hen, pre-ovulatory follicles are arranged and maintained in a size hierarchy (F1-
54 F5), where the largest (F1) follicle will ovulate next and the second largest (F2) will follow on
55 the subsequent day. Daily selection of a single growing follicle from a larger pool of follicles is
56 required to maintain the pre-ovulatory hierarchy. Similar to mammals, there is increasing
57 evidence for the involvement of TGF β superfamily members in initiating follicle selection
58 (Johnson *et al.*, 2008; Kim *et al.*, 2013; Ocón-Grove *et al.*, 2012). Our lab has identified the
59 oocyte as the primary source of GDF9 in the hen ovary (Johnson *et al.*, 2005). Messenger RNA
60 for *BMP15* has been localized by *in situ* hybridization to the hen oocyte in pre-hierarchical
61 follicles (50 μ m to 6 mm) of mature ovaries and in small follicles (50 μ m to 100 μ m) of

immature ovaries (Elis *et al.*, 2007). *BMP15* mRNA (by quantitative RT-PCR) in larger follicles was more abundant in the germinal disc region than in isolated granulosa cells (Elis *et al.*, 2007).

In mammals, BMP15 plays an important role in the differentiation of somatic cells surrounding the oocyte and affects oocyte development (Gilchrist *et al.*, 2008). There is also evidence for species-specific differences in BMP15 function among mammals (Al-Musawi *et al.*, 2013; Yoshino *et al.*, 2006). Mice null for BMP15 are sub-fertile, with little change in folliculogenesis although they have defects in ovulation (Yan *et al.*, 2001). The Inverdale ewe, with two inactive copies of the BMP15 gene, is sterile while a single inactive copy of BMP15 in ewes results in increased ovulation rate and a heightened incidence of twins and triplets (Davis *et al.*, 1992, 1991; Montgomery *et al.*, 2001). BMP15 may be synthesized as a monomer and is able to form noncovalent homodimers or heterodimers with GDF9 (Chang *et al.*, 2002; Liao *et al.*, 2003a). Function of BMP15 is mediated through binding to serine threonine kinase BMP receptor complexes made of a type 1 and type 2 receptor: bone morphogenetic protein receptor, type IB (BMPRII) and bone morphogenetic protein receptor, type II (BMPRII) (Moore *et al.*, 2003; Shimasaki *et al.*, 2004). The BMP15 receptor complex subsequently phosphorylates Smad (mothers against decapentaplegic homolog) proteins, activating the Smad1/5/8 pathway (Moore *et al.*, 2003). Proper function of BMP15 receptors also seems important for maintaining species-specific ovulation rate. Mutations in the BMP15 receptor BMPRII (Alk6) cause increased litter size in sheep (Fabre *et al.*, 2003). The presence of BMP15 and GDF9 receptors has been shown in pre-ovulatory follicles of the hen and mRNA expression was characterized in the granulosa and theca layers of small, 6-8 mm follicles (Al-Musawi *et al.*, 2007; Onagbesan *et al.*, 2003). There are no data, however, on mRNA expression levels of the receptors from smaller follicles.

In the hen, there are distinct developmental changes in granulosa cell mRNA expression of follicle stimulating hormone receptor (FSHR), anti-Müllerian hormone (AMH) and occludin (OCLN), which play important roles in follicle selection. One follicle in the pool of 6-8 mm follicles has a higher abundance of mRNA for *FSHR* which may allow the granulosa cells to become FSH-responsive, resulting in increased cAMP production and expression of genes associated with granulosa cell differentiation (Woods and Johnson, 2005). *AMH* mRNA expression in the hen significantly declines as follicle size increases; it is most abundant in small follicles approximately 1 mm in diameter (Johnson *et al.*, 2008). mRNA expression for *AMH* significantly decreases from 5 mm follicles to 6-12 mm follicles (Johnson *et al.*, 2008), when follicle selection is believed to occur. Although the function of AMH in the hen is not known, the temporal expression of *AMH* in granulosa cells parallels mammalian expression during follicle development with higher mRNA expression in preantral-stage follicles (Baarends *et al.*, 1995). AMH null mice are fertile but develop an increased number of small growing follicles, ultimately resulting in a significant premature depletion of follicles from the ovary (Durlinger *et al.*, 2002). The regulated temporal expression of AMH in the hen could allow orderly selection of a single follicle into the pre-ovulatory hierarchy.

Yolk production and accumulation is imperative for follicle growth. Estradiol (E2) produced in small follicles of the hen stimulates the production of yolk in the liver and the yolk is subsequently incorporated into the oocyte by receptor-mediated endocytosis. Chickens have a unique very low density lipoprotein receptor homolog (LR8; LDL receptor relative with eight binding repeats) localized on the oocyte membrane (Shen *et al.*, 1993). Although small follicles express LR8 mRNA and protein, they fail to take up yolk. Tight junction proteins including occludin (OCLN) regulate paracellular permeability (Anderson and Van Itallie, 1995; Furuse et

al., 1993). It has been suggested that OCLN, present in granulosa cells, may regulate access of yolk to the oocyte surface around the time of follicle selection (Schuster *et al.*, 2004). Expression of protein for OCLN is lower in the granulosa cells of hierarchical follicles actively accumulating yolk as compared to small follicles (Schuster *et al.*, 2004).

To date, the function of BMP15 in the hen has been examined only in later stages of follicle development after selection (Elis *et al.*, 2007). We hypothesize that BMP15 may be important in regulating aspects of early follicle selection in the hen ovary because of its role in follicle development in mammals and the identification of *BMP15* mRNA in the hen ovary.

1. Materials and Methods

2.1. Animals

Single-comb White Leghorn hens (Babcock B300 strain) were used in all the experiments in this study. Hens were housed individually in laying cages and maintained on a lighting schedule of 15 h of light and 9 h of darkness. All hens received *ad libitum* access to feed and water. Egg production was recorded daily. Hens with consistent laying patterns during their first year of lay were selected, euthanized and their ovaries collected within 2 h of ovulation and immediately placed in ice-cold Krebs-Ringer bicarbonate buffer. The Institutional Animal Care and Use Committee of Cornell University approved all animal procedures and techniques.

2.2. Tissue Isolation and Quantitative Real-Time PCR

Ooplasm and granulosa cells from 3 mm follicles were collected from individual hens (n = 4) according to the procedure described by Wang *et al.* (2007). In a separate experiment, granulosa cell layers from 3-5, 6-8, 9-16 mm follicles and whole < 2 mm follicles were isolated (n = 4-6 hens). RNA was extracted using RNeasy Mini Kit 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). Primer pairs (*BMPR1B*, *BMPR2*, *BMP15*, *OCN*) spanning introns were designed using Primer3 software for SYBR green assays (Rozen, Steve Skaletsky, 1998). For *FSHR* and *AMH*, TaqMan primers and probes were designed with Primer Express Software 2.0 (Applied Biosystems, Foster City, CA). Primer and probe sequences can be found in Table 1. Primer efficiencies, determined by the slope of the standard curve, were within 90.4-103.2% for all primers used.

Quantitative real time PCR reactions (AB StepOnePlus Real-Time PCR System) were set up in a volume of 25 µl in duplicate with a final concentration of 1X Power SYBR Green (Applied Biosystems, Foster City, CA, USA) and 300 nM of primer pairs. All samples were run in duplicate and reactions were normalized to *18S* rRNA (Applied Biosystems, Foster City, CA, USA). *18S* has been used as a reference RNA in the ovary (Haugen and Johnson, 2010; Ocón-Grove *et al.*, 2012) and in our experiments, *18S* expression was not significantly different by tissue or treatment. Control reactions containing no template and reactions lacking reverse transcriptase were also run. PCR reactions for the TaqMan assays (*FSHR*, *AMH*) were conducted in a 25 µl reaction in duplicate with 1x TaqMan universal PCR Master Mix (Applied Biosystems, Foster City, CA) and 900 nM of primers and 250 nM of probe or 50 nM of *18S* primers and 200 nM of probe (Applied Biosystems, Foster City, CA)

2.3. Western Blot

Protein lysates were made using Tris-Triton lysis buffer from 2, 6 and 8 mm whole follicles and granulosa cells from 6-8 mm follicles were used from different hens (n = 3 individual hens). Protein concentrations were determined using a BCA Protein Assay Kit (Thermo Scientific Waltham, MA). Forty micrograms of total protein lysate were subjected to

SDS-PAGE under denaturing conditions on a 12% Tris-HEPES gel (Pierce Biotechnology Rockford, IL). Protein was transferred to methanol-activated PVDF membrane and blocked with 5% non-fat dry milk in 1X Tris-buffered saline with 0.1% Tween-20 (TBST). The membrane was incubated with a custom-made primary antibody made in a rabbit against chicken BMP15. The immunizing peptide (15-mer; CLPGYLRDAGGDKSD) was synthesized, purified and then conjugated to KLH for immunization (GenScript, Inc Piscataway, NJ). The primary antibody (at a dilution of 1:750 in TBST with 1% non-fat dry milk) was incubated with the membrane for 2 h at room temperature. The membrane was washed and then incubated with a goat anti-rabbit HRP-conjugated second antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a concentration of 1:5000 in TBST with 5% non-fat milk for 1 h at room temperature. To determine band specificity to cBMP15, the custom cBMP15 antibody was neutralized with 80 µg of the immunizing peptide (10 times the antibody protein concentration) before incubation of blocked blots. Prior to imaging, membranes were incubated with 4 ml LumiGLO chemiluminescence substrate (KPL, Gaithersburg, MD) for 1 min. Western blot images were visualized and imaged using a Fluor Chem HD2 imager (Alpha Innotech, Santa Clara, CA). After immunodetection, equal loading was determined using Coomassie stain according to Welinder and Ekblad, (2011). Membranes were stained with 0.1% Coomassie stain in a methanol and water solution at a ratio of 1:1, then destained in an acetic acid/ethanol/water solution at a ratio of 1:5:4 for 5 minutes. Membranes were washed in water, air dried and examined.

2.4. Function of BMP15 - Granulosa Cell Culture

Granulosa cells were isolated and pooled from 3-5 and 6-8 mm follicles from two hens for each replicate experiment (n = 6 replicates), then washed and dispersed according to our previous report (Davis *et al.*, 1999). Viability and cell number were determined using trypan blue

exclusion and a hemocytometer. Granulosa cells ($2.2-3 \times 10^6$ cells per well) were plated in 6-well culture dishes with 1.5 ml M199 +10% fetal bovine serum (FBS) [+ 1% Antibiotic-Antimycotic (ABAM)] (Life Technologies, Foster City, CA) and were cultured for 24 h at 37° C with 5% CO₂. After 24 h, the medium was replaced with M199 + 0.1% bovine serum albumin (BSA) [+ 1% ABAM] and treatments were administered: 0, 1, 10 or 25 ng/ml rhBMP15 (R&D Systems, Minneapolis, MN). In the hen, recombinant human BMP15 (rhBMP15) has been shown to have biological function as indicated by the ability to phosphorylate Smad1 (Elis *et al.*, 2007). Cells were returned to the incubator for an additional 24 h and subsequently terminated. RNA and protein were collected for qRT-PCR and western blot analysis, respectively. RNA was extracted as described above and relative levels of mRNA for *FSHR*, *AMH* and *OCN* were determined using qRT-PCR. A rabbit anti-chicken occludin antibody (F44 serum), donated by Dr. Mikio Furuse from Kobe University Japan (Sakakibara *et al.*, 1997), was used in western blot analysis (described above). The membrane from each experiment (n = 4) was incubated with the primary anti-chicken occludin antibody (F44 serum) at a dilution of 1:2000 in TBST with 1% non-fat dry milk for 2 h at room temperature. The membrane was washed and then incubated with a goat anti-rabbit HRP-conjugated antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a concentration of 1:5000 in TBST with 5% non-fat milk for 1 h at room temperature. The blots were imaged using a Fluor Chem HD2 imager (Alpha Innotech, Santa Clara, CA) and equal protein loading in each lane was determined using Coomassie stain as described above.

2.5. Regulation of BMP15 - Follicle Culture

Whole follicles (1 mm) from individual hens were isolated under a dissecting microscope. One hen was used for each replicate experiment and three 1 mm follicles were removed from the hen and placed in an individual well in a 96 well plate (n = 5 replicate

experiments). Follicles were cultured at 37° C under an atmosphere of 5% CO₂ in a total volume of 250 µl for 10 h. Treatments consisted of: 0, 1, 10 ng/ml estradiol (E2) (Sigma-Aldrich, St. Louis, Mo). Estradiol was solubilized in 100% ethanol to a concentration of 1 mg/ml and further diluted in M199 to a working concentration of 1 µg/ml E2. The control treatment in the E2 culture experiment consisted of M199 + 0.1% BSA [+ 1% ABAM] and 10 ppm ethanol, the equivalent amount of ethanol in the 10 ng/ml E2 treatment. Follicles were collected and RNA was extracted for analysis of *BMP15* expression by qRT-PCR.

2.6. Statistics

Data were analyzed by a one-way ANOVA and when there was a significant F ratio ($P < 0.05$), means were compared using LS Means test. P values of < 0.05 were considered significant. All statistical analysis was conducted using Jmp 10 software. (SAS Institute Inc., Cary, NC, USA).

2. Results

3.1. *BMP15* mRNA Expression

As expected from previously reported *in situ* hybridization results (Elis *et al.*, 2007), real time PCR analysis of *BMP15* mRNA showed significantly higher steady-state levels ($P < 0.01$) in the ooplasm as compared to the granulosa cells of 3 mm follicles (Figure 1).

3.2. *BMP15* Protein Expression

BMP15 protein was detected in 6 and 8 mm follicles as a single band at approximately 50 kDa in the promature form (Liao *et al.*, 2003a, 2003b; McIntosh *et al.*, 2008; Otsuka *et al.*, 2000). Coomassie staining indicated equivalent protein loading in lanes. No expression was noted in 2 mm follicles or in the granulosa cells from 6-8 mm follicles by Western blot analysis. Pre-

incubation of the antibody with the immunizing peptide markedly reduced detection of BMP15 in 6 and 8 mm follicles (Figure 2).

3.3. *BMP15 Receptor Expression*

Real time PCR analysis indicated that mRNA for both *BMPR1B* and *BMPR2* significantly increased ($P < 0.05$) in granulosa cells during follicle development (Figure 3). There was a gradual increase ($P < 0.05$) in expression of *BMPR1B* (Figure 3A) during follicle development and *BMPR2* (Figure 3B) mRNA expression was significantly greater ($P < 0.05$) in the 6-8 mm and 9-16 mm than in earlier stages.

3.4. *Function of BMP15*

The function of BMP15 was examined using a granulosa cell culture system. Expression of mRNA for *FSHR* was numerically increased by 10 ng/ml rhBMP15 and was significantly increased ($P < 0.05$) at the 25 ng/ml dose in granulosa cells from both 3-5 mm and from 6-8 mm follicles (Figure 4A). *AMH* mRNA decreased with increasing dose of rhBMP15 except at the highest dose of 25 ng/ml rhBMP15 in granulosa cells from both 3-5 and 6-8 mm follicles (Figure 4B; $P < 0.05$). Additionally, *OCN* mRNA (Figure 4C; $P < 0.01$) and *OCN* protein (Figure 4D) decreased in granulosa cells treated with 10 and 25 ng/ml of rhBMP15. *OCN* was more abundant in untreated granulosa cells from 3-5 mm follicles as compared to 6-8 mm follicles (Figure 4D; equivalent protein loading was confirmed by Coomassie staining).

3.5. *Regulation of BMP15*

BMP15 mRNA was significantly increased in the presence of 10 ng/ml of estradiol (Figure 5; $P < 0.05$).

3. Discussion

To our knowledge, this is the first documentation of BMP15 expression, function and regulation in small, growing follicles of the hen. We confirmed that BMP15 mRNA expression is primarily in the oocyte (Figure 1) and we found that BMP15 receptor mRNA expression in granulosa cells increased with follicle development (Figure 3) (Elis *et al.*, 2007). Exogenous BMP15 increased mRNA for *FSHR* and decreased mRNA for both *AMH* and *OCN* (Figure 4), important factors in follicle selection. We also found that *BMP15* mRNA was increased by E2 (Figure 5).

Steady-state levels of mRNA for *BMP15* were approximately 40-fold higher in the ooplasm than the surrounding granulosa layer of 3 mm follicles, consistent with other vertebrate species where the primary source of BMP15 in the ovary was the oocyte (Persani *et al.*, 2014). Al-Musawi *et al.* (2007), found very faint detection of *BMP15* mRNA by semi-quantitative PCR in the theca layer of 6-8 mm follicles, although this was not a pure cell layer (Al-Musawi *et al.*, 2007). We used 3 mm follicles to compare expression of *BMP15* mRNA in the ooplasm and granulosa cells because the granulosa layer cannot be readily isolated from smaller follicles and excessive yolk complicates ooplasm isolation from larger follicles.

Our custom-made BMP15 antiserum detected the predicted promature form of BMP15, a single approximate 50 kDa band present in 6 and 8 mm whole follicles, although the protein was low or undetectable in granulosa cells and in small 2 mm follicles. We did not detect alternate forms of BMP15 on Western blot at any stage of development. By Western blot, BMP15 protein was detected in ovine follicular fluid primarily as the unprocessed promature form, suggesting that the mature form was present at low or undetectable levels in ovarian tissue (McNatty *et al.*, 2006); when in-vitro expression systems were used, however, BMP15 was detected in multiple forms by Western blot (Liao *et al.*, 2003a, 2003b; McIntosh *et al.*, 2008; Otsuka *et al.*, 2000)

Protein expression confirmed that 6 and 8 mm follicles translated *BMP15* mRNA (Figure 2). This pattern of expression was similar to another oviparous vertebrate, the European seabass, in which *BMP15* mRNA was detected during early stages of follicle development but protein expression was at low or undetectable levels until the lipid vesicle stage or beginning of secondary growth phase (García-López *et al.*, 2011). In addition, in the rat ovary, expression of *BMP15* mRNA and protein detected by *in situ* hybridization and immunohistochemistry also increased with follicle development (Otsuka *et al.*, 2000). The expression of mRNA for *BMP15* receptors, *BMPR1B* and *BMPR2*, increased with follicle development in the hen (Figure 3), potentially indicating there is an increase in responsiveness to BMP ligands, including *BMP15*, with follicle development. Other BMP ligands in addition to *BMP15*, interact with *BMPR1B* and *BMPR2* (Shimasaki *et al.*, 2004). The expression patterns of *BMP15*, along with *BMPR1B* and *BMPR2*, support the likelihood of paracrine signaling between the oocyte and the granulosa cells around the time of follicle selection (6-8 mm).

Small follicles (< 1 mm) in the hen ovary grow slowly until they either undergo atresia or are selected at approximately 6-8 mm into the pre-ovulatory hierarchy. Although mechanisms of selection remain unknown, FSH, AMH and OCLN expression change after selection (Johnson *et al.*, 2008; Schuster *et al.*, 2004; You *et al.*, 1996). We suggest that *BMP15* has a role in initiating follicle selection due to alterations in mRNA expression of these genes in response to *BMP15* treatment. *BMP15* significantly increased *FSHR* mRNA and decreased both *AMH* mRNA and *OCLN* mRNA and protein in granulosa cells from 3-5 and 6-8 mm follicles (Figure 4). The abundance of *FSHR* has previously been proposed as an indication of follicle selection (Woods and Johnson, 2005). The highest dose of *BMP15* (25 ng/ml) increased mRNA for *FSHR* with no effect on mRNA levels for *AMH*. Lower doses of *BMP15* decreased *AMH* mRNA potentially

allowing *FSHR* mRNA to increase, suggested by the numerical but not significant increase in *FSHR* at 10 ng/ml BMP15 (Figure 4). Around the time of follicle selection when FSHR increases (Woods and Johnson, 2005), *AMH* expression significantly decreases in the hen, similar to mammals (Johnson *et al.*, 2008; Monniaux *et al.*, 2012). BMP15 has also been shown to increase mRNA for both FSHR and AMH in human granulosa cells (Ogura-Nose *et al.*, 2012). In contrast, in mice BMP15 inhibits FSHR mRNA (Otsuka *et al.*, 2001). These studies suggest that the function of BMP15 signaling in granulosa cells may be species dependent. Other BMPs, including BMP4 and BMP6 have been found to increase both FSHR and AMH in the hen (Kim *et al.*, 2013; Ocón-Grove *et al.*, 2012). Although BMP4 and BMP15 signal through the same receptors, they have opposite effects on FSHR mRNA expression. This could be due to the pattern of expression of the ligand. BMP4 mRNA in the granulosa layer increases with follicle development (Kim *et al.*, 2013) whereas BMP15 is most abundant in smaller follicles. Our studies on in vivo derived tissue support a negative correlation between AMH and FSHR around the time of follicle selection (Johnson *et al.*, 2009). Although the function of AMH in the hen is not clear, in mammals AMH inhibits primordial follicle activation, thus allowing a normal progression of follicle development (Durlinger *et al.*, 2002). Previously, our lab found that an oocyte factor (in oocyte-conditioned media) decreased AMH mRNA in cultured granulosa cells (Johnson *et al.*, 2008). The effect could not be blocked by pre-incubation of the oocyte-conditioned medium with a GDF9 antibody, suggesting the factor was not GDF9, but it could have been BMP15. Similarly, in mammals, *AMH* mRNA has been shown to be regulated by the oocyte (Salmon *et al.*, 2004).

The size of the oocyte in the hen increases dramatically after selection due to yolk accumulation. Prior to follicle selection, access to the yolk receptor on the oocyte surface may be

inhibited by tight junctions formed by adjacent granulosa cells (Schuster *et al.*, 2004). OCLN protein is decreased in the F1 follicle as compared to small follicles (Schuster *et al.*, 2004). In this study, granulosa cells from 3-5 mm and 6-8 mm follicles treated with rhBMP15 had significantly lower OCLN mRNA (Figure 4C) and protein expression (Figure 4D). Therefore, the oocyte may participate in its own growth through BMP15 effects on OCLN, allowing yolk access to LR8, the yolk receptor on the oocyte surface.

We analyzed 1 mm whole follicles to study the regulation of *BMP15* because they have better viability when cultured for extended periods of time and expressed *BMP15* (Elis *et al.*, 2007). In contrast to mammals, small follicles of the hen have a higher concentration of E2 and a greater capacity to produce E2 than larger follicles (Bahr *et al.*, 1983; Huang *et al.*, 1979; Robinson and Etches, 1986; Wang and Bahr, 1983). In the hen, E2 is produced in the theca cells, specifically the theca externa (Nitta *et al.*, 1991). Among other functions, E2 production is necessary during follicle development to stimulate yolk production in the liver (Gruber *et al.*, 1976). The estrogen receptors, estrogen receptor alpha (ESR1) and estrogen receptor beta (ESR2), are found in the ovarian stroma and in small growing and large pre-ovulatory follicles in the hen (Hrabia *et al.*, 2008). We found that E2 significantly increased the production of *BMP15* mRNA (Figure 5A). It is possible that E2 has a paracrine effect to promote BMP15 production in the oocyte. Adequate E2 production from surrounding somatic cells may signal the oocyte to initiate maturational changes in granulosa cells, including decreasing OCLN to allow an increase in yolk accumulation.

In conclusion, this study demonstrates the possible role of the oocyte in promoting follicle selection in the hen. Through the production of BMP15, the oocyte plays an important role in regulating the surrounding somatic cells during follicle selection. BMP15 increases *FSHR*

mRNA and decreases both *AMH* mRNA and OCLN mRNA and protein. The direction of these changes in *FSHR*, *AMH*, and OCLN are hallmarks of follicle selection. This results in further differentiation of the granulosa cell layer, allowing yolk to access the yolk receptor, culminating in rapid growth of the oocyte before ovulation.

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Table 1 Primer and probe sequences used in qRT-PCR analysis.

Figure 1 *BMP15* mRNA levels in the ooplasm and granulosa of 3 mm follicles. RNA was isolated from the ooplasm and granulosa cells of 3 mm follicles (n = 4 replicates). Bars represent the mean $\pm$ SEM and bars with different letters differ significantly ($P < 0.01$).

Figure 2 Representative Western blot of chicken BMP15 (n = 3 replicates). Protein was isolated from whole follicles (2, 6, and 8 mm in diameter) and granulosa cells from 6-8 mm follicles. BMP15 was detected at approximately 50 kDa in 6 and 8 mm follicles with little or no detectable presence in 2 mm follicles or in granulosa cells from 6-8 mm follicles. Specificity of the antibody was determined by pre-incubation of the antibody with the immunizing peptide; blocking the antibody markedly reduced detection of BMP15 in 6 and 8 mm follicles (Blocked).

Figure 3 Messenger RNA for BMP15 receptors (A: *BMPR1B*; B: *BMPR2*) in <2 mm whole follicles and granulosa layers from various sized follicles (3-5 mm, 6-8 mm, 9-16 mm; n = 4-6 replicates). Bars represent the mean $\pm$ SEM and bars with different letters differ significantly ($P < 0.05$).

Figure 4 BMP15 function in small follicles. Messenger RNA for A) *FSHR*, B) *AMH*, and C) *OCN* in response to BMP15 in cultured granulosa cells from 3-5 and 6-8 mm follicles (n = 6 replicate experiments). All mRNA expression levels first were normalized to *18S*; bars represent the mean $\pm$ SEM relative to the control and bars within a follicle size range with different letters (A, B or a, b) differ significantly ($P < 0.05$). Statistical comparisons were made within follicle size groups. D) Representative Western blot analysis of OCN expression with increasing dose of BMP15 in cultured granulosa cells from 3-5 and 6-8 mm follicles (n = 4 replicate experiments).

522 **Figure 5** Effect of estradiol on BMP15 expression in cultured 1 mm whole follicles. *BMP15*
523 mRNA levels in response to estradiol (E2) at 0, 1, or 10 ng/ml. All mRNA expression levels first
524 were normalized to *18S*; bars represent the mean $\pm$ SEM relative to the control and bars with no
525 common letters differ significantly ($P < 0.05$; $n = 5$ replicate experiments).

Table 1

Gene Symbol	Accession Number (GenBank)	Primer Sequence	Probe	Product Size	Primer Efficiency
BMP15	NM_001006 589	forward:5' –ACATGCTGGAGCTGTACCAA-3' reverse: 5'-GACACGGAGAAGGTGCTCA-3'	NA	197	100.9%
BMPR1B	NM_205132	forward 5' –TTCTGGTGGACATTTGGTCA-3' reverse 5'-GACAGTCACCGAGATCAGCA-3'	NA	220	103.2%
BMPR2	NM_001001 465	forward 5'-CATCAGAGCCCTCATTGGAT-3' reverse 5' –GGCAATGTTGTCATGTTCCA-3'	NA	197	101.9%
OCLN	NM_205128	forward 5' –GCTCCGCAGCTCTTCTACA-3' reverse 5' –TGGTTCACCCCTCCGTAGTA-3'	NA	242	90.4%
FSHR	NM_205079	forward 5'-GCACCTTCCAAGCCTCAGATAT-3' reverse 5' -CCCTATGGACGACGGGTAAA-3'	TGTTAATATCAAACACAGGCCT	70	91.5%
AMH	NM_205030	forward: 5'-CCCCTCTGTCCCTCATGGA3'; reverse: 5'-CGTCATCCTGGTGAAACACTTC3'	AGCTCCTCTTTGGCTCA	71	92.3%

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5