THE REGULATION OF ANTI-MÜLLERIAN HORMONE IN THE HEN

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by
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ABSTRACT

Folliculogenesis in the hen is a complex process which involves a number of autocrine, paracrine, and endocrine factors. There is still much that is unknown about folliculogenesis, and the factors involved. We investigated the roles of calcitriol and bone morphogenetic protein 15 (BMP15) in the regulation of folliculogenesis in hen, specifically their effect on anti-Müllerian hormone, which plays a crucial role in activation and selection of follicles. Our goal was to evaluate expression and function of these two factors in the ovary of the hen.

In the first study, calcitriol treatment of granulosa cells 3-5 mm and 6-8 mm follicles resulted in a significant (p<0.05) decrease in AMH mRNA expression and a significant (p<0.05) increase in FSHR mRNA expression. VDR expression was demonstrated in the granulosa layer by western blot and immunohistochemistry. Through quantitative PCR, we found that VDR mRNA expression in granulosa cells significantly (p<0.05) increased as follicle size increased. We were unable to evaluate any effect on calbindin mRNA expression as levels were too low to detect.

We investigated the role of BMP15 in the regulation of folliculogenesis, in a second study. BMP15 was found to be expressed in the granulosa and theca layers of growing follicles. BMP15 mRNA expression significantly (p<0.05) decreased as follicle size increased. Recombinant hBMP15 treatment of granulosa cells from 3-5 mm and 6-8 mm follicles caused a significant (p<0.05) increase in FSHR mRNA expression; AMH mRNA expression was unaffected. BMP15 also caused
a significant (p<0.05) decrease in proliferation of granulosa cells from 3-5 mm and 6-8 mm follicles, but did not affect proliferation in granulosa cells from F1 follicles. Progesterone production by granulosa cells from F1 follicles was significantly (p<0.05) decreased by BMP15 treatment.
BIOGRAPHICAL SKETCH

Jessye Wojtusik was born in Hartford, Connecticut and grew up in Northwood New Hampshire where she attended Coe Brown Northwood Academy. She received her Bachelor of Science degree in Animal Science at Cornell University in May 2008. She began her graduate study at Cornell in January 2009, entering the Master of Science degree program in reproductive physiology.
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CHAPTER 1
LITERATURE REVIEW
1.1 REPRODUCTION IN THE HEN

In the domestic hen, only the left ovary and oviduct are functional. Female embryos initially develop Müllerian ducts on both sides but by embryonic day 3 or 4 an uneven amount of germ cells migrate to the left side and anti-Müllerian hormone (AMH), produced by the right ovary causes the right oviduct to regress by embryonic day 10 (Tran and Josso, 1977). Estrogen prevents AMH from affecting the left side, as the left side has more estrogen receptors (Hutson et al., 1985) and also produces about four to six times more estradiol than the right ovary (Teng et al., 1982).

The ovary has a large resting pool of primordial follicles, from which prehierarchal follicles will arise. The primordial follicles consist of primary oocytes surrounded by a vitelline membrane. As they develop, the oocyte is encompassed by mesodermal cells which become the granulosa cells. The perivitelline layer, thought to be equivalent to the mammalian zona pellucida (Wyburn et al., 1965), forms between the oocyte and the granulosa layer. These growing follicles are less than 2 mm in diameter.

During the transition to the prehierarchal pool, the follicles accumulate white yolk, which is rich in lipo-proteins produced by the liver. The follicles grow to be about 2-4 mm in diameter, they are then known as small white follicles (SWF) (Marza and Marza, 1935; Gilbert et al, 1983). These SWF develop a theca layer, separated from the granulosa cells by the basal lamina. The granulosa cells of SWF have tight junctions between them, maintained by occludin, which prevent the accumulation
of yolk by disallowing yolk components from reaching their receptors on the oocyte (Schuster et al, 2004). As follicles mature, production of occludin decreases allowing yolk accumulation to occur (Schuster et al, 2004).

Through receptor-mediated uptake, the oocyte accumulates vitellogenin and low-density lipoproteins, making up the yellow yolk seen in the hierarchal follicles, and the follicle becomes a small yellow follicle (SYF) (Marza and Marza, 1935; Gilbert et al, 1983). SYF are 6-12 mm in diameter. At this time, the theca layer differentiates into two layers: the theca interna and externa, which become vascularized and innervated.

From this group of prehierarchal follicles, four to six are selected to join a hierarchy of preovulatory follicles depicted in Illustration 1.1 (Etches, 1996; Gilbert et al, 1983). The follicular hierarchy is established at sexual maturity, which typically occurs at 18 to 20 weeks of age in chickens. These follicles range in size representative of the order in which they are selected to ovulate. The largest (up to 40 mm in diameter) of these yolk-filled follicles is designated F1, and is the next to ovulate. The second largest follicle is known as F2 and the hierarchal follicles following by size are similarly ranked (Gilbert et al, 1983). These follicles rapidly accumulate yellow yolk, produced in the liver in response to circulating estradiol, which is produced by the theca cells of smaller follicles (Barber et al, 1991).
Illustration 1.1 Photograph of a normal hen ovary
This photograph depicts the unique follicular hierarchy characteristic of the hen ovary.

As follicles increase in size, a highly specialized group of cells known as the germinal disc region (GDR) becomes more prominent and is easily visualized. The germinal disc is comprised of the nucleus of the oocyte and most of the organelles, and develops into the embryo after fertilization (Tischkau et al, 1997). The proximal granulosa cells are connected to the germinal disc through tight junctions and are not easily separated and are therefore considered part of the GDR. In the hen, the oocyte also includes the yolk. The GDR plays an important role in regulating folliculogenesis and ovulation; when removed, apoptosis is induced and ovulation does not occur (Yoshimura et al, 1994).
The ovulatory cycle is defined as the time from one ovulation to the next. The time between ovulation and oviposition (the laying of an egg) is roughly 24-26 hours (Fraps and Hammond, 1955). A new ovulation occurs 15-45 minutes after oviposition. This ovulation-oviposition rhythm can be greatly affected by external factors including temperature, lighting, and feeding schedules (Fraps and Hammond, 1955). A typical laying pattern is known as a sequence, and is defined by the number of eggs laid in a row followed by a pause day (Etches, 1996). The length of this sequence can last as long as one ovulation – oviposition cycle to several. After ovulation the ovum travels through the reproductive tract and gains layers of membrane, albumin, and finally a shell before the egg is laid.

Ovarian function and the regulation of folliculogenesis are highly regulated by the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH). LH primarily influences granulosa cells of larger follicles. LH receptor (LHR) expression greatly increases as follicles progress past the F3 stage (Zhang et al., 1997). FSHR expression decreased inversely with size in the same follicles (Zhang et al., 1997).

Estradiol (the main steroid produced by SWF), which is produced by theca externa cells, decreases as follicle size increases and is lowest in the F1 follicle (Robinson and Etches, 1986). Androstenedione, a precursor of androgens and estrogens, similarly decreases as follicles mature (Etches and Duke, 1984). Granulosa cells in SYF are not yet steroidogenic, but begin to produce progesterone at 9 to 12 mm in diameter. Progesterone production increases greatly as the follicles enter the hierarchy, specifically in follicles F5 and larger.
Estradiol production peaks four to six hours before ovulation, and primes the hypothalamus for stimulation by progesterone. Also at this time, there is a peak in progesterone, primarily produced by the F1 follicle. This stimulates the release of GNRH by the hypothalamus, which in turn stimulates LH secretion by the anterior pituitary. LH secretion further stimulates progesterone production, creating a positive feedback loop, which induces the preovulatory surge (Johnson and van Tienhoven, 1980). The LH surge causes structural changes in the follicle allowing rupture and thus ovulation.

Prehierarchal follicles appear to be primarily affected by FSH. Smaller follicles have greater FSH receptor (FSHR) expression than in the F1 follicle, but FSHR expression peaks in 6-12 mm follicles (Woods and Johnson, 2005). There is also a slight increase in progesterone production in response to FSH in the smaller follicles as compared to hierarchal follicles (Johnson, 1993). Overall progesterone production increases as follicle size increases and is associated with an increase in LHR expression in granulosa cells (Zhang et al., 1997). LH responsiveness also increases with follicle maturation.

1.2 REGULATION AND FUNCTION OF AMH

Anti-Müllerian hormone (AMH), also known as Müllerian inhibiting substance (MIS), is a dimeric glycoprotein and a member of the transforming growth factor-β (TGF-β) superfamily (Massagué, 1998). In order to exert an effect, AMH binds its type II receptor, and the type I receptor is then also bound, which causes a downstream signaling through the Smad second messenger system (Massague and Chen, 2000). Two
AMH receptors have been found in mammals, but none have been identified in the chicken. In the rat, an AMH type II receptor is expressed in embryonic Müllerian duct mesenchymal cells, postnatal granulosa cells (Baarends et al., 1994; Baarends et al., 1995), and preantral and antral theca cells (Ingraham et al., 2000). In addition, a type I receptor was found in the ovary of fetal and adult mice (Visser et al., 2001). AMH is involved in the regression of the Müllerian ducts in mammalian and avian male embryos as well as the right oviduct in female avian embryos (Romanoff and Romanoff, 1960; Josso, 1971; Tran and Josso, 1977). Estrogen produced by the left ovary, protects the left Müllerian duct from the inhibitory effects of AMH (Hutson et al., 1985; Teng, 2000). Expression of AMH has also been demonstrated in post-natal females, specifically by granulosa cells in the ovary of both mammals and the hen (Ueno et al., 1989; Weenan et al., 2004; Johnson et al., 2008).

**AMH function and regulation in mammals**

In mammals, AMH expression is first seen in granulosa cells of primary follicles and is highest in preantral and small antral follicles (Ueno et al., 1989; Baarends et al., 1995). There is greater expression of AMH mRNA in granulosa cells most proximal to the oocyte (cumulus granulosa) verses mural granulosa cells (Ueno et al., 1989; Hirobe et al., 1992; Munsterberg and Lovell-Badge, 1991). Kit ligand (KITL) and LH-receptor (LHR) have also been shown to be expressed differently in cumulus cells as compared to mural granulosa cells in mice (Eppig et al., 1997; Joyce et al., 1999). These differences in LHR and KITL were shown to be regulated by the oocyte through paracrine factors, suggesting perhaps an oocyte derived factor is also regulating AMH expression. The
oocyte has been shown to increase granulosa AMH mRNA expression in mice, suggesting regulation within the follicle during development (Salmon et al., 2004). In mammals, AMH expression in the female does not seem to be regulated by the same transcription factors implicated in regulation of AMH in the male (Salmon et al., 2005).

In AMH knock-out mice, folliculogenesis is accelerated but the mice remain fertile, suggesting that AMH is primarily involved in follicle recruitment and selection within the ovary (Behringer et al., 1994, Durlinger et al., 1999). Further investigation showed that the number of growing follicles was greatly increased, while the primordial follicle pool was depleted prematurely (Durlinger et al, 1999). It appears that AMH inhibits selection of follicles, maintaining an orderly rhythm for proper timing of ovulation.

There is a proposed inverse pattern of follicular expression of AMH and FSHR; this relationship may regulate follicle selection in mice (Visser and Themmen, 2005). AMH decreases FSH sensitivity; when AMH was added to cultured follicles, FSH stimulated follicle growth was decreased (Durlinger et al., 2001). Mice that are deficient in AMH have increased numbers of growing follicles as compared to wild type mice when they are given FSH injections (Durlinger et al., 2001). AMH has also been shown to inhibit the stimulatory effects of factors known to regulate follicle maturation including kit ligand and keratinocyte growth factor in the mouse (Nilsson et al., 2007). This demonstrates the inhibitory effect that AMH has on follicle activation.
AMH expression and regulation in the hen

In the hen, AMH is expressed in a defined pattern during follicular development, decreasing in expression as follicle size increases (Johnson et al., 2008). There was no significant difference in AMH expression by granulosa cells proximal to the GDR versus those distal (Johnson et al., 2008). This difference between AMH expression in mammals and hens may be due to the fact that the follicle of the hen has no antrum and all granulosa cells are in contact with the oocyte (Perry et al. 1978). Proximity to GDR may not be relevant in this instance.

Oocyte conditioned media (OCM), is a media created by pooling and incubating follicles less than mm in diameter in M199 and 0.1% BSA for 3 days (Johnson et al., 2005). This creates a media rich in factors secreted by the oocyte, as well as granulosa and theca cells, for use in culture. It has been shown that follicles less than 1 mm in diameter are a good source of growth differentiation factor 9 (GDF9) (Johnson et al., 2005). In hen granulosa cells treated with 25% and 50% OCM there was a significant decrease in the expression of AMH mRNA (Johnson et al., 2008). Heat-treating the OCM before use inhibited this decrease. This suggests that the factor in the OCM inhibiting AMH expression is heat labile and likely a protein. GDF9 and bone morphogenetic protein 15 (BMP15), growth factors, were found not to be responsible for the decrease, through the incubation of the OCM with a GDF9 or BMP15 antiserum before treatment (Johnson et al., 2008; Wojtusik et al., unpublished data). The origin of the factor is unknown; it could be secreted by the oocyte, however it may also be coming from the granulosa cells or theca, since they are not separated out when the OCM is made. It
remains unclear whether or not AMH expression by granulosa cells is
influenced by a factor produced by the oocyte.

It was recently found that AMH expression is up-regulated in
human prostate cancer cells treated with calcitriol (1, 25-
dihydroxyvitamin D3) in vitro (Krishnan et al., 2007). A functional
vitamin D-response element (VDRE) was found in the human AMH
promoter, demonstrating that calcitriol directly regulates AMH expression
(Malloy et al., 2009). Vitamin D receptor (VDR) knock-out mice have
been shown to have impaired folliculogenesis: follicles do not progress
past primary or secondary stage, suggesting calcitriol plays an important
role in folliculogenesis (Yoshizawa et al., 1997).

The sequence of AMH in chickens is 52% similar to the
mammalian AMH sequence in the biologically active C-terminal region
(Eusebe et al., 1996). Mammalian AMH, however, is inactive in the hen.
In order to evaluate the possible functions of AMH, testis conditioned
medium (TCM) was used in granulosa cell culture (Johnson et al., 2009).
It was previously shown that AMH is maximally secreted by testes
between days 12 and 14 of embryonic development (Teng, 1987). TCM
was made by removing testes from embryonic chicks aged 12 to 14 days
and incubating them in Medium 199 plus 0.1% bovine serum albumin for
3 days at 37°C and 5% CO₂. TCM was shown to be a source of
immunoreactive AMH in the chicken through western blot, and therefore
was used to evaluate possible functions of AMH (Johnson et al., 2009).
Pre-incubation of the TCM with an AMH antiserum prevented granulosa
cell proliferation usually seen with TCM treatment (Johnson et al., 2009).
This suggests that AMH induces proliferation of granulosa cells. Not
much is known about AMH regulation and function in the ovary of the hen; further investigation of possible roles are needed in order to fully understand the role AMH plays in folliculogenesis and follicular activation.

1.3 OVARIAN FACTORS INVOLVED IN FOLLICULOGENESIS

*BMP15 and GDF9*

Bone morphogenetic protein 15 (BMP15), also known as growth differentiation factor 9B (GDF9B), and growth differentiation factor 9 (GDF9) are members of the transforming growth factor-β (TGF-β) superfamily (Chang et al., 2002). Members of the TGF-β super-family often possess seven conserved cysteines, which allow them to create homodimers through covalent bonds (Vitt et al., 2001). The cysteines fold into a structure known as a cysteine knot, and the remaining cysteine is involved in creating disulfide bonds with another molecule to create a dimer. Interestingly, BMP15 and GDF9 have only six out of the seven cysteines, but are capable of forming non-covalent homodimers and heterodimers with each other (Liao et al., 2003). BMP15 and GDF9 are transcribed as signal peptides, prodomains, and mature regions (Laitinen et al., 1998; Dube et al., 1998).

In mammals, BMP15 is expressed solely by the oocyte beginning at the primary stage of follicular development, and maintains a high level of expression throughout follicular development (Dube et al., 1998). It has been shown to play an important role in granulosa cell development and function in the mammalian ovary (Su et al., 2004; Yan et al., 2001). Rat granulosa cells treated with human recombinant BMP15 (rhBMP15),
showed an increase in proliferation, and an inhibition of FSH-induced progesterone production (Otsuka et al., 2000; Otsuka et al., 2001). FSHR mRNA levels were also shown to be decreased by BMP15 treatment, and BMP15 blocks FSH from stimulating FSHR expression, suggesting BMP15 is an important regulator of FSH in the ovary. FSH action was inhibited due to suppression of FSHR (Otsuka et al., 2000). BMP15 treatment does not affect P450 aromatase (P450arom) or P450 side chain cleavage enzyme (P450scc), but prevents the stimulation of either by FSH.

BMP15 plays an important role in maintaining fertility, though effects vary by species. BMP15 knock-out mice demonstrated sub-fertility represented by a reduced number of oocytes being ovulated and becoming fertilized (Yan et al., 2001). Sheep heterozygous for the BMP15 gene showed an increase in the number of twins and triplets, whereas BMP15 homozygous null sheep were shown to be infertile as their follicles were arrested in the primary stage (Juengel et al., 2002, Galloway et al., 2000, Hanrahan et al., 2004). There is only a 78% similarity between mouse and sheep BMP15 sequences, and the difference may have resulted in BMP15 being more essential for folliculogenesis in sheep. Loss of function of BMP15 in humans leads to ovarian failure, and thus infertility (Di Pasquale et al., 2004).

The role of BMP15 in avian species is less defined than in mammals. BMP15 is expressed in the ovary of the hen, as well as in the brain. BMP15 is expressed in the oocyte (yolk) as shown by in-situ hybridization in follicles 50 μm to 6 mm in diameter (Elis et al., 2007). RT-PCR demonstrated expression of BMP15 mRNA in the germinal disc
as well as granulosa cells of follicles F5-F1, although the expression in the GDR was much greater than in the granulosa cells (Elis et al., 2007; Wang et al., 2007). Overall expression was not different between F5 to F1 follicles, and F6 follicles showed significantly higher expression of BMP15 mRNA, indicating that it decreases as follicle size increases.

Avian BMP15 is 72% similar to human BMP15. Treatment of F1 granulosa cells with human recombinant BMP15 resulted in unchanged proliferation of granulosa cells. Although there was a decrease in LH and FSH stimulated progesterone production (Elis et al., 2007). In the rat, BMP15 treatment did not affect FSH stimulated granulosa cell progesterone production. However, when BMP15 was combined with GDF9, progesterone production was significantly decreased. This suggests there is cooperation between the two proteins.

**GDF9**

GDF9 is expressed solely in the oocyte of many mammalian species including mice (McGrath et al., 1995) and sheep (Juengel et al., 2002); however it has also been found in granulosa cells of some primates (Yamamoto et al., 2002; Duffy, 2003). GDF9 knock-out mice are infertile and follicles do not mature past the primary stage of development (Dong et al., 1996). Growth of preantral rat follicles was greatly stimulated when incubated with GDF9 (Hayashi et al., 1999). GDF9 stimulates granulosa cell proliferation in the rat (Vitt et al., 2000; Elvin et al., 1999) and also stimulates progesterone production by granulosa cells (Elvin et al., 2000). This is different from BMP15 which inhibits progesterone production.

In the hen, GDF9 has been shown to be expressed by granulosa cells as well as the oocyte, though expression in the oocyte is much
greater. Expression in granulosa cells proximal to the GD as compared to those distal was unchanged (Johnson et al., 2005). As follicle size increases, GDF9 expression greatly diminishes, and after ovulation GDF9 expression is minimal (Johnson et al., 2005; Elis et al., 2007). This suggests that GDF9 plays an important role in folliculogenesis and perhaps follicular activation, but is likely not involved in early embryogenesis.

Treatment of granulosa cells collected from follicles 3-8 mm in size with oocyte-conditioned media (OCM), shown to contain GDF9 through western blot, stimulated an increase in proliferation (Johnson et al., 2005). When the OCM was pre-incubated with a GDF9 antibody, proliferation did not occur, suggesting the effect was due to GDF9. Further studies examining the function and regulation of GDF9 and BMP15 are needed in order to better understand their role in folliculogenesis the hen.

1.4 FUNCTION AND REGULATION OF CALCITRIOL

Calcitriol (1,25-dihydroxyvitamin D3 [1,25(OH)2D3]) is the biologically active form of vitamin D3 (Feldman et al., 2005). Vitamin D3 is ingested or synthesized in the skin from 7-dehydrocholesterol. It circulates in the blood and is converted to 25-hydroxyvitamin D3 [25(OH)D3], an inactive hormonal precursor, by 25-hydroxylase in the liver. 25-hydroxyvitamin D3 is then converted into calcitriol in the kidney by 1α-hydroxylase, which is regulated by parathyroid hormone (Feldman et al., 2005).

Calcitriol regulates calcium and phosphate metabolism, and therefore is involved in bone mineralization. One of the primary target
organs with respect to calcium balance is the intestine (Feldman et al., 2005). Calcitriol regulates calcium binding protein (calbindin), and therefore, calcium absorption. Calcitriol has also been shown to stimulate cell differentiation and apoptosis as well as inhibit proliferation and inflammation in a variety of cells. It exerts the majority of its effects through the vitamin D receptor (VDR), a member of the steroid-thyroid-retinoid receptor superfamily of ligand activated transcription factors. Through its receptor, calcitriol can also modify gene transcription, as well as protein and mRNA production (Feldman et al., 2005). VDR binds to specific regions in the promoter regions of appropriate genes, called vitamin D-responsive elements (VDRE) (Ross et al., 1992; Mangelsdorf et al., 1995). VDR creates a heterodimer with retinoid X receptor (RXR) in order to bind VDREs with high affinity. VDR expression is affected by a number of factors including calcitriol, calcium, and estrogen. It has also been shown that calcitriol can exert an effect without binding VDR, by directly binding a VDRE (Ferrari et al., 1994).

The role of calcitriol in reproduction

In mammals, VDR is expressed in the ovary and it is therefore likely that calcitriol plays a role in regulating reproduction (Stumpf, 1995). Calcitriol deficiency greatly affects fertility in rats causing overall fertility to be reduced to about 75% and litter size to decrease to about 30% of normal (Halloran and Deluca, 1980). In vitamin D deficient rats, supplementation with calcitriol restores fertility (Kwiecinski et al., 1989a). Also, matings between vitamin D deficient male and female rats are unsuccessful (Kwiecinski et al., 1989b). VDR knock-out mice have been shown to have impaired folliculogenesis, in that no follicles larger
than primary and secondary were observed. In addition, these mice exhibited uterine hypoplasia (Yoshizawa et al., 1997). When supplemented with estrogen, uterine weight increased, suggesting that a deficiency in estrogen was responsible for the uterine hypoplasia. In VDR knock-out mice there is a decrease in CYP19 expression which encodes P450 aromatase (P450arom) (Kinuta et al., 2000). As the key enzyme involved in the synthesis of estrogen, decreased aromatase greatly influences estrogen levels. A VDRE was found in the promoter of the CYP19 gene, suggesting that calcitriol regulates estrogen synthesis (Sun et al., 1997).

A functional VDRE was recently found in the promoter region of human AMH (Malloy et al. 2009), and treatment of human prostate cancer cells with calcitriol caused an increase in AMH expression (Krishnan et al., 2007; Malloy et al. 2009). This suggests a role for calcitriol in regulating folliculogenesis, as AMH is implicated in follicular activation and maturation in mammalian and avian species (Behringer et al., 1994; Durlinger et al., 1999; Johnson et al., 2008).

The role of calcitriol in the hen

The sequence of the VDR in the hen is 78% similar to mammals. There are two receptor proteins (58 and 60 kDa) that arise from a single mRNA transcript (Mangelsdorf et al., 1987; Lu et al., 1997). The binding domain is almost completely conserved between mammals and birds, as would be expected since they bind the same hormone, calcitriol.

In the hen, calcitriol plays a significant role in the uptake of calcium for use in embryo development. The hen ovary contains a large pool of growing follicles. During follicle development, there is an
accumulation of liver-derived yolk and approximately daily, ovulation of the largest follicle occurs. If fertilization results, the embryo acquires all of the calcium needed for development from the yolk until about embryonic day 10, when the egg shell becomes its main source of calcium for subsequent development (Johnston and Comar, 1955; Tuan, 1987). Calcium mobilization by the embryo from the yolk is regulated by calcitriol (Clark et al., 1989; Lee et al., 1990). Calcitriol enhances calcium uptake in day 3 embryos and stimulates calbindin (a vitamin D-dependent calcium binding protein) expression (Tuan and Ono, 1986; Ono and Tuan, 1991). In the intestine, calbindin facilitates the diffusion of calcium through cells (Wasserman and Fullmer, 1989; Feher et al., 1992). It is thought to play a similar role in the yolk and to aid in the mobilization of calcium from the yolk for use by the embryo. To our knowledge, the possible role of granulosa cells in facilitating calcium transport from the plasma into the yolk during follicle development has not been examined.

Vitamin D- deficient hens lay fewer eggs and have an increase in eggs with thin shells. Calcitriol supplementation reverses this effect (Narbaitz et al., 1987). VDR expression has been shown in the shell gland of hens, and it appears that calcitriol plays an important role in egg shell formation through the stimulation of calbindin (Ieda et al., 1995; Yoshimura et al., 1997). While calcitriol is a very well-studied hormone, there is still much that is not known about its roles in the ovary and in folliculogenesis.
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CHAPTER 2
CALCITRIOL REGULATES ANTI-MÜLLERIAN HORMONE
EXPRESSION IN GRANULOSA CELLS OF THE HEN

2.1 INTRODUCTION

Anti-Müllerian hormone (AMH), also known as Müllerian inhibiting substance (MIS), is a dimeric glycoprotein and a member of the transforming growth factor-β (TGF-β) super-family (Massagué, 1998). AMH is involved in the regression of the Müllerian ducts in mammalian and avian male embryos as well as the right oviduct in female avian embryos (Romanoff and Romanoff, 1960; Josso, 1971; Tran and Josso, 1977). Expression has also been demonstrated in post-natal females, specifically by granulosa cells in the ovary of both mammals and birds (Ueno et al., 1989; Hirobe et al., 1992; Johnson et al., 2008). In AMH knock-out mice, folliculogenesis is accelerated but the mice remain fertile, suggesting that AMH is primarily involved in follicle recruitment and selection within the ovary (Behringer et al., 1994; Durlinger et al., 1999). In the hen, AMH is expressed in a defined pattern during follicular development, decreasing in expression as follicle size increases (Johnson et al., 2008). The oocyte has been shown to increase granulosa AMH mRNA expression in mice, suggesting regulation within the follicle during development (Salmon et al., 2004). In mammals, AMH expression in the female does not seem to be regulated by the same transcription factors implicated in regulation of AMH in the male (Salmon et al., 2005). The factors involved in regulating AMH expression by granulosa cells are not well understood.
It was recently found that AMH expression is up-regulated in human prostate cancer cells treated with calcitriol (1, 25-dihydroxyvitamin D3) \textit{in vitro} (Krishnan \textit{et al.}, 2007; Malloy \textit{et al.}, 2009). A functional vitamin D-response element (VDRE) was found in the human AMH promoter, demonstrating that calcitriol directly regulates AMH expression (Malloy \textit{et al.}, 2009). Vitamin D receptor (VDR) is expressed in the ovary of rodents and vitamin D deficiency leads to 25\% reduction in overall fertility and a 70\% decrease in litter size of female rats (Stumpf, 1995; Halloran and Deluca, 1980). VDR knock-out mice have been shown to have impaired folliculogenesis, in that no follicles develop beyond the primary or secondary stage, suggesting that calcitriol plays a significant role in folliculogenesis (Yoshizawa \textit{et al.}, 1997). In VDR null mice, there is also a decrease in the expression of CYP19 mRNA, which encodes aromatase (Kinuta \textit{et al.}, 2000). As the key enzyme involved in the synthesis of estrogen, this potentially affects estrogen levels. It is likely that calcitriol is involved in the synthesis of estrogen within the ovary, in addition to other possible roles.

Calcitriol is the biologically active form of vitamin D3 (Feldman \textit{et al.}, 2005). It plays a variety of roles throughout the body, including regulating calcium uptake, inhibiting proliferation, and stimulating cell differentiation. Calcitriol primarily exerts its effects through the VDR, a member of the steroid-thyroid-retinoid receptor super-family of ligand-activated transcription factors. Through its receptor, calcitriol can modify gene transcription, as well as protein and mRNA production. VDR creates a heterodimer with retinoid X receptor (RXR) and binds to VDREs in the promoter of the target gene (Ross \textit{et al.}, 1992; Mangelsdorf \textit{et al.}, 1995).
The hen ovary contains a large pool of growing follicles. During follicle development, there is an accumulation of liver-derived yolk and, approximately daily, ovulation of the largest follicle occurs. If fertilization results, the embryo acquires all of the calcium needed for development from the yolk until about embryonic day 10, when the egg shell becomes its main source of calcium for subsequent development (Johnston and Comar, 1955; Tuan, 1987). Calcium mobilization within the yolk is regulated by calcitriol (Clark et al., 1989; Lee et al., 1990). Calcitriol enhances calcium uptake in day 3 embryos and stimulates calbindin-D (a vitamin D-dependent calcium binding protein) expression in the yolk sac (Tuan and Ono, 1986; Ono and Tuan, 1991). In the intestine, calbindin facilitates the diffusion of calcium through cells (Wasserman and Fullmer, 1989; Feher et al. 1992). It is thought to play a similar role in the yolk and to aid in the mobilization of calcium from the yolk for use by the embryo. To our knowledge, the role of granulosa cells in facilitating calcium transport into the yolk has not been examined.

Our goal was to evaluate the effect of calcitriol treatments on AMH mRNA expression. We also examined mRNA expression for follicle stimulating hormone receptor (FSHR) in response to calcitriol, in addition to localizing putative VDREs in the AMH promoter. On the basis of our initial findings, we characterized the expression of VDR (mRNA and protein) within the hen ovary to determine the amount and location of expression during follicle development. Finally, we evaluated the effect of calcitriol treatments on the expression of calbindin in granulosa cells to examine an additional putative function of calcitriol in the hen ovary.
2.2 MATERIALS AND METHODS

Animals

Single-comb White Leghorn hens of the Babcock B300 strain (approximately one year of age) were maintained on a schedule of 15 hours of light and 9 hours of darkness in individual cages. The hens had free access to clean water and food. Hens were selected for consistent laying patterns and were euthanized within one hour of oviposition. The Institutional Animal Care and Use Committee of Cornell University approved all animal procedures and techniques.

Granulosa Cell Culture

Granulosa cell layers were collected from approximately eight 3-5 mm and six 6-8 mm follicles per hen for each experiment (n=7 replicates). The granulosa layers from individual follicles were pooled (according to size), washed, and dispersed (as previously described in Davis et al., 1999). A hemocytometer was used to estimate cell number and viability; viability was 95% or greater at the start of each culture. Granulosa cells at a density of 3x10^6 cells per well, were plated in 6-well plates and cultured in 1.5 ml of Medium 199 with 5% fetal bovine serum and 1% Penicillin/Streptomycin at 37° C with 5% CO₂ for 24 hours. Medium was removed and replaced with Medium 199 with 0.1 % bovine serum albumin, 1% Penicillin/Streptomycin and calcitriol (at doses of 0, 10, 100 nM). Calcitriol was reconstituted with 100% ethanol to a concentration of 10μg/ml (24 μM). It was further diluted with PBS to a working stock of 1μg/ml (2.4 μM) in M199. The control treatment consisted of M199 with
the equivalent amount of ethanol as in the 100 nM treatment. Treated cells were cultured for 24 hours and then collected into Buffer RLT (supplied in RNeasy micro kit, Qiagen) plus 10% β-mercaptoethanol (supplied in RNeasy micro kit, Qiagen). Samples were stored at -80°C until later use. At the initiation of each culture, a 96-well plate was set up with granulosa cells at a density of 1x10^5 cells per well and treatments were added parallel to the 6-well plates to assess cell proliferation. Proliferation was determined at culture termination using AQueous One Solution Cell Proliferation Assay (Promega).

**Quantitative Real Time PCR**

RNA was extracted from the cultured granulosa cells using a Qiagen RNeasy micro kit according to manufacturer’s instructions and then subjected to reverse transcription to cDNA. Quantitative real time PCR (qPCR) was used to assess AMH and FSH receptor (FSHR) mRNA expression using Taqman primers and probes. Primers were designed with Primer Express Software 2.0 (Applied Biosystems, Foster City, CA). The program defined a product of 71 bp for AMH (GenBank accession number U61754) that spanned an intron (forward: 5’CCCCCTCTGCTCCTCATGGA3’; reverse: 5’CGTCATCCTGGTGAAACACTTC3’; probe: 6FAMAGCTCCTCTTTTGCTCAMGBNFQ). For FSHR, (GenBank U51097) a product of 70 bp was defined, which spanned an intron (forward: 5’GCACCTTCCAAGCCTCAGATAT3’; reverse: 5’CCCTATGGACGACGGGTA3A3’; probe: 6FAMTGTATATATCAACACACAGGCTMGBNFQ). All samples
were run in duplicate and were normalized to 18S ribosomal RNA (18S) using Taqman 18S primers and probes. Using an ABI 7000 Sequence Detection System, the PCR reactions were conducted in a 25 µl volume of reaction buffer (1x TaqMan Universal PCR Master Mix; Applied Biosystems) with 900 nM of AMH and FSHR primer pairs and 250 nM of the probes or 50 nM of 18S primers and 200 nM of the probe. Control reactions without cDNA and reactions without reverse transcriptase were also run.

Granulosa cells from 3-5 mm, 6-8 mm, 9-16 mm, and from F1 follicles were collected directly into buffer RLT plus 10% β-mercaptoethanol (n=4-5 replicates). RNA was extracted and subjected to reverse transcription for qPCR using SYBR green to evaluate VDR and calbindin mRNA expression. Primers were designed as stated above; both sets spanned an intron; for VDR (GenBank Accession number NM205098) (forward: 5’AGGGCTGCAAGGGCTTCT3’; reverse: 5’GTTGAACGGGAGGTGAACATC3’) and for calbindin (GenBank Accession number 205513) (forward: 5’CGGCGGCCCCAGTCTCTCGAG3’; reverse: 5’CCGTCGCAACACCTGAGCA3’). Samples were run in 25 ul with a final concentration of 1X SYBR green master mix (Qiagen), 0.15µM of each primer or .1µM of 18S primers. Controls are the same as stated above.

Western Blot

Protein lysates were made from chicken granulosa cells from 3-5 mm, 6-8 mm, and 9-16 mm follicles. Samples were subjected to SDS-
PAGE under denaturing conditions. The nitrocellulose membrane was blocked in TBST with 5% dry milk. The membrane was then incubated with primary antibody against chicken VDR (1:1000; MA1710, Thermo Scientific) for 2 hours at room temperature. Subsequently, the membrane was incubated for 1 hour with a goat anti-rat HRP conjugated secondary antibody diluted 1:1000 (Santa Cruz Biotechnology). Protein bands were detected using enhanced chemiluminescence and examined using a Fluor Chem HD2 imager (Alpha Innotech).

*Immunohistochemistry*

Sections of intestine and whole follicles (3-5 mm and 6-8 mm) were collected from euthanized hens and fixed in 10% formalin for 24 hours. The tissue was embedded in paraffin and sectioned at 4-5 μm by the Cornell University Histology lab, and then rehydrated, deparaffinized, and boiled in 0.1 M citrate for 20 minutes for antigen retrieval. The sections were blocked in 10% goat serum in PBS for 30 minutes at 37°C. The primary antibody (monoclonal; MA1-710, Thermo Scientific) was made in a rat against partially purified VDR protein from chicken intestine. The antibody was diluted 1:100 and incubated with the sections for 1 hour at 37°C. Control slides were similarly incubated with rat IgG (1:50; Rockland Inc). Alexa Fluor 488 goat anti-rat IgG was used as the secondary antibody (diluted 1:850) and incubated with sections for 1 h at 37°C. A Nikon Eclipse E600 microscope with fluorescence capability was used to examine the slides.
**Electrophoretic Mobility Shift Assay (EMSA)**

Sequences of known VDREs, found in the promoter regions of chicken carbonic anhydrase II (ggggga---agtca) (Quelo et al., 1998) and human AMH (gggtga---gggaca) (Malloy et al., 2009) were used to search for putative VDREs in the promoter of chicken AMH. VDREs are made up of two sections of 6 base pairs connected with a hinge 3-4 base pairs in length. Due to variability between known VDREs, sequences having 8 out of the 12 base pairs in common with the known VDREs, were considered putative. Three sequences in the chicken AMH promoter were considered to be putative on this basis, and primers were created for use in EMSA. EMSA was conducted according to manufacturer’s instructions (Pierce Light Shift Chemiluminescent EMSA Kit). Bands were visualized using enhanced chemiluminescence and a Fluor Chem HD2 imager (Alpha Innotech).

**Statistics**

Statistics were performed with SAS using Proc GLM with protected least significance difference. Means were compared using Duncan’s multiple range test and p values of <0.05 were considered significant.

**2.3 RESULTS**

Quantitative real time PCR (qPCR) was performed to assess expression of AMH and FSHR mRNA in granulosa cells after calcitriol treatment. There was a significant decrease in the expression of AMH mRNA in granulosa cells from 3-5 mm and 6-8 mm follicles (Figure 2.1)
and 2.2; p<0.05). There was also a significant increase in expression of FSHR at 100 nM in granulosa cells from 3-5 mm follicles (Figure 2.3; p<0.05) and at 10 and 100 nM in granulosa cells from 6-8 mm follicles (Figure 2.4; p<0.05). We also used qPCR to evaluate VDR mRNA expression in the granulosa layer in relation to follicle size. There was a significant increase in VDR mRNA expression in the granulosa layer with increasing follicle size (Figure 2.5; p<0.05).

![Graph](image_url)

**Figure 2.1 AMH mRNA expression after treatment with calcitriol in granulosa cells from 3-5 mm follicles**

AMH expression is significantly decreased by 100 nM calcitriol, (n=7, p<0.05).
Figure 2.2 AMH mRNA expression after treatment with calcitriol in granulosa cells from 6-8 mm follicles
AMH expression is significantly decreased by treatment with 10 nM and 100 nM calcitriol, (n=7, p<0.05).

Figure 2.3 FSHR mRNA expression after treatment with calcitriol in granulosa cells from 3-5 mm follicles
FSHR expression is significantly increased by treatment with 100 nM calcitriol, (n=7, p<0.05).
**Figure 2.4** FSHR mRNA expression after treatment with calcitriol in granulosa cells from 6-8 mm follicles
FSHR expression is significantly increased by treatment with 100 nM calcitriol, (n=7, p<0.05).

**Figure 2.5** VDR mRNA expression in follicles of different sizes
VDR expression is significantly increased in 9-16 mm and F1 follicles (n=4-5, p<0.05).
Figure 2.6 Proliferation of Granulosa Cells
A. Relative absorbance of granulosa cells from 3-5 mm follicles relative to control treatment (n=3, p<0.05). B. Granulosa cells from 6-8 mm follicles (n=3, p<0.05).

Proliferation of granulosa cells collected from 3-5 mm and 6-8 mm follicles was significantly increased by calcitriol at doses of 10 and 100 nM (Figure 2.6 A and B). We were unable to detect calbindin mRNA expression in granulosa cells or whole ovary using RT-PCR, western blot or immunohistochemistry as levels were too low. Primers and antibody were validated through the use of intestine as a positive control in each technique. A protein band at the expected size of 28 kDa was observed in the intestine (Figure 2.7). RT-PCR and immunohistochemical data not shown.
Figure 2.7 Western blot protein expression of calbindin
Protein band is seen at expected size of 28 kDa in the intestine.

Western blot analysis of lysates from the granulosa layer from 3-5 mm follicles and from 6-8 mm follicles showed two bands at approximately 58 kDa and 60 kDa (Figure 2.8). This doublet is representative of VDR in avian species (Mangelsdorf et al., 1987; Lu et al., 1997). EMSA evaluation of putative VDRE’s in AMH promoter region, did not show a band in the appropriate position (data not shown).

Nuclear expression of VDR was demonstrated by immunohistochemistry in the intestine (Figure 2.9A) as well as in the granulosa layer (Figure 2.9 B). Negative controls, substituting rat IgG for the primary antibody, did not show specific staining (Figures 2.9 C and D).
Figure 2.8 Western blot protein expression of VDR
Protein bands are seen at the expected sizes of 58 kDa and 60 kDa in granulosa cells from 3-5 mm, 6-8 mm, 9-16mm follicles.

Figure 2.9 Immunohistochemistry of VDR expression
2.4 DISCUSSION

Our goal was to determine the effect of calcitriol on the expression of AMH by granulosa cells in the ovary of the hen. Previous studies have shown that AMH expression in human prostate cancer cells was increased by calcitriol (Krishnan et al., 2007; Malloy et al., 2009); however, we found that treating granulosa cells with calcitriol caused a significant decrease in the expression of AMH. An inverse pattern of AMH and FSHR expression within the follicles of mammals has been proposed (Visser and Themmen, 2005). Interestingly, we found that calcitriol treatment also caused a significant increase in the expression of FSHR mRNA, supporting the proposed inverse pattern with AMH in the avian ovary. The decrease in AMH expression due to calcitriol treatment occurred at a lower dose as compared to the increase in FSHR expression. It appears that calcitriol inhibits AMH expression, allowing an increase in FSHR expression.

We also found that as follicle size increases, VDR expression in the granulosa also increases. It was previously shown that AMH mRNA expression in the hen decreases as follicle size increases (Johnson et al., 2008). In mammals, AMH inhibits follicular activation, as demonstrated by accelerated folliculogenesis in AMH knockout mice (Behringer et al., 1994; Durlinger et al., 1999). It could be that the increase in VDR expression allows calcitriol to progressively exert an inhibitory effect on AMH expression. A decrease in AMH allows follicle selection and maturation to occur through an increase in FSHR expression. Treatment with calcitriol caused granulosa cells to proliferate, which was
unexpected, as calcitriol has often been shown to have anti-proliferative effects (Feldman et al., 2005). These effects suggest a significant role of calcitriol in the regulation of folliculogenesis within the hen.

The increase in VDR as follicles matured suggested to us that perhaps calcitriol plays a role in regulating calcium uptake by the oocyte for later use by the developing embryo. Calcitriol regulates calcium homeostasis through calbindin in the embryonic yolk sac (Clark et al., 1989; Lee et al., 1990). Quantitative RT-PCR was used to evaluate calbindin expression in granulosa cells from follicles of different sizes and after granulosa cell treatment with calcitriol. In all cases, mRNA levels for calbindin in granulosa cells were too low for detection. Calbindin expression was not detected in whole ovary or granulosa cells, but was clearly expressed in the intestine. It appears that the primary role of calcitriol in the ovary is not through increase of calbindin expression.

Since calcitriol most often regulates gene expression through its receptor, which must bind a VDRE, we searched for sequences within the AMH promoter similar to known VDREs. We found three sequences that had at least 8 base pairs out of 12 in common with a known VDRE. These sequences were used to create primers for use in EMSA. We were unable to verify that the sequences were VDREs. It is possible that the signal was not strong enough to be detected using this method. In addition, the known VDREs, even within the same species for various regulated genes are highly variable. It is possible, therefore, that we did not locate the VDRE in the chicken AMH promoter. It remains unclear if the effect that calcitriol has on AMH is direct or indirect.
Through western blot and immunohistochemistry, we showed that the vitamin D receptor is expressed in the granulosa cells of the hen ovary. This suggests that calcitriol plays a functional role within the ovary. The western blot showed two bands at the expected sizes of 58 kDa and 60 kDa in the granulosa layer (Mangelsdorf et al., 1987; Lu et al., 1997). Immunohistochemical staining appears to be nuclear, which is expected, as VDR is a nuclear receptor (Feldman et al., 2005).

Future studies will evaluate other possible roles of calcitriol in the ovary of the hen, including its effect on steroidogenesis. In addition, further evaluation of the putative VDREs in the AMH promoter is needed to determine if the effect of calcitriol on AMH is direct.
REFERENCES


CHAPTER 3
THE EXPRESSION AND FUNCTION OF BMP15 IN THE OVARY OF THE HEN

3.1 INTRODUCTION

Folliculogenesis, the growth and development of somatic and germ cells in preparation for ovulation, requires complex autocrine, paracrine, and endocrine input. Communication between the oocyte and surrounding somatic cells is vital for proper and timely growth and function. In order for this to occur, the mechanisms in control must be highly regulated, although the regulation of folliculogenesis is not entirely understood. In mammals, the oocyte plays a significant role in regulating the development and function of the surrounding granulosa cells and signals from the granulosa cells are also received by the oocyte (Eppig et al., 2002; Matzuk et al., 2002).

Bone morphogenetic protein 15 (BMP15), also known as growth differentiation factor 9B (GDF9B), is a member of the transforming growth factor-β (TGF-β) super-family (Chang et al., 2002). In mammals, BMP15 is expressed solely by the oocyte beginning at the primary stage of follicular development, and is maintained at a high level of expression throughout follicular development (Dube et al., 1998). It has been shown to play an important role in granulosa cell development and function in the mammalian ovary (Su et al., 2004; Yan et al., 2001). Rat granulosa cells treated with human recombinant BMP15 (rhBMP15), showed an increase in proliferation; however, FSH-induced progesterone production was inhibited (Otsuka et al., 2000; Otsuka et al., 2001). FSHR mRNA levels were also shown to be decreased by BMP15 treatment in vitro.
(Otsuka et al., 2001). FSH action was inhibited due to suppression of FSHR.

BMP15 plays an important role in maintaining fertility, although effects vary by species. BMP15 knock-out mice demonstrate sub-fertility as fewer oocytes are ovulated and subsequently fertilized as compared to wild-type mice (Yan et al., 2001). Sheep heterozygous for the mutated BMP15 gene showed an increase in the number of twins and triplets, whereas BMP15 homozygous mutant sheep were shown to be infertile, as ovarian follicles were arrested in the primary stage (Juengel et al., 2002, Galloway et al., 2000, Hanrahan et al., 2004). Loss of function of BMP15 in humans leads to ovarian failure, and thus infertility (Di Pasquale et al., 2004).

The role of BMP15 in avian species is less well-defined than in mammals. The mammalian preovulatory follicle has a fluid-filled antrum and the oocyte is surrounded by granulosa cells, those most proximate making up the cumulus cells. The avian follicle does not have an antrum, but rather an accumulation of yolk. A specialized region on the surface of the oocyte, known as the germinal disc region (GDR), is comprised of the germinal disc (GD) and the overlying granulosa cells. The GD is comprised of the nucleus of the oocyte and most of the organelles. The GD is in very close contact with the granulosa layer and granulosa cells most proximal proliferate more rapidly and produce more progesterone (Marrone et al., 1990; Tischkau et al., 1997; Yao and Bahr, 2001).

BMP15 is expressed in the ovary of the hen, as well as the brain. It was shown to be expressed in the oocyte by in situ hybridization in follicles 50 µm to 6 mm in diameter (Elis et al., 2007). RT-PCR
demonstrated expression of BMP15 mRNA in the GDR as well as in granulosa cells of follicles F5-F1, although the expression in the GDR was much greater than in the granulosa cells (Elis et al., 2007; Wang et al., 2007). Overall expression was not different between granulosa cells from F5 to F1 follicles. F6 follicles however, showed significantly higher expression of BMP15 mRNA, compared to larger follicles, supporting a decrease in BMP15 expression with an increase of follicle size. Similar to what is seen in mammals, treatment with recombinant human BMP15 decreased both luteinizing hormone (LH)-induced and follicle stimulating hormone (FSH)-induced progesterone production, although there was no effect on proliferation of granulosa cells from F1 follicles (Elis et al., 2007).

Although BMP15 has been demonstrated in the hen and shown to affect steroidogenesis in the large preovulatory follicles (Elis et al., 2007), little is known concerning BMP15 expression and regulation in small pre-hierarchal follicles. This is the stage at which follicle selection occurs (Woods and Johnson, 2005). Therefore, our goal was to assess mRNA expression for BMP15 throughout follicle development and to localize protein expression. Additionally, we evaluated the effect of recombinant human BMP15 on granulosa cell proliferation, AMH and FSHR mRNA expression, as well as steroidogenesis.

3.2 MATERIALS AND METHODS

Animals

Single-comb white Leghorn hens of the Babcock B300-strain were maintained on a lighting schedule of 15 hours of light: 9 hours of
darkness, housed in individual cages, and used within the first year of lay. The hens were fed ad-libitum and had continual access to fresh water. Hens were euthanized and follicles removed within 2 hours of lay. All procedures were approved by Cornell University Institutional Animal Care and Use Committee.

*Granulosa Cell Collection*

To evaluate BMP15 mRNA expression, granulosa cells from 3-5 mm, 6-8 mm, 9-16 mm, and F1 follicles were collected directly into buffer RLT + 10% β-mercaptoethanol (n=4 to 6 replicates). RNA was extracted and subjected to reverse transcription to cDNA. cDNA was used in quantitative real time PCR (qPCR) using Taqman primers and probes. Primers were designed with Primer Express Software 2.0 (Applied Biosystems). A product of 54bp was defined for chicken BMP15 (GenBank AY729025), (forward: 5’GGGCGAAGCGGACAT3’; reverse: 5’CCTCCTGAGCTGCTGTTCG3’; probe: 6FAMACCCCTATTTATCCCMGBNFQ). All samples were run in duplicate and were normalized to 18S ribosomal RNA (18S) using Taqman primers and probes. Using an ABI 7000 Sequence Detection System, the PCR reactions were conducted in a 25 µl volume of reaction buffer with 1x Taqman universal PCR Master Mix (Applied Biosystems) and 900 nM of BMP15 primers and 250 nM of probe or 50 nM of 18S primers and 200 nM of probe. Control reactions without cDNA and reactions without reverse transcriptase were also run.

*Western Blot*

Protein lysates were made from chicken granulosa cells (from 1 mm and 3-5 mm) and the ovarian body. Protein concentrations were
determined using a BCA Protein Assay Kit (Pierce) and 40mg of total protein for each sample was loaded onto a 12% polyacrylamide gel (Pierce) along with a Biotinylated protein ladder (Cell Signaling). These lysates were subjected to SDS-PAGE under denaturing conditions and the gel was transferred to a nitrocellulose membrane (Thermo Scientific). The membrane was blocked in 1X Tris-buffered saline with 0.1% Tween-20 (TBST) containing 5% milk for 30 minutes at room temperature. A custom-made primary antibody was created. The immunizing peptide (15-mer; CLPGYLRDAGGDKSD) was synthesized, purified and conjugated to KLH for immunization (GenScript, Inc). The membrane was incubated in the primary antibody (1:750) in TBST with 1% milk for 2 hours at room temperature. The membrane was washed and then incubated with a HRP-conjugated goat anti-rabbit IgG diluted 1:850 and an anti-biotin antibody (1:1000) in TBST with 5% milk for 1 hour at room temperature. Protein bands were detected using ECL and examined using a Fluor Chem HD2 imager (Alpha Innotech).

Immunohistochemistry (IHC)

Sections of whole ovary and 3-5 mm follicles were fixed in 10% formalin for 24 hours, embedded in paraffin and sectioned at 4-5um by Cornell University Histology lab. The slides were rehydrated and deparaffinized and then boiled in 0.1M citrate (pH 6.0) for 20 min for antigen retrieval. The sections were blocked in 10% goat serum for 30 min at 37°C. The primary antibody used was the custom-made antiserum as described above (GenScript). The antibody was diluted 1:50 and incubated with the sections for 24 hours at 4°C. Control slides were similarly incubated with rabbit IgG. Alexa Fluor 488 anti-rabbit IgG was
used as the secondary antibody (diluted 1:850) and incubated with sections for 1 hour at 37°C. Slides were then incubated with propidium iodide (1 µg/ml) in 1XPBS for 15 min at room temperature, in order to identify nuclei. Subsequently, sections were examined with a Nikon Eclipse E600 microscope with fluorescence capability.

*Granulosa Cell Culture*

Granulosa cell layers, from follicles 3-5 mm and 6-8 mm in size, were pooled and dispersed as previously described (Davis *et al.*, 1999). Approximately eight 3-5 mm follicles and six 6-8 mm follicles were collected from one hen for each replicate experiment (n=5 and 4 replicate experiments respectively). Cell number and viability were estimated using a hemocytometer. Cell viability was 95% or greater at the beginning of each experiment. Cells at a density of 3x10⁶ cells per well were plated in six-well plates and cultured in 1.5 ml of Medium 199 with 5% fetal bovine serum and 1% penicillin/streptomycin at 37°C with 5% CO₂. After 24 hours, the medium was removed and Medium 199 with 0.1% bovine serum albumin, 1% penicillin/streptomycin and rhBMP15 (at doses of 0, 0.1, 1, 10, and 50 ng/ml; R&D Systems) were added. The cells were cultured for an additional 24 hours and then harvested. Medium was removed and stored at -80°C for later assay of progesterone production and cells were collected into Buffer RLT (supplied in RNeasy micro kit) plus 10% β-mercaptoethanol. Cells were stored at -80°C until RNA was extracted using Qiagen RNeasy micro kit according to manufacturer’s instructions. At the initiation of each culture, a 96-well plate was set up with cells at a density of 1x10⁵ cells per well and treatments were added parallel to the six-well plates to assess cell proliferation. Proliferation was
determined at culture termination using Aqueous One Solution Cell Proliferation Assay (Promega). In preliminary experiments using higher doses of rhBMP15 (50 and 100ng/ml), we observed an unusual morphology in cultured granulosa cells from 3-5 mm and 6-8 mm follicles. Therefore, we documented morphology of treated cells as well as proliferation in response to rhBMP15 treatment.

Real Time PCR

RNA extracted from cultured granulosa cells from 3-5 mm and 6-8 mm follicles was subjected to reverse transcription for cDNA. Quantitative real time PCR (qPCR) was used to assess AMH and FSH receptor (FSHR) mRNA expression. Primers were designed as stated above. The program defined a product of 71 bp for AMH (GenBank accession number U61754) that spanned an intron (forward: 5’CCCCTCTGTCCCTCATGGA3’; reverse: 5’CGTCATCCTGGAACACTTC3’; probe: 6FAMAGCTCCTCTTTGGCTCAMGBNFQ). For the FSHR, (GenBank U51097) a product of 70 bp (which also spanned an intron) was defined (forward: 5’GCACCTTCCAAGCCTCAGATAT3’; reverse: 5’CCCTATGGACGACGGTAAA3’; probe: 6FAMTGTATATATCAAACACAGGCTMGBNFQ). Samples were analyzed as described above with the same volumes. Similar controls were also run.

Theca Cell Culture

The theca cell layer was isolated from 6-8 mm follicles. For each repetition (n=4 replicates), the theca shells from one hen were pooled and cut up into small pieces. Theca pieces were weighed and approximately
equal weights (18-23 mg) were allocated to wells in a 24-well plate with 0.5 ml of media plus 0.1% BSA and rhBMP15 (at doses of 0, 0.1, 1, 10, and 50 ng/ml) was added. After 24 hours of incubation at 37°C in a humidified atmosphere of 5% CO2, the medium was collected and stored at -80°C for later use in estradiol assay.

Radioimmunoassay

Media samples were assayed to determine granulosa cell progesterone production or theca cell estradiol production using assays previously validated in our lab (Correa et al., 2005). Steroid hormone concentrations were estimated using Coat-A-Count radioimmunoassay kits for estradiol and progesterone (Diagnostics Product Corporation, Los Angeles, CA). The analytical sensitivity of the kits is 8.0 pg/ml for estradiol and 0.02 ng/ml for progesterone. Granulosa culture media was diluted 1:10 prior to assay. For both assays, plasma from immature roosters was used as a low pool and plasma from ovulating hens treated with exogenous progesterone was used as a high pool. Standards provided in the kit were assayed in triplicate and all culture media samples were assayed in duplicate. The coefficient of variation (CV) for the progesterone assay was 6.8% and the mean CV for the estradiol assays was 4.4%.

Statistics

Statistics were performed with SAS using Proc GLM with protected least significant difference. P values of <0.05 were considered significant, and means were compared using Duncan’s multiple range test.
3.3 RESULTS

Quantitative PCR was used to evaluate BMP15 mRNA expression in the granulosa layer in relation to follicle size. There was a negative relationship between follicle size and BMP15 mRNA expression. With increasing follicle size, granulosa cell BMP15 expression is significantly decreased (Figure 3.1).

![Graph showing BMP15 mRNA expression in the granulosa layer](image)

**Figure 3.1 BMP15 mRNA expression in the granulosa layer**

BMP15 expression is significantly decreased in the granulosa layer of follicles 9-16 mm and F1 (n=4-5, p<0.05).

Western blot analysis was performed on lysates which were made from medium of cultured whole oocytes (OCM), the ovary body (WO), 1 mm follicles, and the granulosa layer from 3-5 mm follicles (Figure 3.2). Several bands were repeatedly observed at ~16-17, 34, and 50 kDa. The band at approximately 50 kDa is believed to represent a precursor form (Liao et al., 2003; Guéripel et al., 2006), while the 16-17 kDa form is thought to represent the mature peptide (Liao et al., 2003; McNatty et al., 2005). It is hypothesized that the 34 kDa form represents a homodimer.
**Figure 3.2 Western blot protein expression of BMP15**
Protein bands seen at the expected sizes of ~16, 17, 34, and 50 kDa, in oocyte conditioned media (OCM), ovary body (WO), 1 mm follicles and granulosa cells of 3-5 mm follicles.

Positive BMP15 immunostaining (green) was demonstrated in the granulosa and theca layers of a 3-5 mm follicle (**Figure 3.3 A**). There is no specific staining in the negative control, where rabbit IgG is substituted for the primary antibody (**Figure 3.3 B**). PI staining demonstrated nuclei (red) in the follicular section (**Figure 3.3 C**) while co-localization of BMP15-Ab and PI is depicted in yellow (**Figure 3.3 D**).

The morphology of cultured granulosa cells from 3-5 mm (**Figure 3.4**) and 6-8 mm (data not shown) follicles was altered by treatment with 10 and 50 ng/ml rhBMP15. In contrast the morphology of cultured granulosa cells from F1 follicles was unchanged at these doses (**Figure 3.4**).
Figure 3.3 Immunohistochemistry of BMP15

A. Section stained with BMP15 antibody showing primarily nuclear staining in granulosa and theca cells of follicle. B. Negative control with rat IgG used instead of primary antibody. There is some autofluorescence due to red blood cells and edge effect. C. Negative control stained with PI. D. Section stained with BMP15 antibody as well as PI, to indicate nuclei.

Proliferation of granulosa cells collected from 3-5mm and 6-8mm follicles was significantly decreased by BMP15 at doses of 10ng/ml and 50ng/ml (P<0.05) (Figure 3.5 A) and in 6-8mm (P<0.05) (Figure 3.5 B). There was no significant change in F1 granulosa proliferation (Figure 3.5 C).
Figure 3.4 Comparison of granulosa cells cultured with various doses of BMP15
Representative photographs (magnification 20x) of granulosa cells from 3-5 mm and F1 follicles treated with BMP15 doses of 0, 10, and 50 ng/ml.
Figure 3.5 Proliferation of granulosa cells
Data is represented as absorbance relative to control treatment (n = 4). A. Relative absorbance of granulosa cells from 3-5mm follicle (p<0.05) B. 6-8mm (p<0.05) C. F1follicle.
FSHR mRNA expression was significantly increased in granulosa cells from 3-5 mm and 6-8 mm follicles treated with 10 and 50 ng/ml rhBMP15 (Figure 3.6 and 3.7; p<0.05). rhBMP15 treatment did not significantly affect the expression of AMH mRNA expression (data not shown).

Progesterone production by granulosa cells of F1 follicles was significantly increased by 10 and 50 ng/ml of rhBMP15 (Figure 3.8; p<0.05). Estradiol production by the theca layer in follicles of either size was not significantly altered by BMP15 treatment (data not shown).

Figure 3.6 FSHR mRNA expression in response to BMP15 treatment in granulosa cells from 3-5 mm follicles
FSHR expression is significantly increased by treatment with 10ng/ml and 50ng/ml BMP15 (n=5, p<0.05).
Figure 3.7 FSHR mRNA expression in response to BMP15 treatment in granulosa cells from 6-8 mm follicles
FSHR expression is significantly increased by treatment with 50ng/ml BMP15 (n=4, p<0.05).

Figure 3.8 Progesterone production by F1 granulosa cells after BMP15 treatment
Progesterone production is significantly decreased by treatment with 1, 10, and 50 ng/ml rhBMP15 (n=4, p<0.05).
2.4 DISCUSSION

In this study we showed that BMP15 is expressed in the granulosa cells of the hen. To our knowledge, no such expression has been shown before. In mammalian species, expression has been shown to be solely located in the oocyte. The hen oocyte is set up differently as compared to the mammalian oocyte; it includes the GDR, but the bulk of the oocyte is comprised of the yolk.

Our expression data showed that BMP15 expression in granulosa cells decreases as follicle size increases. This decrease in granulosa cell expression was also shown in larger follicles (Elis et al., 2007). Our data suggest that the relative amount of mRNA for BMP15 is at least 15-20 fold higher in granulosa cells from 6-8 mm follicles as compared to granulosa cells from the F1 follicle (Figure 3.1). The decrease in expression as follicle size increases suggests that BMP15 is involved in early folliculogenesis, and may regulate follicular activation but is less important in the later stages. It is unlikely BMP15 plays an important role in late follicular development, ovulation, or early embryogenesis, as levels are so diminished in the larger follicles. Additionally, our immunohistochemistry results support the mRNA data and indicate that BMP15 is expressed in the granulosa and theca layers.

BMP15 was previously shown by in situ hybridization to be expressed in the oocyte of the hen (Elis et al., 2007). We have no explanation for the difference in expression between our study and the previous study. We would note that the techniques used were different: qPCR, IHC verses in situ hybridization. In addition, our lab has
previously demonstrated, by qPCR and IHC, oocyte localization of GDF9 with minimal expression in the granulosa layer (Johnson et al., 2005).

As previously reported (Elis et al., 2007), we saw no change in proliferation of granulosa cells treated with BMP15 in the F1 follicle. However, in granulosa cells collected from 3-5mm and 6-8mm follicles, proliferation was significantly inhibited by the 10 and 50ng/ml treatments. In addition, the appearance of the cells from smaller follicles was altered in contrast to those from the F1. This suggests that granulosa cells from large preovulatory follicles are less sensitive to BMP15.

BMP15 treatment had a significant stimulatory effect on FSHR at the two higher doses (10, 50ng/ml) in the 3-5 mm granulosa cells and at 50 ng/ml in 6-8 mm granulosa. FSHR expression is low in preovulatory follicles, perhaps due to lack of stimulation by BMP15, as BMP15 expression is greatly diminished in preovulatory follicles. Interestingly, there was no effect on AMH expression which in mammals has been shown to be inversely related to FSHR mRNA expression (Visser and Themmen, 2005). The relationship between AMH and FSHR expression is still not well understood. FSHR expression may be influenced by other factors that do not alter AMH expression.

Treatment with BMP15 significantly decreased progesterone production by granulosa cells. This is similar to results seen in rats; BMP15 treatment decreased FSH stimulated progesterone production (Otsuka et al., 2000). The decrease in BMP15 expression as follicles mature, may progressively remove the inhibiting effect BMP15 has on progesterone production. Progesterone production has been shown to be greatest in the preovulatory follicles, especially the F1. Progesterone is
involved in a positive feedback loop with LH, which is necessary to induce ovulation (Johnson and van Tienhoven, 1980). The removal of BMP15 inhibition may allow progesterone production to increase and to induce the positive feedback loop. BMP15 treatment did not affect estradiol production by theca cells, which is similar to the results seen in mammals (Otsuka et al., 2000).

In conclusion, BMP15 may play an important role in folliculogenesis in the hen, specifically through regulation of FSHR mRNA expression and progesterone production. Future studies will further investigate the role of BMP15 in the ovary by examining its effects on other factors involved in the communication between the oocyte and granulosa cells.
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CHAPTER 4
SUMMARY AND CONCLUSIONS

Our goal in completing the previously described studies was to gain further insight into the regulation of folliculogenesis and the function and regulation of factors thought to be involved in this process in the hen. We focused on examining possible regulators of anti-Müllerian hormone (AMH), a hormone known to be crucially involved in the inhibition of activation of follicle activation and selection (Behringer et al., 1994; Durlinger et al., 1999). We examined the roles of calcitriol and BMP15 in follicular maturation, as well as their effect on the expression of AMH by granulosa cells in the hen.

The first study focused on the function of calcitriol in the ovary. Calcitriol has been shown to play a crucial role in the regulation of folliculogenesis in mammals, demonstrated by VDR knock-out studies in mice (Yoshizawa et al., 1997; Kinuta et al., 2000). However, the role of calcitriol in the ovary of the hen is not well studied. We found that when granulosa cells from follicles 3-5 mm and 6-8 mm in diameter were treated with calcitriol, AMH mRNA expression was significantly inhibited. In addition, we found that follicle stimulating hormone receptor (FSHR) mRNA expression in granulosa cells, from both follicle sizes, was significantly increased. This inhibition of AMH mRNA expression occurred at a lower dose than does the increase in FSHR expression leading us to believe that calcitriol inhibits AMH expression, which allows FSHR expression to increase.
Through western blot and immunohistochemistry, we demonstrated VDR expression in granulosa cells, suggesting calcitriol has a functional role in the ovary. We also found that as follicle size increases, vitamin D receptor (VDR) mRNA expression increases. Our lab previously found that AMH expression decreases as follicle size increases (Johnson et al., 2008). It appears that as follicles mature there is an increase in VDR which could promote a more effective decrease of AMH expression by calcitriol. We showed that calcitriol treatments also result in an increase in FSHR mRNA expression. This is not unexected, as there is a proposed inverse pattern of AMH and FSHR expression in mammals (Visser and Themmen, 2005). However, we were unable to determine if calcitriol was directly affecting AMH expression as we were unsuccessful in verifying a VDRE in the AMH promoter. In response to the increasing VDR expression in larger follicles, we also attempted to evaluate the possible role calcitriol may have in calcium uptake by the yolk. We did so by investigating calbindin mRNA expression in the granulosa cells. Calbindin mRNA levels were too low to detect.

In the second study, we evaluated the possible role of BMP15 in folliculogenesis in prehierarchal follicles as well as in the regulation of AMH. We localized expression of BMP15 to the granulosa and theca layers in the hen; to our knowledge no such expression has been shown before. BMP15 mRNA expression in the granulosa cells decreased as follicle size increased, suggesting a greater role in early folliculogenesis as compared to later stages of follicular growth. With BMP15 treatment, proliferation of granulosa cells was unaffected in the F1 follicle as previously reported (Elis et al., 2007); however in granulosa cells from 3-
5 mm and 6-8 mm follicles, proliferation was significantly decreased by the two highest doses of BMP15. Morphology was altered but viability was maintained, suggesting that granulosa cells from prehierarchal follicles are more sensitive to BMP15 treatment. Treatment with BMP15 significantly decreased progesterone production by granulosa cells of the F1 follicle. Since progesterone production is significantly greater in the F1 follicle, perhaps the removal of inhibition by BMP15 is needed before follicles can increase progesterone production.

Treatment of granulosa cells, from 3-5 mm and 6-8 mm follicles, with BMP15, resulted in a significant increase in the expression of FSHR mRNA. FSHR mRNA expression by granulosa cells is greatest in follicles 6-12 mm in diameter (Woods and Johnson, 2005). Our study suggests that BMP15 stimulates FSHR expression in small follicles, at a critical time when follicle selection occurs. When BMP15 mRNA expression drops past a certain threshold, it is no longer able to stimulate FSHR expression.

Table 4.1: Functions of Calcitriol and BMP15 in the ovary of the hen. X represents effects not investigated and --- represents no effect.

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<tr>
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<th>Calcitriol</th>
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<td>FSHR</td>
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<td>P4</td>
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Illustration 4.1 Schematic of mRNA expression of factors by the granulosa cells in the ovary of the hen.

In summary, we were able to gain better insight into the process of folliculogenesis in the hen, an overview of findings can be found in Table 4.1 and Illustration 4.1. Additional investigation into the roles of calcitriol and BMP15 in the regulation of folliculogenesis and their interactions with other ovarian factors is necessary.
REFERENCES


