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Integral Role of GDF-9 and BMP-15 in Ovarian Function

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Abstract

The oocyte plays an important role in regulating and promoting follicle growth, and thereby its own development, by the production of oocyte growth factors that predominantly act on supporting granulosa cells via paracrine signaling. Genetic studies in mice demonstrated critical roles of two key oocyte-derived growth factors belonging to the transforming growth factor-β (TGF-β) superfamily, growth and differentiation factor-9 (GDF-9) and bone morphogenetic protein-15 (BMP-15) in ovarian function. The identification of *Bmp15* and *Gdf9* gene mutations as the causal mechanism underlying the highly prolific or infertile nature of several sheep strains in a dosage-sensitive manner also highlighted the crucial role these two genes play in ovarian function. Similarly, large numbers of mutations in the *GDF9* and *BMP15* genes have been identified in women with premature ovarian failure and in mothers of dizygotic twins. The purpose of this article is to review the genetic studies of GDF-9 and BMP-15 mutations identified in women and sheep as well as describing the various knockout and over-expressing mouse models, and to summarize the molecular and biological functions that underlie the crucial role of these two oocyte factors in female fertility.

Keywords

BMP; GDF; oocyte; ovary; female reproduction

2. Introduction

There is a large amount of evidence supporting the concept that the oocyte plays a central role in regulating follicle growth and development (Gilchrist et al. 2008; Matzuk et al. 2002; Su et al. 2009). Pioneer work by Falck and colleagues demonstrated in 1959 that intact rabbit preovulatory follicles do not luteinize when transplanted into the anterior chamber of the eye, in contrast to oocyte-free explants of either the follicle wall or granulosa cells that do undergo morphological luteinization (Falck 1959). Therefore, the oocyte acted to inhibit luteinization of the other follicular cells. Subsequently, Nalbandov and colleagues confirmed this work using rabbit dominant follicles *in situ*, showing that removal of the oocyte caused spontaneous luteinization of granulosa and theca cells and elevated secretion of progesterone to levels produced by corpora lutea (El-Fouly et al. 1970; Nalbandov 1970). These studies were integral in establishing that the oocyte is involved in governing follicle luteinization, possibly by secretion of the putative luteinizing inhibitor(s) that has been of persistent interest in the field.

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Decades later, it has been established that ovary-derived transforming growth factor-β (TGF-β) superfamily members play an integral role in inhibiting granulosa cell progesterone production. Initial work showed that two theca cell-derived bone morphogenetic proteins (*e.g.,* BMP-4 and BMP-7) inhibit FSH-dependent progesterone production while stimulating FSH-dependent estradiol production in rat granulosa cells *in vitro* (Shimasaki et al. 1999). This finding is consistent with *in vivo* steroidogenesis during the follicular phase of the estrus/menstrual cycle. Subsequent studies have demonstrated that other members of the TGF-β superfamily (*e.g.,* oocyte-derived BMP-15 and BMP-6) also inhibit FSH-stimulated progesterone production without changing estradiol production in rat granulosa cells (Otsuka et al. 2001b; Otsuka et al. 2000). Therefore, it appears that the long-sought oocyte-derived luteinization inhibitors are most likely BMP family members (Shimasaki et al. 2004).

Following the identification of the BMP system in the ovary, remarkable progress has been made in understanding at the molecular level how oocyte factors regulate the function of granulosa cells during follicle growth and ovulation (Gilchrist et al. 2008; Matzuk et al. 2002; Su et al. 2009). Among oocyte-derived BMP family members, the biological and physiological activities of growth and differentiation factor-9 (GDF-9) and BMP-15 have been most extensively researched as genetic studies revealed essential roles for both factors in female fertility in several mammalian species (Gilchrist et al. 2008; Juengel et al. 2004a; McNatty et al. 2005d; Moore et al. 2004; Su et al. 2009). In this review, we focus on the role of the two most homologous oocyte-factors, GDF-9 and BMP-15, in ovarian function.

3. Genetic studies of mutations in the *Gdf9* gene

3.1 *Gdf9* knockout mice

Generation of a *Gdf9* null mouse model by the Matzuk lab provided a new avenue for studying the role of GDF-9 in ovarian function (Dong et al. 1996). Homozygous *Gdf9* null mice were grossly indistinguishable from heterozygous or wild type mice. A complete loss of GDF-9 in homozygous males showed that GDF-9 is not required for male fertility. This is in contrast to homozygous null females that were infertile, while heterozygous *Gdf9* null female mice were phenotypically normal (Dong et al. 1996). Ovaries from adult female homozygous mice were significantly smaller than wild type ovaries and exhibited a complete absence of normal follicles beyond the primary stage. The block in follicle growth appeared to be attributed to the loss of granulosa cell mitotic ability at the end of the primary stage.

Differentiation of all follicle compartments, including the oocyte, granulosa and theca cells, is influenced by GDF-9 (Carabatsos et al. 1998; Dong et al. 1996; Elvin et al. 1999b). GDF-9-deficient oocytes grow more rapidly than control oocytes and attain a larger size despite the halt in follicle growth at the primary stage (Carabatsos et al. 1998). While GDF-9-deficient oocytes displayed relatively normal meiotic competence for early stage follicles, there were some ultrastructural meiotic defects including an absence of cortical granules and clustering of organelles around the germinal vesicle. This suggests that late secretory events in oogenesis are affected by the absence of GDF-9. In the immature primary follicles with degeneratedoocytes, granulosa cells showed characteristics associated with dominant preovulatory follicles (Elvin et al. 1999b). Of note, *Cyp19a1* expression that is normally most upregulated in preovulatory follicles was 50% of wild-type levels in *Gdf9* null ovaries despite the lack of follicles past the primary stage (Dong et al. 1996), suggesting that GDF-9 may normally act to inhibit aromatase expression. In addition, theca cell recruitment was impaired in *Gdf9* null ovaries, as determined by the lack of CYP17, LH receptor (LHCGR) and c-kit expression in theca cells (Elvin et al. 1999b). Therefore, the

oocyte-derived GDF-9 appears to be essential for normal oocyte and granulosa cell function as well as theca cell formation.

Further evidence for the important paracrine role of GDF-9 comes from the finding that in GDF-9 deficient mice granulosa cell expression of kit ligand (KL) and inhibin-α is upregulated (Elvin et al. 1999b). Generation of double-mutant mice lacking both GDF-9 and inhibin-α revealed that the block at the primary follicle stage in *Gdf9* null ovaries was overcome by co-deletion of the *Inha* gene. Thus, despite the absence of GDF-9, follicle growth proceeds normally until the type 5b (late preantral) follicle stage in *Inha*/*Gdf9* double-null ovary. Therefore, in mice it appears that one of the critical functions of GDF-9 that promotes early follicle growth is down regulation of inhibin-α.

3.2 Ewes with *Gdf9* mutations

Sheep have proved to be a valuable model to elucidate the importance of GDF-9 in female reproductive function. Normal ewes ovulate one or two oocytes per cycle (Montgomery et al. 1992). The Cambridge and Belclare sheep breeds were known to have highly prolific natures, although there was extreme variation in the fertility of both breeds with some ewes ovulating up to 3–6 oocytes per cycle, some ovulating a normal number of oocytes and other ewes were infertile. Analysis of the inheritance patterns of the ovarian hypoplasia present in the infertile ewes in these strains suggested an autosomal gene was likely to be involved. Although ewes from a different breed (called Inverdale) that have a similar phenotype were previously identified to carry a mutation in the *Bmp15* gene (addressed in Section 4.2) histological analysis of the hypoplastic ovaries from Cambridge and Belclare ewes revealed different abnormalities to those associated with the Inverdale ewes, such as the presence of follicles with an antrum and oocytes with thickened zona pellucidae (ZP) surrounded by abnormally dispersed layers of cells (Hanrahan et al. 2004).

Therefore, since GDF-9 is most homologous to BMP-15 in primary structure as well as spatio-temporal expression in the ovary, a potential involvement of this gene in the peculiar phenotypes observed in Cambridge and Belclare ewes was investigated. Consequently, genetic studies uncovered eight single nucleotide polymorphisms across the coding region of the *Gdf9* gene (Galloway et al. 2000; Hanrahan et al. 2004), of these one mutation, GDF-9^{S77F}, was directly associated with the sterility phenotype in both the Cambridge and Belclare breeds (Table 1). This mutation from Ser to Phe replaces an uncharged polar group with a non-polar group, and is considered to affect the binding to BMP type I receptors. Interestingly, ewes homozygous for the GDF-9^{S77F} mutation are infertile, while heterozygous careers have an increased ovulation rate associated with a larger litter size than normal. In addition to the *Gdf9* polymorphisms, four *Bmp15* polymorphisms were also identified in Cambridge and Belclare sheep, two of which were linked to the infertility phenotype in homozygotes or double heterozygotes. Double heterozygotes (GDF-9^{S77F}/BMP-15^{S99I} or GDF-9^{S77F}/BMP-$15^{Q-29Ter}$) exhibit an additive effect on ovulation rate, thus the biological effects of BMP-15 and GDF-9 are likely to be distinct (Table 1).

More recently, another point mutation in the *Gdf9* gene resulting in a non-conservative amino acid change (GDF-9^{S109R}) in the C-terminus of the mature GDF-9 protein was identified in Icelandic Thoka sheep (Nicol et al. 2009). Similar to the GDF-9^{S77F} mutation, this mutation was also associated with increased fecundity in heterozygous ewes and infertility in homozygotes. There is a complete absence of follicle development in GDF-9^{S109R} homozygotes, despite normal activation of oocytes and expression of a number of oocyte-specific genes involved in ZP formation (Nicol et al. 2009). These studies provided the evidence that GDF-9 is a critical player in ovine follicle growth.

3.3 Women with *GDF9* mutations

The identification of variant GDF-9 sequences in non-syndromic premature ovarian failure (POF) patients (Dixit et al. 2006; Kovanci et al. 2007; Laissue et al. 2006; Zhao et al. 2007) suggests that altered GDF-9 function may also be involved in ovarian dysfunction in women. Further, GDF-9 missense and nonsense mutations have also been detected in mothers of dizygotic twins, suggesting that some variants may also be linked to a polyovulatory phenotype (Montgomery et al. 2004; Palmer et al. 2006). Three missense mutations, GDF-9^{P103S}, GDF-9^{P374L} and GDF-9^{R454C}, occur in conserved amino acid positions and are significantly related to an increased ovulation rate in women (Palmer et al. 2006). Intriguingly, the GDF-9^{P103S} mutation is detected both in mothers of dizygotic twins and in women with POF. Therefore, GDF-9 is likely to alter the ovulation rate in women as well as in ewes.

Interestingly, many of these mutations were found in the proregion of the GDF-9 proprotein. In this regard, one of the important features in the posttranslational processing of the TGF-β superfamily members is that the proregion is necessary for dimerization of the mature protein (Shimasaki et al. 2004). As such, proregion mutations may influence dimerization of the proprotein, and thereby negatively impact the production of functional mature protein dimers. Therefore, GDF-9 proregion mutations identified in POF women and mothers of dizygotic twins may cause impaired processing of the proproteins by forming mis-folded proprotein dimers.

It is notable that ovaries from 25- to 31-week-old female *Gdf9* null mice contained either single unilateral or bilateral ovarian follicular cysts lined by several layers of flattened granulosa cells (Dong et al. 1996). This cystic ovarian phenotype bears some resemblance to the histological characteristics seen in polycystic ovary syndrome (PCOS) in women. PCOS is one of the most common causes of anovulation, infertility, and menstrual irregularities in women, affecting between 5 to 10% of women of reproductive age (Knochenhauer et al. 1998). Analysis of human ovary tissues from women with PCOS revealed that GDF-9 mRNA expression is substantially delayed and reduced during the growth and differentiation phase (Teixeira Filho et al. 2002). In contrast, PCOS/PCO had no detectable effect on the expression pattern of BMP-15 (Teixeira Filho et al. 2002). The disparity between alterations in these two critical oocyte factors, with changes in GDF-9 but not BMP-15 expression, may be due to the specific role of GDF-9 in maintaining follicle structure, although there are controversial data showing no significant change in GDF-9 or BMP-15 protein levels associated with PCOS (Zhao et al.). Further studies are needed to determine whether changes in BMP-15/GDF-9 can directly trigger follicular arrest in PCOS/PCO women or whether altered expression is secondary to ovarian dysfunction.

4. Biological functions of GDF-9 in regulating folliculogenesis

In vitro studies using recombinant GDF-9 protein have clarified the biological roles and importance of GDF-9 actions in follicle growth and development at all stages of folliculogenesis. There is compelling evidence in several mammalian species that GDF-9 is essential for early stages of follicle development. *Gdf9* null mice (Dong et al. 1996) and ewes either homozygous for naturally occurring mutations in the *Gdf9* gene (Hanrahan et al. 2004) or immunized against GDF-9 (Juengel et al. 2002) all exhibit a block in follicle growth at the primary stage. Further, GDF-9 also elicited a reduction in primordial follicle number *in vivo* (Vitt et al. 2000b), and *in vitro* exposure of rodent (Hayashi et al. 1999; Nilsson and Skinner 2002) or human (Hreinsson et al. 2002) ovarian tissue to GDF-9 promotes primary follicle progression.

At the preantral stage, GDF-9 has been shown to be effective in stimulating growth of *in vitro* cultured rat preantral follicles (Hayashi et al. 1999). GDF-9 also promotes early preantral follicle growth in human ovaries (Hreinsson et al. 2002). In the transition to the antral stage, it appears that GDF-9 promotes follicular survival by suppressing granulosa cell apoptosis and follicular atresia (Orisaka et al. 2006). This may be achieved in part by GDF-9 stimulation of follicular FSH receptor (FSHR) expression, as adequate FSHR levels in granulosa cells is essential for FSH-dependent antral follicle growth.

Subsequently, in rat granulosa cells collected from early antral and preovulatory follicles GDF-9 promotes granulosa cell proliferation while inhibiting FSH-induced steroidogenesis and LHCGR expression (Vitt et al. 2000a). The GDF-9 inhibition of FSH action was postulated to occur via modulation of FSHR level or FSHR coupling level to Gs protein (Vitt et al. 2000a). However, findings in rats that GDF-9 stimulates FSHR expression in preantral follicles (Orisaka et al. 2006) and in cultured granulosa cells from early antral follicles (our unpublished findings) suggest that GDF-9 inhibition of FSH-actions acts via modification of post-receptor signaling.

GDF-9 also plays an important role during the final stages of follicle growth prior to ovulation. Prior to the LH surge, cumulus cells require GDF-9 to support the metabolic cascades such as glycolysis and sterol biosynthesis (Sugiura et al. 2005). Evidence for this comes from findings of reduced cholesterol synthesis from acetate in cumulus/oocyte complexes of *Bmp15* null mice, and to a greater extent, of *Bmp15*$^{-/-}$*Gdf9*$^{+/-}$double mutant mice. As mouse oocytes are deficient in cholesterol synthesis they depend on cumulus cells to provide newly synthesized cholesterol. Importantly, oocyte-derived GDF-9 seems to act to promote cholesterol biosynthesis in cumulus cells thereby ensuring the oocyte receives an adequate supply of essential metabolic precursors (Su et al. 2008).

In addition to regulation of cumulus cell metabolism, GDF-9 regulates diverse processes and gene expression during the preovulatory stage. In mouse preovulatory follicles, GDF-9 inhibits FSH-induced steroidogenesis while promoting cumulus cell progesterone production by stimulating the expression of an intrinsic prostaglandin-E2/EP2 receptor signaling pathway (Elvin et al. 2000). GDF-9 also enhances cumulus cell expansion in the presence of FSH (Elvin et al. 1999a), but not without FSH (Dragovic et al. 2005), which may relate to the GDF-9 enhancement of hyaluronan synthase 2 (HAS2) and cyclooxygenase 2 (PTGS2) mRNAs (Elvin et al. 1999a). Addition of the extracellular domain of the type II BMP receptor (BMP-RII) to granulosa cell cultures suppressed GDF-9-induced cumulus cell expansion but only partially neutralized oocyte-induced expansion (Dragovic et al. 2005). This suggests that GDF-9 is not the sole oocyte-derived cumulus expansion-enabling factor, and that other factors are also involved in cumulus expansion. Thus, GDF-9 plays an important role in regulating several aspects of granulosa cell function during the preovulatory stage of follicle development.

Oocyte-secreted factors possess anti-apoptotic actions. In a study using bovine cumulus-oocyte complexes (Hussein et al. 2005), oocyte removal significantly increased cumulus cell apoptosis compared with intact cumulus-oocyte complexes. Increased apoptosis in oocytectomized cumulus cells could be reversed in a dose-dependent manner by co-culture with denuded oocytes. In the study, GDF-9 had no significant effect on cumulus cell apoptosis whereas two other oocyte-secreted factors (*i.e.*, BMP-15 and BMP-6) reduced cumulus cell apoptosis (Hussein et al. 2005). However, in a study using rat preantral follicles, GDF-9 was shown to exert anti-apoptotic effects in preantral follicles and protects granulosa cells from undergoing apoptosis via activation of the phosphatidylinositol 3-kinase/Akt pathway (Orisaka et al. 2006). Differences between the anti-apoptotic actions of GDF-9 in these two models may suggest that there are differences in GDF-9 anti-apoptotic

activity between species or may suggest that GDF-9 has anti-apoptotic actions during early but not late stages of follicle development.

As primary human granulosa cells are difficult to obtain in large quantities, most studies have utilized luteinized human granulosa cells to examine GDF-9 actions in the human ovary. In luteinized human granulosa cells, GDF-9 has no direct effects on basal or FSH-induced progesterone production, which is inconsistent with the effect in rodent granulosa cells. Rather, GDF-9 reverses activin A suppression of FSH-induced progesterone secretion (Shi et al. 2010), while enhancing activin A-induced inhibin B secretion (Shi et al. 2009b). GDF-9 has been detected in luteinized human granulosa cells in addition to oocytes, and appears to be involved in an autocrine-enhancement of activin A-induced inhibin B secretion from the luteinized granulosa cells (Shi et al. 2009a). Thus in humans, it appears that increasing GDF-9 expression during folliculogenesis enhances granulosa cell response to activin A, leading to an upregulation of inhibin B levels.

GDF-9 also plays a role during *in vitro* maturation of oocytes and subsequent fetal viability (Yeo et al. 2008). The addition of exogenous GDF-9 to mouse cumulus-oocyte complexes cultured with FSH prior to *in vitro* fertilization and transfer to recipient females had no effect on implantation rate or fetal and placental weights but increased the number of viable fetuses. This suggests GDF-9 supports embryo development and fetal viability.

GDF-9 has been reported to regulate granulosa cell mitosis through both Smad-dependent and independent signaling pathways (Huang et al. 2009). In human luteinized granulosa cells, GDF-9 upregulated cyclin D1 and E mRNA and protein expression and phosphorylation of cyclin D1 and Rb protein via ERK1/2 activation, and suppressed the negative cell cycle regulators $p15^{INK4B}$ and $p16^{INK4A}$ via Smad3 activation. Consistent with the general consensus that GDF-9 signals through the type I receptor (ALK-5), SB431542 (an ALK-4/5/7 inhibitor) was able to block the mitotic activity of GDF-9 (Huang et al. 2009).

GDF-9 also plays a key role in controlling theca cell functions. GDF-9 effects on theca cells were initially uncovered by an in vivo study, in which intra-peritoneal injections of GDF-9 into immature rats promoted the progression of primordial and primary follicles to small preantral follicles and caused an increase in the expression of the theca cell-specific marker CYP17 (Vitt et al. 2000b). GDF-9 stimulates testosterone production in cultured rat preantral follicles. Taken together with the finding that the androgen receptor antagonist flutamide blocks GDF-9-induced preantral growth, it is suggested that GDF-9 actions in theca cells as well as granulosa cells are important for promoting preantral follicle growth to the early antral stage (Orisaka et al. 2009). GDF-9 actions in theca cells were also reported in bovine follicles (Spicer et al. 2008). GDF-9 stimulates the proliferation of theca cells derived from small follicles, inhibiting theca cell IGF-1- and LH-induced progesterone and androstenedione production. Hence, GDF-9 may play a role in increasing proliferation but suppressing differentiation of theca cells from small follicles in bovine ovary.

GDF-9 actions in theca cells to regulate androgen production subsequently affect granulosa cells at a number of levels, with androgen acting as a substrate to facilitate granulosa cell estrogen production from androgen aromatization and also via direct effects of androgens on granulosa cell growth. Direct effects of androgens are mediated by the androgen receptor that is expressed in granulosa cells. Granulosa cells also have signaling pathways for FSH and IGF-1, which are functionally linked to each other and also appear to be modulated by androgens. In porcine granulosa cells, GDF-9 potently enhances IGF-1-stimulated proliferation and inhibits FSH-stimulated progesterone secretion (Hickey et al. 2005). Androgens dose-dependently enhance the mitotic activity of oocytes or GDF-9, and this

enhancement is opposed by co-treatment with anti-androgen drugs. Therefore in the pig, androgens interact with GDF-9 to amplify granulosa proliferation at the early antral stage.

As GDF-9 and BMP-15 are both present in follicles throughout most stages of follicular growth, it is important to consider whether these growth factors are synergistic or redundant in processes where they have similar activity alone. GDF-9 can act co-operatively with BMP-15, with some studies in granulosa cells showing the effects of a combined GDF-9/BMP-15 treatment are different from either growth factor alone (McNatty et al. 2005b). In addition, GDF-9 and BMP-15 have species-dependent effects. In ovine granulosa cells, ovine BMP-15 given together with mouse GDF-9 or ovine GDF-9 was more potent in stimulating ovine granulosa cells mitosis compared with each growth factor independently, while in bovine granulosa cells there is little or no co-operative action between ovine BMP-15 and ovine GDF-9 in terms of mitotic activity (McNatty et al. 2005c). The species of origin of GDF-9 also affected the progesterone response as well as inhibin production by ovine granulosa cells. Therefore, the effects of GDF-9 and BMP-15 when combined can be co-operative compared with each growth factor alone; however, the response of granulosa cells from different species to GDF-9 from a particular species can vary in addition to variation in response of granulosa cells to different sources of GDF-9.

The use of recombinant GDF-9 in *in vitro* assays is critical for determining the biological actions of GDF-9 in the ovary. However, it appears that different preparations of recombinant GDF-9 protein may have different activities. Specifically, the inclusion of a C-terminal affinity-purification tag may affect GDF-9 bioactivity (Mottershead et al. 2008), although *in vitro* GDF-9 studies to date have mostly utilized N-terminal tag or non-tag preparations. Moreover, whether FSH is required for the activity of GDF-9 in cumulus cell expansion remains to be determined as discussed previously (Pangas and Matzuk 2005). Future studies should ideally adopt the use of a standard method of production of recombinant GDF-9

5. Genetic studies of mutations in the *Bmp15* gene

5.1 *Bmp15* knockout and *Bmp15* transgenic mice

Knocking-out the *Bmp15* gene unexpectedly revealed minimal alterations in follicle development and fertility in mice (Yan et al. 2001). Heterozygous and null males and females were all viable and developed normally. *Bmp15* null males were fertile with normal testis size, whereas *Bmp15* homozygous mutant females were subfertile with reduced litter size compared with heterozygous and wild type females. *Bmp15* null ovaries typically contained follicles at all stages of development and multiple corpora lutea. However, on occasion *Bmp15* null ovaries contained very few follicles and increased ZP remnants indicating increased follicle atresia. Further, in response to ovulation stimuli *Bmp15* null females ovulated less oocytes, with some oocytes remaining trapped in follicles. Also these mice have defects in early embryonic development, resulting in subfertility. Overall, in mice folliculogenesis is largely unaffected by the loss of BMP-15 and the defects are confined to the ovulation process and subsequent fertilization.

GDF-9 expression was not altered in *Bmp15* null ovaries, therefore compensation by GDF-9 most likely does not explain the less severe phenotype of the Bmp15 knockout mice, compared with *Gdf9* null mice. Generation of double *Gdf9* and *Bmp15* mutant mice ($Bmp15^{-/-}/Gdf9^{+/-}$) facilitated analysis of biological interactions between the two molecules (Yan et al. 2001). Normal folliculogenesis and corpora lutea were occasionally observed in $Bmp15^{-/-}/Gdf9^{+/-}$ ovaries, similar to *Bmp15* null ovaries. However, several abnormalities including reduced numbers of late-stage follicles, increased oocyte loss and an accumulation of ZP remnants, and an absence of corpora lutea were observed. While early

phases of folliculogenesis were relatively normal and *Bmp15*$^{-/-}$/*Gdf9*$^{+/-}$ mice ovulated a high number of oocytes in response to exogenous hormones, the ability of the oocytes to be fertilized in vivo was dramatically reduced. This was also observed though less pronounced in *Bmp15*$^{-/-}$ mice. Similar to *Bmp15*$^{-/-}$ mice, cumulus cells failed to stably adhere to eggs isolated from the oviducts of *Bmp15*$^{-/-}$/*Gdf9*$^{+/-}$ mutant mice. Analysis of Bmp15$^{-/-}$ and *Bmp15*$^{-/-}$/*Gdf9*$^{+/-}$ovaries from mice stimulated with PMSG/hCG revealed a frequent absence of cumulus expansion and the existence of larger oocytes, suggesting that BMP-15 is functionally involved in the process of cumulus expansion and in the functional interaction between cumulus cells and oocytes. Ovaries of the *Bmp15*$^{-/-}$/*Gdf9*$^{-/-}$ mice at early time-points resembled *Gdf9*$^{-/-}$ ovaries with a block at the type 3B primary follicle stage, thus the block in follicle development in *Gdf9*$^{-/-}$ mice is not due to unopposed BMP-15 actions (Yan et al. 2001). At later ages, additional unique abnormalities were observed in *Bmp15*$^{-/-}$/*Gdf9*$^{-/-}$ mouse ovaries compared with *Gdf9*$^{-/-}$ ovaries, including enhanced oocyte loss, increased ZP remnants, and interstitial cell proliferation. Thus, BMP-15 appears to play synergistic but independent roles in oocyte survival with GDF-9.

A major defect detected in female mice lacking BMP-15 is during the ovulation process, which supports findings that functional mature BMP-15 protein level in mouse oocytes is very low until after the LH surge *in vivo* (Gueripel et al. 2006; Yoshino et al. 2006). In addition, the recombinant mouse BMP-15 proprotein was not readily processed into the functional mature protein in transfected HEK293 cells (Hashimoto et al. 2005), although in a different experimental setting the mature protein was detected (McIntosh et al. 2008). Interestingly, however, a chimeric protein consisting of the human proregion, human cleavage site, and mouse mature region (termed hhmBMP-15) could be processed leading to the secretion of mature protein (Hashimoto et al. 2005). On this basis, the role of BMP-15 in folliculogenesis was investigated by generation of transgenic (TG) mice overexpressing hhmBMP-15 exclusively in oocytes (McMahon et al. 2008a). BMP-15 TG mice carrying a flox-stopped hhm*Bmp15* transgene were generated using a Cre/loxP system. Female transgenic hhmBMP-15-flox mice were mated with males expressing Cre recombinase under control of the oocyte-specific ZP3-promoter. BMP-15 mature protein levels were five-fold higher in BMP-15 TG than wild ovaries, with BMP-15 protein detected exclusively in oocytes from the primary follicle stage throughout folliculogenesis (McMahon et al. 2008a).

Granulosa cells from immature BMP-15 TG mice displayed an increased mitotic potential and reduced FSHR mRNA expression. Primordial follicle formation and primordial to primary transition occurred normally in BMP-15 TG mice. However, a significant decrease in primary follicles with a concomitant increase in secondary follicles was observed, which may reflect elevated granulosa cell mitosis in primary follicles. Although secondary follicles are not dependent on FSH, they are sensitive to FSH, thus increased secondary follicle atresia in BMP-15 TG mice may be the result of suppressed FSHR signaling. Although comparable numbers of dominant follicles reach the preovulatory stage initially, the ovarian reserve is exhausted earlier in BMP-15 TG mice due to the accelerated follicle development and increased atresia, resulting in a premature onset of acyclicity. Therefore, BMP-15 TG mice confirmed *in vivo* important BMP-15 functions, including the ability to stimulate granulosa cell mitosis (Otsuka et al. 2000) and inhibit FSHR expression (Otsuka et al. 2001c). Further, the early onset of acyclicity in BMP-15 TG mice shows suppression of BMP-15 actions during early folliculogenesis is important for restraining follicle development to prevent premature loss of the follicle reserve.

Collectively these mouse models show that BMP-15 plays a key role during late stages of follicle development and following ovulation. Importantly, the biological importance of BMP-15 versus GDF-9 during folliculogenesis may differ between mice and other species,

and this may reflect inherent differences between mono- and poly-ovulatory mammals (Galloway et al. 2002; Yan et al. 2001).

5.2 Ewes with *Bmp15* mutations

Variation in ovulation rate in sheep due to genetic differences is well known. Inverdale and Hanna sheep, naturally occurring strains, provided a major breakthrough in the reproductive biology of BMP-15 (Galloway et al. 2000). The gene responsible for the ovulation variability in these sheep was known to be X-linked, then in 2000 the two causal independent point mutations were localized to the *Bmp15* gene, namely BMP-15^{V31D} and BMP-15^{E23Term} for Inverdale and Hanna strains, respectively. (Galloway et al. 2000). Homozygous carriers of the Inverdale and Hanna *Bmp15* mutations are infertile with streak ovaries showing folliculogenesis arrested at the primary stage, whereas heterozygous careers exhibit higher ovulation rate and larger litter size than controls (Galloway et al. 2000; Shimasaki et al. 2003).

The importance of BMP-15 in sheep fertility was confirmed with the identification of two additional *Bmp15* point mutations, BMP-15$^{Q-29Term}$ and BMP-15^{S99I}, in Cambridge and Belclare sheepthat resulted in the same phenotype as the Inverdale and Hanna ewes (Hanrahan et al. 2004) (Table 1). Subsequently, two more new BMP-15 mutants (BMP-15$^{17bp-del}$ and BMP-15^{C53Y}) have been identified in Rasa Aragonesa and Lacaune sheep, respectively (Bodin et al. 2007; Martinez-Royo et al. 2008; Monteagudo et al. 2009). Both Rasa Aragonesa and Lacaune sheep display a similar phenotype to other mutant ewes with increased ovulations in the heterozygotes and sterility in the homozygotes (Table 1). *In vitro* studies showed that the BMP-15^{C53Y} mutation impairs the maturation process of BMP-15 protein, resulting in defective secretion of both the mature and proprotein (Bodin et al. 2007). In addition, another sheep strain with increased ovulation rate, Woodlands sheep, was reported to bear a putative genetic mutation (FecX2^{W}) that is different from known *Bmp15* and *Gdf9* mutations (Feary et al. 2007). Ovaries of Woodlands ewes express reduced levels of BMP-15 in oocytes and ALK-6 in both oocytes and granulosa cells, while the expression patterns of GDF-9 and BMP-RII were not altered. Therefore, functional defects in BMP-15 may be involved in the increased ovulation rate in FecX2^{W} mutant ewes (Feary et al. 2007). The prevalence of BMP-15 mutations identified in sheep breeds with altered ovulation rates convincingly shows the important role this oocyte protein plays in reproductive function in sheep.

The infertile and superfertile phenotypes of the *Bmp15* mutant ewes were both reproducible by immunizing wild-type ewes against synthetic peptides derived from sheep BMP-15 mature protein (Juengel et al. 2002; Juengel et al. 2004b). Specifically, the use of a short term immunization protocol achieved an increase in ovulation rates (Juengel et al. 2002; Juengel et al. 2004b), while a longer term and higher dose protocol typically resulted in an anovulatory phenotype (Juengel et al. 2002; Juengel et al. 2004b). Further studies showed that antibodies generated against the N-terminal region of BMP-15 or GDF-9 were most effective at inhibiting the paracrine actions of these oocyte-secreted factors in vivo and in vitro, inducing an anovulatory phenotype (McNatty et al. 2007). Similarly in cattle, immunization against BMP-15 and/or GDF-9 altered follicle development (Juengel et al. 2009). However in terms of fertility the outcome varied, with some cattle immunized to BMP-15 peptide alone becoming anovulatory, whereas others immunized with BMP-15 or BMP-15 with GDF-9 peptides had increased ovulation rates (Juengel et al. 2009). Overall, BMP-15 appears to be important for promoting early stages of follicle growth while restraining the number of dominant preovulatory follicles, and is a key determination of ovulation quota and litter size in sheep and cattle (Shimasaki et al. 2004).

In vitro studies provided an interesting interpretation regarding the role of endogenous GDF-9 in the *Bmp15* mutant sheep. When recombinant human BMP-15 with a mutation at the 31st amino acid from Ile to Asp (BMP-15^{I31D}), to mimic ovine BMP-15^{V31D} identified in Inverdale sheep (Galloway et al. 2000), was expressed in HEK293 cells, the BMP-15^{I31D} proprotein was processed and the mature dimeric BMP-15^{I31D} was normally secreted. However, when BMP-15^{I31D} was co-expressed with GDF-9, the processing of proprotein and thus secretion of the mature protein were concomitantly disrupted (Liao et al. 2003). Aberrant processing and secretion are suggested to occur by formation of BMP-15^{I31D}/GDF-9 heterodimers that are not susceptible to normal proteolytic cleavage leading to degradation within cells expressing these molecules (Liao et al. 2003). Given the negative impact of the BMP-15^{I31D} mutation on secretion of normal GDF-9, it is reasonable to propose that decreased secretion of GDF-9 may be, at least in part, functionally involved in the aberrant phenotype of the homozygous Inverdale ewes.

5.3 Women with *BMP15* mutations

Critical roles of BMP-15 in female fertility have also been demonstrated in women. The discovery of *BMP15* mutations in female patients with reproductive defects provided the opportunity to translate basic BMP-15 knowledge to the clinic. The first BMP-15 mutation in women that was associated with hypergonadotropic ovarian failure, due to ovarian dysgenesis, was an A-G transition at position 704 of *BMP15* gene (Di Pasquale et al. 2004). This mutation results in a non-conserved substitution of Y235C in the proregion of BMP-15 proprotein (BMP-15^{Y235C}), which acts in a dominant negative fashion by altering the processing of the wild-type BMP-15 proprotein (Di Pasquale et al. 2004). The carriers of this mutation were two sisters who inherited the mutation from their father. Interestingly, they were heterozygous carriers yet their streak ovaries resembled the ovarian phenotype of homozygous mutant BMP-15 ewes. The exaggerated phenotype may indicate this mutation has dominant negative activity.

A number of other mutations in the *BMP15* gene have been identified in women with POF (Di Pasquale et al. 2004; Di Pasquale et al. 2006; Dixit et al. 2005; Dixit et al. 2006; Laissue et al. 2006). In addition, rare deletions and missense mutations in the coding region of BMP-15 have also been identified in mothers of dizygotic twins (Montgomery et al. 2004; Palmer et al. 2006). Therefore, these phenotypes associated with *BMP15* gene mutations are similar to *GDF9* mutations. However, in contrast to the finding that GDF-9^{P103S}, GDF-9^{P374L} and GDF-9^{R454C} mutations significantly increased ovulation rate in women (Palmer et al. 2006), no significant relationship was detected between 35 *BMP15* gene SNPs and dizygotic twinning (Zhao et al. 2008).

To explore the biological significance of the *BMP15* and *GDF9* gene mutations found in women with POF, two representative BMP-15 (BMP-15^{R76C} and BMP-15^{R206H}) and GDF-9 (GDF-9^{K67E} and GDF-9^{P103S}) mutants that occur with a high incidence in POFpatients but have not been identified in normal cases (Dixit et al. 2005; Dixit et al. 2006; Kovanci et al. 2007) were selected for study (Inagaki and Shimasaki 2010). All these mutations are located in the proregion of the proprotein, thus the mature protein should be indistinguishable from wild type if the posttranslational proprotein processing occurs normally. Interestingly, these mutations impaired proprotein posttranslational processing, leading to reduced production of mature BMP-15 and GDF-9 proteins (Inagaki and Shimasaki 2010). As a consequence, these BMP-15 and GDF-9 mutations may be associated with a short early period of enhanced fertility, leading to the increased likelihood of dizygotic twins and/or rapid exhaustion of the ovarian reserve leading to POF (Inagaki and Shimasaki 2010). Further phenotype-genotype analysis is necessary to conclude the biological impact of human BMP-15 mutations on the occurrence of POF and dizygotic twinning.

Overall, parallels between phenotypes in women and sheep with BMP-15 mutations suggest that reduced BMP-15 and/or GDF-9 levels are linked to increased ovulation rate and litter size. Further, proper posttranslational processing of the BMP-15 proprotein is a critical aspect of female fertility in sheep as well as in humans.

6. Biological functions of BMP-15 in regulating folliculogenesis

BMP-15 is exclusively expressed in the oocyte within the ovary, with expression increasing in relation to follicle growth and development (Otsuka et al. 2000). Consistent with responsiveness to BMP ligands throughout folliculogenesis, expression of the type I (ALK-6) and type II (BMP-RII) receptors utilized by BMP-15 has been confirmed in granulosa cells from the primordial to primary stages onwards (McNatty et al. 2005a; Moore et al. 2003). *In vitro* studies using recombinant human BMP-15 showed that BMP-15 stimulates proliferation of undifferentiated rat granulosa cells in an FSH-independent manner (Otsuka et al. 2000). However, BMP-15 can also inhibit FSH actions by suppressing rat granulosa cell FSHR expression (Otsuka et al. 2001c). BMP-15 inhibits FSH-induced granulosa cell expression of steroidogenic acute regulatory protein (StAR), Cyp11a1, 3β-hydroxysteroid dehydrogenase (HSD), LH receptor and inhibin/activin subunits (Otsuka et al. 2001c). Moreover, BMP-15 stimulates kit ligand (KL) expression in granulosa cells (Otsuka and Shimasaki 2002a). Interestingly, KL inhibits BMP-15 expression in the oocytes. Thus, BMP-15 and KL form a negative feedback loop between the oocyte and surrounding granulosa cells; when the oocyte produces BMP-15 this stimulates granulosa cells to produce KL that in turn signals back to the oocyte via c-kit to inhibit further oocyte BMP-15 expression. The combination of increased KL expression and subsequent reduction in BMP-15 inhibition of FSH actions allows effective induction of granulosa cell proliferation (Otsuka and Shimasaki 2002a).

BMP-15 actions are regulated by follistatin, a binding protein that can bind BMP-15 and negate its activity (Otsuka et al. 2001a). Follistatin is strongly expressed in dominant follicles, with very low or undetectable levels in atretic follicles. As BMP-15 inhibits FSH receptor expression, follistatin regulation of BMP-15 actions is likely important for maintaining granulosa cell responsiveness to FSH.

Studies to explore BMP-15 bioactivity were initiated using recombinant human BMP-15 tagged with a Flag epitope at the C-terminus (Otsuka et al. 2000), and mounting biological data were reported (Moore et al. 2003; Otsuka et al. 2001a; Otsuka and Shimasaki 2002a; Otsuka and Shimasaki 2002b; Otsuka et al. 2001c; Otsuka et al. 2000). Recently, N-terminally tagged recombinant human BMP-15 protein was also produced and showed activity in stimulating the transcription of genes including Pentraxin3 and HAS2, inhibitory Smad6/7, BMP antagonists, and inhibin/activin βA and βB subunits (Li et al. 2009). Additionally, recombinant ovine BMP-15 and recombinant ovine or mouse GDF-9 were shown to exhibit a co-operative regulation on proliferation and FSH-induced differentiation of ovine, bovine and rat granulosa cells (McNatty et al. 2005b; McNatty et al. 2005c).

BMP-15 is also involved in the regulation of cumulus cell apoptosis. In bovine ovaries, removal of the oocyte from cumulus-oocyte complexes triggers cumulus cell apoptosis. The induction of cumulus cell apoptosis can be prevented by treatment with BMP-15, but not with GDF-9 (Hussein et al. 2005). Thus, BMP-15 may contribute to decreasing the incidence of apoptosis within cumulus-oocyte complexes until the point of ovulation. BMP-15 is also involved in stimulating cumulus expansion (Yoshino et al. 2006), by enhancing cumulus cell expression of epidermal growth factor (EGF)-like growth factors. Oocytes promote the expression of EGF receptors on cumulus cells, which is crucial for cumulus cells to be capable of responding to LH-induced mural granulosa cell signals.

Oocyte removal reduces EGF receptor expression in mouse cumulus cells, and treatments with either GDF-9 or GDF-9 plus BMP-15 can restore EGF receptor levels which suggests that both GDF-9 and BMP-15 are crucial for the process of cumulus cell expansion through induction of the EGF receptor (Su et al. 2010).

A link between BMP and fibroblast growth factor (FGF) signaling has been established in a number of tissues. FGFs play an important role during embryonic development and in adult tissues in the regulation of the nervous system, tissue repair and tumor angiogenesis. In the ovary, FGF-8 is expressed specifically in oocytes, and FGF receptors in granulosa cells (Berisha et al. 2004; Buratini et al. 2005; Miyoshi et al. 2010). Since mammalian oocytes are unable to initiate glycolysis, companion cumulus cells must transmit to the oocyte glycolysis products necessary for oocyte development. Expression of cholesterol biosynthesis enzymes is reduced in both *Bmp15*$^{-/-}$/*Gdf9*$^{+/-}$ double mutant cumulus cells and in wild-type cumulus cells after removal of oocytes (Su et al. 2008); therefore it appears that BMP-15 and GDF-9 regulate cumulus cell cholesterol biosynthesis as a compensation for the deficiency in oocytes (Su et al. 2008), thus BMP-15 and FGF-8 cooperate to promote glycolysis in cumulus cells (Sugiura et al. 2007). Moreover, the interaction between the FGF and BMP systems is also important for the maintenance of FGF receptor signaling in granulosa cells. FGF-induced SPRY2 expression negatively affects FGF receptor signaling; however BMP-15 and GDF-9 suppress FGF-stimulated SPRY2 expression allowing FGF signals to continue to act in cumulus cells (Sugiura et al. 2009).

A mass-spec analysis of the mature protein of recombinant human BMP-15 revealed that the Ser residue at the 6th position is phosphorylated (Saito et al. 2008). Further studies demonstrated that Golgi-apparatus casein kinase phosphorylates the Ser^{6th} of recombinant human BMP-15 as well as GDF-9 (Saito et al. 2008). Importantly, the phosphorylation of these proteins is essential for their biological activities (McMahon et al. 2008b). Moreover, de-phosphorylated BMP-15 and GDF-9 exhibited antagonistic activity toward not only their phosphorylated counterparts but also toward each other, as well as BMP-7, which shares the same type II receptor, BMP-RII. Thus, phosphorylation may be a means of regulating the bioactivity of BMP-15 and GDF-9, as well as other members of the TGF-β superfamily. Identification of the posttranslational modification of BMP-15 and GDF-9 is an important step toward understanding the molecular basis of their function. However, it remains unknown whether their phosphorylation is directly involved in the physiology of BMP-15 actions *in vivo*.

Recently, Foxo3a an oocyte transcription factor involved in the PI3K/Akt signaling pathway has been shown to influence follicular development and female fertility. Female transgenic mice with constitutively active Foxo3a are infertile, due to impaired oocyte growth and follicular development leading to anovulation (Liu et al. 2007). Constitutively active Foxo3a in oocytes causes a dramatic reduction in the expression of BMP-15, connexin 37 and connexin 43, which are important molecules for the establishment of paracrine and gap junction communications in follicles (Liu et al. 2007). This suggests that Foxo3a is a key signaling molecule that can reduce oocyte growth and follicular development by inactivating BMP-15.

Overall, these studies have shown BMP-15 plays an important role in female fertility. While BMP-15 is not absolutely required for fertility of female rodents, a poly-ovulatory species, it does contribute to the process of ovulation. In contrast, in several mono-ovulatory species, including sheep, cattle and humans, BMP-15 is critical for female fertility.

7. Implications for the translational studies

The clear association between mutations and deletions in the *Bmp15* and *Gdf9* genes and altered reproductive function, in addition to characterization of the biological functions of BMP-15 and GDF-9 in follicular cells has established the importance of these two molecules for normal female fertility. In order to drive translation of the basic research on the role of BMP-15 and GDF-9 in ovarian function to potential clinical therapeutic options there is a requirement for translational research that will further define the involvement of BMP-15/GDF-9 in ovarian physiology and pathophysiology in women.

There are potential clinical implications for BMP-15 and GDF-9 as a potential tool to help overcome female infertility. POF is a common cause of infertility in women, which can manifest as primary amenorrhea or secondary amenorrhea after pubertal development. POF occurs either through follicle dysfunction or follicle depletion, where patients with follicle dysfunction are infertile despite follicles remaining in the ovary while follicle depletion reflects premature loss of the primordial pool due to accelerated recruitment or increased atresia. As BMP-15 and GDF-9 play a role both in promoting early follicle growth as well as restraining dominant/pre-ovulatory follicle development in mono-ovulatory mammals, if identified early enough there is potential for BMP-15 and/or GDF-9 to help both overcome follicle dysfunction and to slow further follicle depletion.

The immunization studies in sheep and cattle showed that the anovulatory phenotype of homozygous mutant sheep could be reproduced by multiple high dose injections of BMP-15 or GDF-9 peptides. Therefore, there is the potential to adopt this technique to use as a permanent, low cost, non-surgical and safe contraceptive option for women or domesticated mammalian species. As this would arrest follicle growth at the primary stage this would disrupt normal estrogen production, thus, it may be considered to be a too radical contraceptive option for normal women of a reproductive age. However, this could be a safe non-surgical option for women who otherwise would undergo surgical ovary removal for more serious and persistent conditions that failed to be helped by traditional contraceptives or pharmaceuticals; such as endometriosis or uterine fibroids which are both estrogen responsive conditions and a common reason for surgical ovary removal in women. However, before treatment with BMP-15 or GDF-9 could be used as a treatment to assist with infertility, or immunization with GDF-9/BMP-15 peptides utilized as a contraceptive option, translational studies evaluating the effects of treatment doses and timing, as well as the effectiveness of exogenously administering mature protein versus designing a treatment to effectively promote enhanced endogenous production of the oocyte factors would need to be performed.

In summary, the elucidation of species-dependent genetic differences in ovulation rate provided novel insight into potential target molecules for manipulating female reproduction. Utilizing the BMP-15/GDF-9 system could potentially facilitate the development of a unique strategy that may help overcome infertility in women with reproductive defects such as POF.

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Abbreviations

ALK	activin receptor-like kinase
BMP	bone morphogenetic protein
BMP-RII	BMP type II receptor
PTGS2	cyclooxygenase 2
EGF	epidermal growth factor
ERK	extracellular signal-regulated kinase
FGF	fibroblast growth factor
FSHR	follicle-stimulating hormone receptor
GDF	growth and differentiation factor
HAS	hyaluronan synthase
3βHSD	3β-hydroxysteroid dehydrogenase
IGF	insulin-like growth factor
KL	kit ligand
LHCGR	Luteinizing hormone/choriogonadotropin/receptor
MAPK	mitogen-activated protein kinase
PCOS/PCO	polycystic ovary syndrome/polycystic ovary
PI3K	phosphatidylinositol 3-kinase
POF	premature ovarian failure
Cyp19a1	P450 aromatase
Cyp11a1	P450 steroid side-chain cleavage enzyme
StAR	steroidogenic acute regulatory protein
TG	transgenic
TGF	transforming growth factor
ZP	zona pellucida

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Table 1

Naturally occurring mutations in ovine *Gdf9* and *Bmp15* genes

Sheep breeds	Gene allele	Nucleotide changes	Amino acid changes	Mature protein	References
Belclare/Cambridge	$FecG^{H}$	c.1184 (230)C>T	Ser395Phe	GDF-9^{S77F}	Hanrahan et al. (2004)
Icelandic Thoka	FecTT	c.1279 (326)A>C	Ser427Arg	GDF-9^{S109R}	Nicol et al. (2009)
Inverdale	$FecX^{I}$	c.896 (92) T>A	Val299Asp	BMP-15^{V31D}	Galloway et al. (2000)
Hanna	$FecX^{H}$	c.871 (67) C>T	Gln291Term	BMP-$15^{Q23Term}$	Galloway et al. (2000)
Lacaune	$FecX^{L}$	c.962(160)G>A	Cys321Tyr	BMP-15^{C53Y}	Bodin et al. (2007)
Rasa Aragonesa	$FecX^{R}$	c.525–541 del (17 bp)	208Term	BMP-$15^{17bp-del}$	Martinez-Royo et al. (2008) Montaegudo et al. (2009)
Belclare/Cambridge (double heterozygotes)	$FecG^{H}$ $FecX^{G}$	c.1184 (230) C>T c.718 C>T	Ser395Phe Gln23.9Term	GDF-9^{S77F} BMP-$^{Q-29Term}$	Hanrahan et al. (2004)
Belclare/Cambridge (double heterozygotes)	$FecG^{H}$ $FecX^{B}$	c.1184 (230) C>T c.1100 (296) G>T	Ser395Phe Ser367Ile	GDF-9^{S77F} BMP-15^{S99I}	Hanrahan et al. (2004)
Woodlands	$FecX^{W}$	unknown	unknown	unknown	Feary et al. (2007)