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Bone morphogenetic protein signaling transcription factor (SMAD) function in granulosa cells

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Abstract

The transforming growth factor β (TGFβ) family of proteins are key regulators of growth and differentiation. Members of this family, including multiple TGFβs, activins, bone morphogenetic proteins (BMPs), and growth and differentiation factor 9 (GDF9), are expressed from oocytes or their associated follicular somatic cells (granulosa and thecal cells) with cell-type and stage-dependent specificity. Granulosa cells are the target cells for many of these ligands. Granulosa cell-specific knockout mice for all of the receptor-regulated SMADs, as well as the common regulatory SMAD4, have recently been generated and highlight the importance of this family in most stages of folliculogenesis. These models have also uncovered a novel role for the BMPs in suppression of granulosa cell tumor development and metastasis. This review summarizes the phenotypes of these mouse models and their contribution to our understanding of the complexity of BMP function during follicle development.

Keywords

reproduction; ovary; fertility; SMAD; knockout; bone morphogenetic protein; cancer

1. Introduction

Many of the mechanisms underlying ovarian dysfunction that lead to infertility in women are poorly understood. Complicating our understanding of female reproductive dysfunction is the interdependent relationship between the developing oocyte and its surrounding somatic cells that form the ovarian follicle. The oocyte and follicular somatic cells (granulosa and thecal cells) develop in consort, and each cell type produces a number of growth factors with key paracrine, autocrine, or endocrine functions. Without their associated somatic cells, oocytes cannot develop [1–3]. The co-dependence between ovarian cell types may pose a unique constraint on the development of artificial reproductive technologies (ART), which seek to develop means of growing eggs from earlier developmental stages [4–7]. Thus, understanding growth factor interactions between somatic

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cells and oocytes is critical not only in understanding normal ovarian physiology, but for the ability to treat or possibly prevent female infertility.

The pool of primordial follicles begins to form midgestation in humans and perinatally in mice, and each follicle will contain an immature non-growing oocyte arrested in early prophase I (Fig. 1). Oocytes are separated into individual follicles by a small number of somatic cells forming a flattened epithelium that will eventually become the granulosa cells. In addition, the oocyte and pre-granulosa cells are separated from the ovarian stroma and other follicles by a basement membrane [8]. Once established, the size of the quiescent primordial follicle pool is thought to limit the reproductive lifespan of the female. Primordial follicles activate by largely unknown mechanisms to enter a growth phase characterized by coupled oocyte and granulosa cell growth, and development of a third somatic cell component, the thecal cells. Activation of oocyte-specific transcription factor cascades can cause unregulated primordial follicle activation and result in premature ovarian failure [9–14]. Whether initial primordial follicle activation is stochastic or regulated, or driven by internal (oocyte) or external (somatic cell) cues, is unclear. While there are excellent mouse models to study oocyte function at early stages of oogenesis and folliculogenesis [15–17], mouse models to analyze granulosa cell function at the transition of pregranulosa cells to cuboidal granulosa cells are lacking. This is due in part to the unavailability of conditional knockout mice to make somatic cell-specific deletions at this stage. Widespread embryonic and perinatal lethality for mice null for components of major developmental signal transduction pathways, including those for the BMPs, are additional confounding issues (Table 1).

2. Growth and differentiation of murine granulosa cells

Little is known about the origin and initial growth of granulosa cells, or about the signal transduction pathways that control granulosa cell specification and development. Embryologically, cells that give rise to granulosa cells are thought to originate from the embryonic coelomic epithelium, similar to Sertoli cell precursors in the male [18,19]. When a primordial follicle activates, the oocyte begins to increase in size, and pre-granulosa cells transition to a cuboidal shape and undergo mitosis [8] (Fig. 1). Interestingly, primordial follicles that are positive or negative for Ki-67 (a marker of proliferation), have similarly sized oocytes, and while granulosa cells undergo the transition to the cuboidal shape, there is no initial increase in oocyte size [20]. These data suggest that granulosa cell proliferation might occur independently from the oocyte at this early stage [20], though how this is regulated is unclear. Pathologically, granulosa cell growth can also be uncoupled from oocyte growth as demonstrated in the *Inha KO* mouse, though the resultant phenotype is the development of granulosa cell tumors [21,22].

During early folliculogenesis, a glycoprotein rich matrix called the zona pellucida forms between the oocyte and developing granulosa cells, and granulosa cells closest to the oocyte (at later stages termed cumulus cells) remain coupled to the oocyte via transzonal projections [23,24]. Granulosa cells continue to divide, forming multiple layers, and a third cell type, the thecal cells, differentiates and surrounds the basement membrane around the perimeter of the follicle at the secondary follicle stage (Fig. 1). Further growth and differentiation occurs in both the granulosa cells and thecal cells throughout the remainder of folliculogenesis (Fig 1). Finally, under the influence of pituitary gonadotropins [follicle stimulating hormone (FSH) and luteinizing hormone (LH)], ovulation occurs and the remaining cells of the follicle terminally differentiate in a process known as luteinization to form the corpus luteum, a transient progesterone-secreting endocrine organ necessary for the establishment of pregnancy.

3. BMPs in early murine folliculogenesis

There are no studies as of yet that indicate whether the BMPs play a role in the breakdown of germ cell cysts (GCC) or the formation of primordial follicles. Oocytes contained in GCCs prior to their breakdown express oocyte-restricted members of the TGFβ family, *Gdf9* and *Bmp15* [10], although protein production of GDF9 is not detectable by immunohistochemistry until the early primary follicle stage (3a) [25]) [26]. *Gdf9* null ovaries contain follicles arrested at the primary follicle stage (Fig. 1), suggesting that GDF9 function is critical at this stage. However, double mutant female mice containing one copy of *Gdf9* but null for *Bmp15* (*Gdf9*$^{+/-}$*Bmp15*$^{-/-}$) are subfertile due to reduced ovulation and fertilization. Interestingly, their ovaries also contain developing follicles with multiple oocytes [27], and this suggests that GCC breakdown may be compromised when the copy number of these oocyte-expressed members of the TGFβ family is reduced.

While BMP15 may act in concert with GDF9 or possibly regulate GDF9 activity [27–29], the mechanism(s) by which it may do so is unclear. *In vitro* studies have shown that these ligands utilize different signaling pathways; BMP15 signals via the SMAD1/5 pathway, while GDF9 signals through SMAD2/3 [30–34]. In contrast to *Gdf9* or *Bmp15*, deletion of *Bmp6*, another oocyte-expressed TGFβ-related ligand, has minimal effects on female fertility, while *Bmp6 Bmp15* double knockout mice have a phenotype similar to the double heterozygous controls (*Bmp6*$^{+/-}$ *Bmp15*$^{+/-}$)[35]. These latter data along with additional published studies [31,36], suggest that BMP15 and BMP6 likely do not have the same role in granulosa cell function. Furthermore, based on the similarity between the double heterozygous to the homozygous *Bmp6 Bmp15* mutant mice, it has also been suggested that deletion of *Bmp6* may in fact rescue some of the fertility defects demonstrated in *Bmp15* KO females, though this remains speculative [35].

There is sufficient data to suggest that the BMP family plays a likely role at the primordial to primary follicle transition (Fig. 1). BMP4 and BMP7 are produced from the ovarian stroma and thecal cells [37,38]. Studies using *in vitro* cultures of isolated postnatal rat ovaries show that BMP4 treatment promotes the development of primary follicles, while treatment of ovaries with a BMP4 neutralizing antibody show progressive lose of oocytes in primordial follicles [39]. *In vivo*, passive immunization in postnatal mice against BMP4 results in fewer primary follicles than control mice, though also in this study, there was an increase in the number of primordial follicles and no associated apoptosis [40]. BMP7 increases the percentage of primary follicles (as well as secondary and antral) but reduces primordial follicles, when injected into the ovarian bursa of adult rats [41]. However, early follicle development cannot be studied in embryos null for either *Bmp4* or *Bmp2* because they die midgestation and either lack germ cells (*Bmp4*) [42], or have reduced numbers (*Bmp2*) [43]. *Bmp7* null mice die perinatally [44,45]. Overexpression of *Bmp15* in oocytes causes accelerated follicle growth, with decreased numbers of primary follicles and increases in secondary follicles, increases in the mitotic index of granulosa cells, and though adult mice have normal litter sizes, they also display an earlier onset of acyclicity [46].

While preantral follicles grow independent of extraovarian factors [47], the pituitary gonadotrophins, FSH and LH, are required for continuation of antral stage growth and ovulation, respectively [15,48,49] (Fig. 1). The TGFβ family, including the BMPs, modulates the effects of both FSH and LH. BMP4 and BMP7 promote FSH-induced estrogen synthesis, while inhibiting progesterone production [38]. Some BMPs can also modulate gonadotropin action by regulating expression of their receptors; BMP15 inhibits FSH receptor (*Fshr*) expression [46,50], while BMP7 and BMP2 increase it [51,52]. In contrast, BMP6 has no effect of *Fshr* expression [50]. BMPs appear to suppress expression of the LH receptor (*Lhcgr*), including mouse BMP15 [53], human BMP7 [52], and human

BMP2 [51]. BMPs also play a role during ovulation and cumulus cell function, and mice with null mutations in the BMP type I receptor, *Bmpr1b* (*Alk6*) have defects in cumulus expansion [54]. The BMP system appears to be downregulated during ovulation, further suggesting that their important role may be as regulators of luteinization [55,56]. *Bmp2* expression, in particular, is dynamically regulated during later stages of follicle development [55]; it is highly expressed in mural granulosa cells to the preovulatory stage and suppressed during ovulation, but re-expressed during luteolysis of corpora lutea, thus suggesting that BMP2 might also play a critical role in terminal granulosa cell differentiation and as a luteinization inhibitor [55]. Interestingly, *Bmp2* expression is repressed in cumulus cells and periantral granulosa cells [55]. This differential expression pattern of mural versus cumulus is typical of genes regulated by oocyte-expressed factors such as GDF9 and BMP15 [57–59].

4. The role of the SMADs in ovarian function

The canonical signaling pathway for the TGFβ family is through the intracellular SMAD transcription factors. There are three general subcategories of SMADs: (1) the receptor-associated SMADs, which are phosphorylated upon formation of specific serine-threonine kinase receptor complexes [SMAD1, SMAD2, SMAD3, SMAD5, SMAD9 (formerly referred to as SMAD8)]; (2) the common mediator SMAD4, which forms part of the heterotrimeric transcription factor complex along with the phosphorylated R-SMADs; and (3) the inhibitory SMADs, SMAD6 and SMAD7, which act to negatively regulate SMAD signaling by competing for SMAD4 or by blocking receptor phosphorylation of the R-SMADs. KO mice for the R-SMADs and *Smad4* are embryonic lethal except for *Smad3* and *Smad9* (Table 1). The majority of *Smad6* null mice die postnatally with cardiac defects [60], while mice homozygous for a *Smad7* hypomorphic allele have reduced body size and small litter sizes [61].

The first conditional SMAD deletion generated in the mouse ovary was for *Smad4* [62] (Table 2). This mouse was created using cre-loxP technology [63], with cre recombinase expression from the endogenous *Amhr2* locus [64]. *Amhr2cre* shows expression in the embryonic Müllerian duct and ovarian stroma [64,65], as well as in granulosa cells of the postnatal and adult mouse ovary with variable expression in thecal cells [65,66]. Herein, these mice are referred to as “ovarian” or “granulosa cell” conditional knockout mice. The expectation for these mice was that *Smad4* deletion would result in ovarian tumors because *SMAD4* [also called *deleted in pancreatic carcinoma 4*, (*DPC4)*] is a well-known tumor suppressor in humans [67]. However no ovarian tumors ever develop in these mice [62]. Instead, *Smad4* conditional knockout mice (termed *Smad4* cKOs) are initially subfertile, but half of the females are infertile by six months of age [62].

The ovarian phenotype of *Smad4* cKO mice is complex, in part due to the breadth of TGFβ family functions in granulosa cells, which includes activity of TGFβ isoforms, GDF9, activin isoforms, BMP15, and other BMPs. It is interesting, however, that the phenotype of the *Smad4* cKO in many ways closely resembles the phenotype of the *Smad2 Smad3* double cKO mice (dKO) that were subsequently generated with *Amhr2cre* [68] (Table 2). SMAD2 and SMAD3 signal for TGFβ, activin, and GDF9 and may be referred to as AR-SMADs [69]. Conditional deletion of either *Smad2* or *Smad3*, when generated as single mutations, has little effect on ovarian function [68] (Table 2). Therefore double conditional (*Smad2 Smad3* dKO) knockouts were produced [68]. These mice show progressive defects in litter size, resulting in infertility by 5 months of age. Similar to *Smad4* cKO mice, the *Smad2 Smad3* dKO mice show fewer antral follicles, luteinized follicles with trapped oocytes, reduced ovulation rates, and impaired cumulus expansion [62,68]. Interestingly, one of the differences between *Smad4* cKO and *Smad2 Smad3* dKO mice is that progesterone levels

increase in the former by 12 weeks of age [62], but not in the latter at the same age [68]. Deletion in granulosa cells of the BMP type I receptors, *Bmpr1a* and *Bmpr1b*, which signal though *Smad1* and *Smad5*, also do not show an increase in progesterone [70]. One possible explanation may be that both the SMAD2/3 and SMAD1/5 pathways act similarly with respect to suppression of progesterone production. Thus, only when all SMAD signaling is suppressed (*i.e.* in *Smad4* cKO cells) is progesterone increased. This is supported by data that show that although they utilize different SMADs, GDF9 (or activin) and BMP15 have been shown to suppress basal, or in some cases FSH-induced, expression of the same genes involved in granulosa cells luteinization and progesterone production including *Lhcgr*, *Star*, and *Cyp11a1* [26,50,71–75]. However, the mechanisms by which they do so need not be identical. A full comparison of the defects in *Smad4* deficient and *Smad2 Smad3* dKO would be necessary to understand the similarities and differences between these mouse models.

5. SMADs in granulosa cell tumor development

SMAD1, SMAD5, and SMAD9 signal in association with ligands using the type I receptors ACVRL1 (ALK1), ACVR1A (ALK2), BMP1A (ALK3), and BMP1B (ALK6). Typically, these are BMP receptors, though they are also used by anti-Müllerian hormone (AMH) and some GDFs [69]. SMAD1, SMAD5 and SMAD9 may be referred to as the BR-SMADs. TGFβ may also utilize SMAD1 or SMAD5 in a limited number of cell types [76,77]. As with *Smad2* or *Smad3* single cKOs using *Amhr2cre*, no phenotype is observed in mice with *Amhr2cre*-driven granulosa cell deletion of *Smad1* or *Smad5* when generated as single mutations. [78] (Table 2). In addition, no phenotype is detected when either of these mutations is generated in a *Smad9* homozygous null background [78]. However, mice with conditional mutations in granulosa cells using *Amhr2cre* to delete both *Smad1* and *Smad5* (*Smad1 Smad5* dKO) or the combination of *Smad1 Smad5* dKO in a *Smad9* homozygous null background (*Smad1 Smad5 Smad9* tKO), have a phenotype distinct from *Smad4* cKO or *Smad2 Smad3* dKO. For clarity, these mouse models for the SMADs with *Amhr2cre* are referred to as BR-SMAD cKOs (for *Smad1 Smad5* deletion, both with or without *Smad9*), while *Smad2 Smad3* deletion is referred to as AR-SMAD cKOs.

Female BR-SMAD cKOs are initially subfertile, but most become infertile as young adults (Table 2). Litter sizes are the same between the *Smad1 Smad5* dKO and the *Smad1 Smad5 Smad9* tKO, which suggests that *Smad9* is not redundant with *Smad1* or *Smad5* in granulosa cells. This hypothesis is in line with embryological data that also do not show a genetic interaction between *Smad1* and *Smad5* with *Smad9* [79]. BR-SMAD cKO female mice develop granulosa cell tumors by 8 weeks of age, and showed an increasing incidence of peritoneal metastases with age. Because the onset of granulosa cell tumor development mirrors the time frame for the development of infertility, it is unclear if the fertility defects are simply secondary to tumor development or whether there are additional underlying defects related to loss of BMP signaling in granulosa cells. Cumulus cell defects might be expected, particularly because female mice null for *Bmpr1b (Alk6)*, which phosphorylates the BR-SMADs, are sterile in part due to impaired cumulus expansion [54].

The target genes for BR-SMADs implicated in tumorigenesis of granulosa cells are unknown. Partly this is due to incomplete knowledge of direct BR-SMAD target genes regulated by BMPs in normal folliculogenesis. However, gene expression arrays have been carried out for WT granulosa cells compared to *Smad1 Smad5* dKO tumors, and indicate that a set of genes known to be BMP-regulated in other tissues are also altered in *Smad1 Smad5* dKO tumors, including gremlin-1 (*Grem1*) and inhibitor of differentiation-1 (*Id1*) [57,78,80]. These were confirmed as likely downstream target genes for BMP signaling by treatment of WT granulosa cells with BMP4 [78]. A role of *Grem1* in the ovary has been suggested [57,81], but *Grem1* KO mice die perinatally [82], and thus, conditional knockout

mice will have to be generated to study its role in postnatal folliculogenesis. IDs function as negative regulators of basic helix-loop-helix transcription factors, and mediate cell growth, differentiation, and cancer development [83–85]. Very little is known about their role in reproduction or reproductive cancers. In sheep ovaries, all four *ID* genes are expressed in granulosa and thecal cells, and BMPs and activin differentially regulate (up and down, respectively) their expression [86]. Currently, any role of the *Id* genes in mouse granulosa cell differentiation or their contribution to BR-SMAD cKO tumor development is unknown.

Because TGFβ has been shown to signal via SMAD1 and SMAD5 in other cell types [76,87] (though this has not been demonstrated in granulosa cells), and loss of function in TGFβ signaling is implicated in tumor development in epithelial cells, it is possible that deletion of *Smad1* and *Smad5* causes tumors in granulosa cells due to disruption of TGFβ signaling though SMAD1 and SMAD5. However, mice with deletion of the BMP type I receptors, *Bmpr1a* and *Bmpr1b*, in granulosa cells (herein termed BMP-RI cKO), which were subsequently generated after the BR-SMAD cKOs, also develop granulosa cell tumors [70]. Similar gene expression changes occur in both the BR-SMAD and BR-RI cKO mouse models. These data firmly establish that it is loss of signaling via the BMP receptor-SMAD pathway that is critical in granulosa cell tumor development in these mouse models. However, there are some differences between the BR-SMAD cKO and the BMP-RI cKO. In contrast to the development of tumors in the BR-SMAD cKO in young adult mice, BMP-RI cKO do not develop granulosa cell tumors until late in age (+16 months) and rarely show metastases [70]. Because an additional BMP type I receptor [*Acvr1a (Alk2)*], which also phosphorylates SMAD1 and SMAD5, is expressed in granulosa cells and is increased when *Bmpr1a* and *Bmpr1b* are deleted, residual BR-SMAD signaling may be sufficient to delay tumorigenesis in the BMP-RI cKO [70]. Interestingly, *Id1* is one of the genes that does not change in the BMP-R1 cKO tumors. Thus, it is tempting to speculate that changes in *Id1* status may be linked to the development of metastases in granulosa cell tumors of BR-SMAD cKO (which have reduced levels of *Id1*) and the granulosa cell tumors of BMP-R1 cKO, in which it is unchanged.

6. Questions regarding BR-SMAD loss and additional signaling pathways leading to granulosa cell tumorigenesis

Granulosa cell tumors in humans are classified into either adult (AGCT) or juvenile (JGCT) forms, with JGCT being the more rare type. Recent studies have identified a mutation in *FOXL2* associated with the majority of AGCT but not in JGCT [88], and the etiology of JGCT is still unknown. Because of the relative rarity of JGCT, it is difficult to obtain a large patient population or tissues for analysis. However, histologic and hormone analysis of BR-SMAD cKO tumors suggest these tumors are more similar to JGCT than to AGCT [89] and as such, allow for a model to study this rare disease.

There are a number of mouse models that develop granulosa cell tumors, including mice with deletion of the inhibin α subunit (*Inha KO*) [22], mice with granulosa cell overexpression of β-catenin with and without deletion of *Pten* [90,91], mice with chronic overexpression of LH [92], transgenic mice with granulosa cell expression of SV40 T-antigen [93], as well as a number of spontaneous mutations [94]. It is not yet clear if these mouse models share a common mechanism that leads to GCT. Analysis of gene expression changes in BR-SMAD cKO tumors indicated that significant changes were found for ligands, receptors, and downstream target genes related to the TGFβ pathway [78]. This led us to hypothesize that part of the phenotype of the BR-SMAD cKO is due to dysregulation of TGFβ signaling via SMAD2 and SMAD3 when the BR-SMADs are deleted [78]. This is supported by immunohistochemical data that show BR-SMAD cKO tumors contain high levels of nuclear and phosphorylated SMAD2 and SMAD3, indicative of an active signaling

pathway. Similar phospho-SMAD2 and SMAD3 immunostaining is also found in granulosa cell tumors from *Inha* KO mice as well as samples of human JGCTs [89], suggesting that AR-SMADs are active in GCT, though their function within the tumor is currently not known.

TGFβ signaling in other human cancers is also known to upregulated the transcription factors glioma associated oncogene (*Gli1)* and *Gli2*, via a SMAD3 dependent pathway [95], and high *Gli2* expression is correlated with increased invasiveness and bone metastases in mice [96]. Interestingly, *Gli1* and *Gli2* are upregulated in response to TGFβ1 treatment in granulosa cells [70], and both are significantly increased in BMP-R1 cKO and BR-SMAD cKO tumors [55](S.A. Pangas, unpublished data). GLI1 and GLI2 serve as transcription factors for the hedgehog signaling pathway, and may play an as yet uncharacterized role in JGCT [70]. Besides TGFβ and hedgehog signaling, there is likely other signaling that is misregulated when the BR-SMADs are deleted. Future studies will be necessary to determine what changes contribute to the infertility in BR-SMAD cKO mice or are involved in GCT development and tumor dissemination.

7. Conclusion

There are a large number of related BMP ligands expressed in the ovary that have cell-type, as well as follicle-stage, specificity. Because the same conserved developmental signal transduction pathways that drive embryogenesis also control ovarian folliculogenesis, many of the TGFβ and BMP-related mouse knockout models cannot be used because of embryonic or perinatal lethality. All of the receptor-related SMADs and the common regulatory SMAD4 have been conditionally deleted in granulosa cells of the ovary, with varying effects on female fertility. Granulosa cell deletion of the AR-SMADs (*Smad2 Smad3* dKO) or *Smad4* (*Smad4 cKO*) generates the most similar phenotype. Ovary-specific receptor and SMAD conditional knockouts also demonstrate that the BMP ligands are important for maintenance of female fertility, but also surprisingly for the suppression of granulosa cell tumor growth. The mechanism(s) by which the BR-SMADs do so are still unknown. Data from mouse and human JGCT suggest that the TGFβ pathway is active in these tumors, and thus is a potential target for inhibition. While it is necessary to understand the genes regulated by the SMADs, it is also important to study the interaction of the SMADs with other signal transduction pathways, such as the hedgehog pathway, that control granulosa cell growth and differentiation. These are key steps for uncovering candidate genes underlying female infertility and possibly cancer development in the ovary as well as other tissues.

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We review BMP signaling via the SMAD pathway in female reproductive biology.

We emphasize the phenotype of SMAD ovarian conditional knockout models.

BMPs regulate stage-specific ovarian follicle growth and tumor suppression.

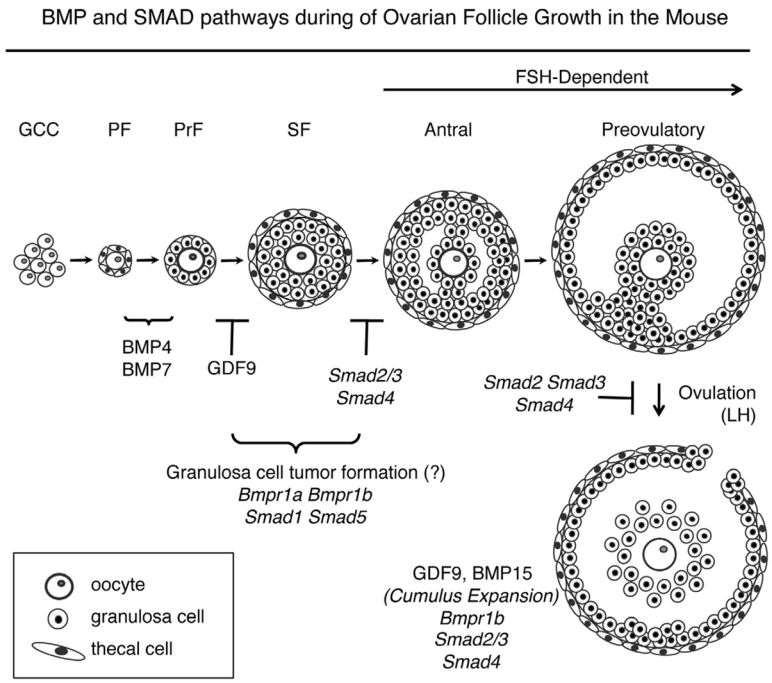

Figure 1.

Ovarian follicle development in the mouse. Oocytes are found as syncytia (germ cell cysts, GCC) in the newborn mouse ovary and GCCs breakdown to form the pool of primordial follicles (PF). Upon activation, primordial follicles transition to primary (PrF) and secondary (SF) stages. BMP4 and BMP7 are implicated in the primordial to primary transition. Follicles in mice null for *Gdf9* do not progress past the primary follicle stage. In wild type mice, secondary follicles acquire a fluid-filled space (antrum) upon stimulation by FSH, and undergo cumulus expansion and ovulation under stimulation by LH. Granulosa cell-specific knockouts for *Smad4* and *Smad2/3* have similar defects at the terminal stages of follicle development. In contrast, granulosa cell-specific knockouts for the BMP type I receptors, *Bmp1a* and *Bmpr1b* and downstream SMAD transcription factors, *Smad1* and *Smad5*, develop granulosa cell tumors. The follicle stage at which GCT tumors form is unknown, but based on their expression pattern [78,89], likely occurs prior to antrum formation.

Table 1

Summary of the general and reproductive phenotypes of mouse knockout (KO) models for the BMP ligands, receptors, and signaling SMADs that are expressed in the mouse ovary. N/D, no data

Ligands	**KO**	**Overall Phenotype**	**Reproductive Phenotype**	**Reference**
Bmp2	Embryonic Lethal	Malformation of the amnion/chorion	Fewer primordial germ cells	[97]
Bmp3	Viable and Fertile	Increased bone density	None noted	[98]
Bmp3b (Gdf10)	Viable and Fertile	Regulates osteoblast differentiation	None noted	[99]
Bmp4 (Bmp2b)	Embryonic Lethal	Failure to form mesoderm	No primordial germ cells	[42,100,101]
Bmp5	Embryonic Lethal	Failure to form mesoderm	N/D	[102]
Bmp6 (Vgr1)	Subfertile	Regulates osteoblast function	Fewer oocytes ovulated	[35]
Bmp7 (Op1)	Perinatal Lethal	Bone, kidney, eye defects	N/D	[103]
Bmp15 (Gdf9b)	Subfertile	Reproductive	Fewer oocytes ovulated; cumulus function	[27]
Gdf9	Infertile	Female infertility	Folliculogenesis blocked after primary stage	[104]
Receptors				
Acvr2a	Perinatal Lethal (25%)	Female infertility	FSH suppression	[105]
Acvr2b	Perinatal Lethal	Lateral asymmetry; cardiac defects	N/D	[106]
Bmpr2	Embryonic Lethal	Failure to form mesoderm	N/D	[107]
Acvr1a (Alk2)	Embryonic Lethal	Failure to form mesoderm	No primordial germ cells	[108,109]
Bmpr1a (Alk3)	Embryonic Lethal	Failure to form mesoderm	N/D	[110]
Bmpr1b (Alk6)	Infertile	Brachydactyly; retinal defects	Cumulus expansion defect; irregular estrous cycle	[54,111]
Smads				
Smad1	Embryonic Lethal	Failure to connect to placenta	Fewer primordial germ cells	[112]
Smad2	Embryonic Lethal	Multiple defects including failed gastrulation and mesoderm development	N/D	[113,114]
Smad3	Subfertile	Defective immune response, colorectal tumors,	Defective follicle development	
Smad4	Embryonic Lethal	Defective visceral endoderm	No primordial germ cells	[115,116]
Smad5	Embryonic Lethal	Gut, cardiac, brain defects	Fewer primordial germ cells	[117,118]
Smad6	Postnatal lethality (majority)	Cardiac defects	N/D	[60]
Smad7	Embryonic lethality (partially penetrant)	Reduced body size, immune defects, cardiac development (in null model)	Smaller litter sizes (hypomorphic allele)	[61,119]
Smad9 (Smad8)	Viable and Fertile	Defective vascular remodeling	None noted	[78,79]

Table 2

Summary of granulosa cell phenotypes in female mice with SMAD conditional (cKO) deletion in granulosa cells of the ovary. Mice were generated either as heterozygotes with one floxed allele and one null allele ($Smad^{flox/-}$), or homozygotes of floxed alleles ($Smad^{flox/flox}$), with or without cre recombinase, as indicated in the original references, except for *Smad9*, which was generated as a homozygous null ($Smad9^{-/-}$). Recombination of floxed alleles was carried out using mice with cre recombinase expression from the *Amhr2* promoter [64].

Conditional KO	Fertility	Ovarian Defects	Reference
Smad1 cKO	Normal	None noted	[78]
Smad2 cKO	Normal	None noted	[68]
Smad3 cKO	Normal	None noted	[68]
Smad4 cKO	Subfertile, then 50% infertile by 6 months	Reduced antral follicles and ovulation rates, cumulus cell defects, increased luteinization, increased serum progesterone	[62]
Smad5 cKO	Normal	None noted	[78]
Smad9 KO	Normal	None noted	[78]
Smad2 Smad3 dKO	Subfertile, then infertile at 5 months	Reduced antral follicles and ovulation rates, cumulus cell defects	[68]
Smad1 Smad9 dKO (formerly *Smad1 Smad8*)	Normal	None noted	Unpublished data, S.Pangas
Smad5 Smad9 dKO (formerly *Smad5 Smad8*)	Normal	None noted	[78]
Smad1 Smad5 dKO	Subfertile, then infertile at 4–6 months	Granulosa cell tumors	[78]
Smad1 Smad5 Smad9 tKO (formerly *Smad1 Smad5 Smad8*)	Subfertile, then infertile at 4–6 months	Granulosa cell tumors	[78]