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Minireview

Inhibitory SMADs: Potential Regulators of Ovarian Function[1]

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ABSTRACT

Transforming growth factor beta (TGFB) superfamily signaling regulates essential reproductive functions. Dysregulation of TGFB signaling results in cellular and molecular deficiencies in the ovary, leading to reproductive diseases and cancer development. SMAD proteins are canonical TGFB signaling components consisting of receptor-regulated SMADs (SMAD1/2/3/5/9), a common SMAD (SMAD4), and inhibitory SMADs (SMAD6/7). Inhibitory SMADs are negative regulators of TGFB and bone morphogenetic protein signaling, and their reproductive functions are poorly defined. Emerging evidence supports that inhibitory SMADs are potential regulators of ovarian function. Further efforts and new genetic models are needed to unveil the role of inhibitory SMADs in the ovary.

development, growth factors, ovary, SMAD6, SMAD7

INTRODUCTION

Transforming growth factor beta (TGFB) superfamily signaling regulates essential female reproductive processes and is indispensable for ovarian development and function [1–8]. SMAD proteins are intracellular transducers of TGFB superfamily signaling. In this *Minireview*, we summarize the role of SMADs in development and female reproduction, with an emphasis on the recent progress on inhibitory SMADs. The emerging role of inhibitory SMADs as potential regulators of ovarian function and the direction of future studies are highlighted.

SMAD PROTEINS

SMA and mother against decapentaplegic (MAD)-related proteins (SMADs) are intracellular components of TGFB signaling pathway. In mammalian species, eight SMAD proteins have been identified. SMADs consist of receptor-regulated SMADs (R-SMADs; SMADs1/2/3/5/9), a common SMAD (Co-SMAD; SMAD4), and inhibitory SMADs (I-SMADs). For R-SMADs, SMAD2/3 generally mediate TGFB/activin signaling, whereas SMAD1/5/9 transduce bone morphogenetic protein (BMP) signals. I-SMADs are comprised of SMAD6 and SMAD7, which antagonize TGFB and/or BMP signaling in vitro [9–11]. SMADs usually share two conserved domains, the N-terminal Mad homology (MH) 1 and the C-terminal MH2 domains, connected by a linker region. MH1 domain binds to DNA [12], whereas MH2 domain interacts with receptors to elicit signal transduction [13]. Of note, I-SMADs have conserved MH2 domains, but divergent MH1 domains [14]. Moreover, these SMADs seem to lack many secondary structural elements compared with R-SMADs, but have distinct tertiary structure characterized by extended loops [14].

SMAD6 can be induced by BMP signaling via SMAD1/5, which serves as a negative feedback loop to terminate BMP signaling [15]. Mechanistically, SMAD6 inhibits BMP signaling by antagonizing activin receptor-like kinase (ALK6)-mediated SMAD1 phosphorylation [16]. SMAD6 also acts as a SMAD4 decoy to suppress BMP-induced SMAD1 activation [17]. In the nucleus, SMAD6 inhibits BMP signaling by interacting with homeobox C8 (Hoxc8) or binding to DNA and recruiting histone deacetylases (HDACs) to repress gene transcription [18, 19]. Furthermore, the associated molecule with the SH3 domain of signal-transducing adaptor molecule (AMSH), a binding partner for SMAD6, inhibits the binding of SMAD6 to activated BMP type 1 receptor or SMAD1/5 [16]. In contrast, SMAD7 inhibits TGFB, activin, and BMP signaling [10, 20]. SMAD7 interacts with TGFB type 1 receptor (TGFBR1/ALK5) to inhibit R-SMAD activation. The stability/association of SMAD7-TGFBR1 complex can be increased by serine-threonine kinase receptor-associated protein (STRAP), atrophin 1-interacting protein 4 (AIP4), and Yes-associated protein (YAP65) [21–23]. Moreover, SMAD7 recruits SMAD ubiquitin regulatory factor 1 (SMURF1) and SMURF2 to TGFBR1, resulting in receptor ubiquitination and degradation [24, 25]. Thus, the activity of TGFB signaling can be controlled via modulation of the stability or degradation of SMAD7 [21–27]. For example, in the nucleus, transcriptional coactivator p300 acetylates SMAD7, preventing its ubiquitination [28], while HDAC1 or silent information regulator 1 (SIRT1)-mediated deacetylation of SMAD7 promotes degradation [29, 30]. In addition, SMAD7 binds to SMAD-binding element (SBE; CAGA)-containing DNA sequence and affects the formation of TGFB signaling-induced functional SMAD-DNA complex [31]. Recent studies showed that *Smad7* can be targeted by micro-RNAs (miR), such as miR-106b-25 cluster,

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during epithelial-to-mesenchymal transition in breast cancer development [32].

ROLE OF R-SMADS AND CO-SMAD IN DEVELOPMENT AND FEMALE REPRODUCTION

The role of R-SMADs and Co-SMAD in development has been clearly demonstrated by genetic studies. *Smad1* knockout (*Smad1*$^{\Delta ex3}$) mice are embryonically lethal (~E9.5) with defective allantois formation [33]. The developmental function of SMAD2 is supported by the fact that *Smad2* null mice die during embryonic development, with defects in a number of developmental events, including, but not limited to, mesoderm formation, left-right patterning, craniofacial development, and egg cylinder elongation [34–38]. *Smad3*$^{\Delta ex1}$ mice show reduced body size, but are viable [39]. *Smad3*$^{\Delta ex2}$ mice are viable and fertile, but develop metastatic colorectal cancer [40]. In contrast, impaired fertility and mucosal immunity have been found in *Smad3*$^{\Delta ex8}$ mice [41], accompanied by accelerated wound healing, but impaired intestinal mucosal healing [42, 43]. *Smad3*$^{\Delta ex8}$ mice demonstrate ovulatory defects and lack corpora lutea in the ovary. Altered ovarian cell differentiation and compromised follicular growth and atresia have been identified in these mice [8]. Furthermore, granulosa cells from *Smad3*$^{\Delta ex8}$ mice express lower levels of FSH receptors and respond poorly to FSH [6]. *SMAD4* is frequently deleted in pancreatic cancer [44], and *Smad4* mutant mice show gastrulation defects [45]. *Smad5* null mice die at midgestation, resulting from both embryonic and extraembryonic abnormalities [46, 47]. Moreover, SMAD5 is indispensable for primordial germ cell proliferation [48]. Although *Smad9* knockout mice are viable and fertile, they demonstrate defects in pulmonary vascular remodeling, characterized by medial thickening and vascular smooth muscle hyperplasia [49].

The functions of several R-SMADs and SMAD4 in reproduction have been recently identified using a conditional knockout strategy to overcome the embryonic lethality resulting from ubiquitous disruption of these genes (Table 1). It has been recognized that SMAD1 and SMAD5 are tumor suppressors with redundant roles in the ovary and testis. Loss of SMAD1 and SMAD5 in the somatic cells of the gonad leads to metastatic granulosa cell/testicular tumor formation [50]. To explore the role of SMAD2 and SMAD3 in ovarian granulosa cells, we have generated three independent conditional knockout mouse lines using *Smad2*$^{\Delta ex9,10}$, *Smad3*$^{\Delta ex2,3}$, and *Smad2/3* and anti-Müllerian hormone receptor type 2 (*Amhr2*)-Cre recombinase. We show that SMAD2 and SMAD3 function redundantly to maintain female fertility by regulating follicular development, ovulation, and cumulus cell expansion [5]. Disruption of SMAD4 signaling in the ovary using *Amhr2*-Cre causes premature granulosa cell luteinization and impaired ovulation and cumulus expansion [51]. Most recently, deletion of *Smad4* in late-stage follicles using cytochrome P450 aromatase (*Cyp19*)-Cre results in multiple ovulatory defects, with no manifestation of premature luteinization [52]. It should be noted that *Amhr2*-Cre and *Cyp19*-Cre lines delete conditional alleles at different stages during folliculogenesis [51, 52], suggesting that the ovarian function of SMAD4 depends on the differentiation stage of the granulosa cells. A difference has also been observed during oocyte-specific deletion of *Smad4*, where growth differentiation factor 9 (*Gdf9*)-iCre, but not zona pellucida glycoprotein 3 (*Zp3*)-Cre, shows an effect on female fertility [53]. Since the expression of *Gdf9*-iCre and *Zp3*-Cre starts from the respective primordial and primary follicle stages, these findings argue for a role of SMAD4 in oocytes during primordial follicle development.

FUNCTION OF INHIBITORY SMADS

In mouse embryos, SMAD6 and SMAD7 are coexpressed in the developing cardiovascular system [54–56]. In noncardiovascular tissues, *Smad6* is expressed in intramembranous bone, whereas *Smad7* is expressed in the seminiferous tubules of testis [55]. *Smad6* and *Smad7* are also highly expressed in the kidney and lung [9, 10]. To elucidate the role of SMAD6, Galvin et al. generated *Smad6* mutant mice, which show partial lethality due to cardiovascular complications [54]. However, little is known about the role of SMAD6 in the reproductive system.

To date, there are four knockout mouse lines for *Smad*7 generated by independent research groups (Table 2):

1) *Smad7*$^{\Delta ex1}$ mice. Deletion of exon 1 of *Smad7* eliminates the N-terminal 204-aa residues of SMAD7 protein. However, these mice can produce a truncated protein that suppresses TGFB signaling, as demonstrated by a luciferase activity assay in vitro. Phenotypically, the *Smad7*$^{\Delta ex1}$ mice are viable and fertile. The mutant mice are smaller than controls, and their B cell responses are altered due to an increase in phosphorylated SMAD2 that activates TGFB signaling and B cell apoptosis [57]. Moreover, SMAD7 is a negative regulator of TGFB and NF-κB pathways that promote renal fibrosis and inflammation following a unilateral ureteral obstruction [58]. Consistently, enhanced renal fibrosis and inflammation, along with increased activation of TGFB and NF-κB, have been demonstrated in streptozotocin-induced diabetic *Smad7* knockout mice [59].

TABLE 1. Conditional knockouts of *Smads* in ovarian somatic cells and oocytes.

Conditional allele	Cre-recombinase	Major reproductive phenotype in females [reference]
Smad1^{flox}	*Amhr2*-Cre	None [50]
Smad2^{flox}	*Amhr2*-Cre	None [5]
Smad3^{flox}	*Amhr2*-Cre	None [5]
Smad4^{flox}	*Amhr2*-Cre	Impaired fertility; premature granulosa cell luteinization; defective cumulus expansion and ovulation [51]
Smad4^{flox}	*Cyp19*-Cre	Impaired fertility; increased follicular atresia; defective ovulation and luteal formation [52]
Smad4^{flox}	*Zp3*-Cre	None [53]
Smad4^{flox}	*Gdf9*-iCre	A minor fertility reduction [53]
Smad5^{flox}	*Amhr2*-Cre	None [50]
Smad1^{flox}; *Smad5*^{flox}	*Amhr2*-Cre	Impaired fertility; metastatic granulosa cell tumor development [50]
Smad1^{flox}; *Smad5*^{flox}; *Smad9*$^{-/-}$	*Amhr2*-Cre	Impaired fertility; metastatic granulosa cell tumor development [50]
Smad2^{flox}; *Smad3*^{flox}	*Amhr2*-Cre	Impaired fertility; defective follicular development and ovulation [5]

TABLE 2. Currently available *Smad7* mutant mice.

Targeted deletion	*Smad7* transcript/protein	Major phenotype [reference]
Smad7 exon 1	A truncated protein detected	Reduced body size and abnormal B cell responses [57]
Smad7 exon 4	Transcripts corresponding to exons 1–3 detected	Cardiovascular defects with severe embryonic lethality [60]
Smad7 promoter-exon 1	Deletion of *Smad7* transcript	Embryonic lethality [62]
Smad7 exon 4	No SMAD7 protein detected	Postnatal survival of the mutants depends on genetic backgrounds; an ICR background is permissive to postnatal development of the mutant mice to adulthood with growth retardation [63]

2) *Smad7*$^{\Delta ex4}$ mice. Deletion of the exon 4 of *Smad7* eliminates the entire MH2 domain. The majority of *Smad7*$^{\Delta ex4}$ mice died in utero due to multiple cardiovascular defects [60]. The surviving adults demonstrate enhanced SMAD2/3 phosphorylation in the heart, accompanied by increased apoptosis, impaired cardiac functions, and severe arrhythmia.
3) *Smad7* promoter-exon 1 knockout mice. Kleiter et al. generated a *Smad7* conditional allele by flanking the promoter region and exon 1 of the *Smad7* gene with two LoxP sites. A *Smad7* null allele was subsequently produced using Cre-recombinase driven by a ubiquitously expressed human cytomegalovirus minimal promoter [61]. *Smad7* homozygous mice are embryonically lethal. Using *CD4*-Cre, the authors generated *Smad7* conditionally deleted mice in T cells and showed that SMAD7 drives T helper 1 responses in multiple sclerosis and experimental autoimmune encephalomyelitis [62].
4) *Smad7* mutant mice lacking MH2 domain and the poly (A) sequence. This is the most recently developed *Smad7* knockout mouse [63]. Interestingly, *Smad7* null mice on a C57BL/6 background die within a short time after birth. However, mice on an ICR background survive to adulthood with growth retardation. Counterintuitively, levels of phospho-SMAD2/3, indicators of TGFB signaling activity, are reduced in mouse embryonic fibroblast (MEF) cells from the knockout mice, with no alterations in gene expression for the plasminogen activator inhibitor-1 (*PAI-1*), a known target of TGFB. Moreover, the activity of BMP signaling does not appear to be altered, as evidenced by comparable levels of phospho-SMAD1/5/9 and mRNA expression for inhibitor of DNA binding 1 (*Id1*; a BMP target gene) between *Smad7*-deficient MEF cells and controls [63]. The discrepancies in the phenotype of these mice are currently unknown, and are potentially associated with the different targeting strategies utilized by different investigators and the genetic background of the mice. Nevertheless, these studies reveal a complex role of SMAD7 in vivo, suggesting potential compensatory mechanisms that antagonize TGFB signaling activity in the absence of SMAD7. Further exploiting the available *Smad7* knockout mouse models will be beneficial to define the function of inhibitory SMADs.

ARE INHIBITORY SMADS REGULATORS OF OVARIAN FUNCTION?

The ovarian function of inhibitory SMADs is poorly understood. In the ovary, SMAD6 protein is strongly expressed in oocytes of primordial follicles, but weakly expressed in growing oocytes, and theca and granulosa cells [64]. *Smad6* and *Smad7* mRNA levels are lower in fully grown oocytes compared with those of growing oocytes [65]. In addition, SMAD6 and SMAD7 are subject to gonadotropin regulation [64, 66]. A comprehensive quantification analysis of SMAD6 and SMAD7 expression at different stages of follicular development is currently unavailable. Inconsistent results of SMAD6 and SMAD7 expression in mouse oocytes have been reported, which may reflect the differences of antibodies, experimental approaches, and models utilized [64–67]. A definitive role of TGFB signaling in the oocyte remains elusive, and conditional deletion of SMAD4 in the oocyte produces minimal reproductive phenotype [53], suggesting that TGFB signaling is tightly controlled during development. It is tempting to speculate that SMAD6 and SMAD7 may act as guardians in the oocyte to keep TGFB signaling in check and prevent its overactivation, which could result in a catastrophic outcome. However, this speculation must be experimentally tested. The expression of inhibitory SMADs in ovarian tissues/cells provides circumstantial evidence that SMAD6 and SMAD7 may play a role in the ovary.

In 2012 and 2013, two reports, one of which is from our group, suggested that SMAD7 is a potential regulator of ovarian function [66, 67]. In one study, the McGee laboratory elegantly demonstrated that SMAD7 serves as a mediator of apoptosis induced by TGFB signaling in ovarian granulosa cells in vitro [67]. TGFB activates *Smad7* promoter and induces *Smad7* gene expression in granulosa cells via interaction with a SMAD response element, the mutation of which impairs TGFB action. By manipulating the expression levels of *Smad7* in mouse granulosa cells using overexpression and a small interfering RNA (siRNA) knockdown approach, the authors were able to provide compelling evidence supporting that SMAD7 is essential for TGFB-induced apoptosis. Since apoptosis is known to influence follicular development, results of this study further imply that SMAD7 regulates ovarian folliculogenesis and function.

In the other report, we examined the potential function of SMAD7 in the mouse ovary using siRNA knockdown and primary mouse granulosa cell culture [66]. We showed that SMAD7 is expressed in mouse granulosa cells and acts as a negative regulator of TGFB1 and GDF9 signaling. As GDF9 is a key oocyte-derived factor that regulates oocyte-granulosa cell interaction, follicular development, and ovulation [1, 68], our findings indicate that SMAD7 may function to precisely control TGFB superfamily signaling essential for oocyte-granulosa cell communication within the ovary. More specifically, induction of SMAD7 may serve as a negative feedback to limit the extent and duration of TGFB and GDF9 signaling and ensure optimal biological responses by ovarian cells. To further define the function of SMAD7 in the ovary, we generated a conditional knockout of *Smad7* using mice harboring a conditional allele of *Smad7* [62] and *Amhr2*-Cre. However, the *Smad7* conditional knockout mice did not show

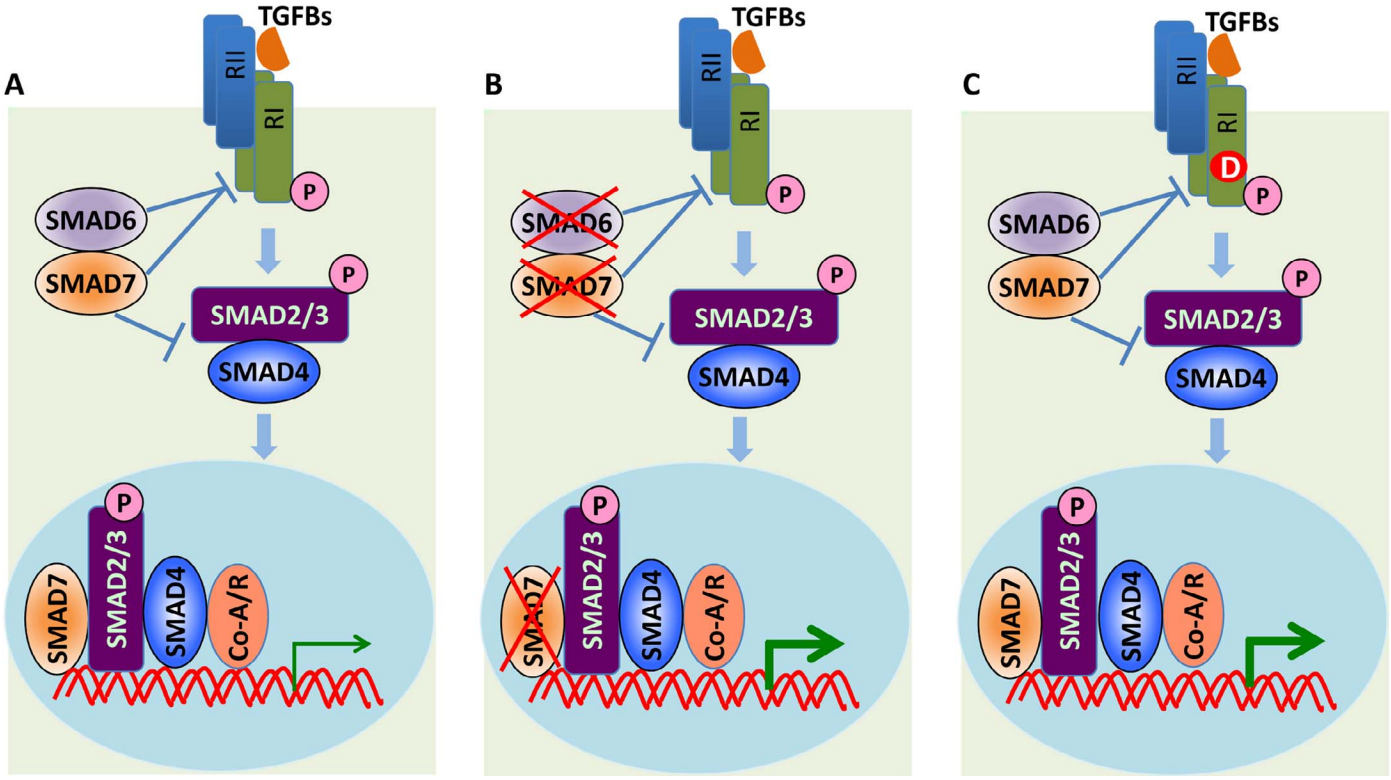

FIG. 1. A hypothetical model depicting the role of inhibitory SMADs and the strategy to enhance TGFB signaling in the ovary. TGFB ligands bind to the type 2 (RII) and type 1 (RI) receptors, which activate downstream SMAD2/3 proteins. SMAD2/3 then complex with SMAD4 and translocate into the nucleus to regulate gene transcription in concert with coactivators and corepressors (Co-A/R). The TGFB signaling activity is controlled by inhibitory SMADs (i.e., SMAD6 and SMAD7). SMAD6/7 inhibit TGFB signaling by promoting R1 ubiquitination and degradation, interfering with SMAD2/3-SMAD4 complex formation, and antagonizing SMAD2/3 action in the nucleus. In a physiologic condition, the TGFB signaling activity is finely tuned, with an optimal signaling output in response to ligand stimulation (**A**). In the absence of ovarian SMAD6 and SMAD7, the suppressive effect imposed by inhibitory SMADs is lost, and TGFB signaling activity is expected to be enhanced. Consequently, potentiated gene expression and/or altered ovarian development/function will be anticipated (**B**). **C**) An alternative approach to induce enhanced TGFB signaling in the ovary. A glycine/serine-rich (GS) domain mutation (i.e., T204D) renders the TGFBR1 constitutively active. Mice containing a latent constitutively active *TGFBR1* allele [76] and Cre recombinase targeting the ovary can be used to achieve such a goal. D, aspartic acid; P, phosphorylation.

an overt reproductive defect. Moreover, alterations of known target genes of TGFB signaling were not detected in granulosa cells isolated from these mice (data not shown). These data argue for a potential compensatory mechanism that operates to control the TGFB signaling activity in the absence of SMAD7. Of note, a redundancy between SMAD6 and SMAD7 has been hypothesized in heart development and function [60]. Since the *Smad6* mutant mice show partial lethality, and the viable ones develop numerous cardiovascular abnormalities [54], a conditional knockout approach appears to be a better strategy to elucidate the potential redundancy between SMAD6 and SMAD7 in the ovary.

PERSPECTIVES

TGFB superfamily signaling regulates essential female reproductive functions. Dysregulation of TGFB signaling leads to reproductive disorders and cancer development [50, 69–72]. The role of inhibitory SMADs in the ovary and female reproductive tract remains elusive. Mouse models have been widely used to interrogate the function of TGFB signaling. However, the majority of models have been created to inactivate genes of this superfamily pathway (i.e., loss of function). Relevant mouse models with tissue/cell-specific overactivation of TGFB signaling (i.e., gain of function) are needed in view of the enhanced, instead of suppressed, TGFB signaling in a number of diseases (e.g., fibrosis, Marfan syndrome, and cancer at certain stages) [73–75]. Thus, development of a mouse model with simultaneous disruption of *Smad6* and *Smad7* may help to identify the function and redundancy of inhibitory SMADs. Such a model is important not only to elucidate the reproductive function of inhibitory SMADs, but also to clarify the consequence of overactivated TGFB signaling in the ovary. It is tantalizing to speculate that conditional deletion of *Smad6* and *Smad7* in the ovary may enhance TGFB signaling activity, which could potentially affect cell cycle gene expression, leading to abnormal granulosa cell proliferation, differentiation, and apoptosis. Additional mouse models with enhanced TGFB signaling in the ovary could be generated by taking advantage of a latent constitutively active TGFBR1 [76] and specific Cre-recombinases targeting the ovary. A hypothetical model has been proposed to illuminate the function of inhibitory SMADs and the consequence of enhanced TGFB signaling in the ovary (Fig. 1). We envision that the availability of these novel mouse models will help us to gain a more comprehensive understanding of TGFB signaling in both physiologic and pathological conditions.

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