Genetic evidence that SMAD2 is not required for gonadal tumor development in inhibin-deficient mice

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Abstract

Background: Inhibin is a tumor-suppressor and activin antagonist. Inhibin-deficient mice develop gonadal tumors and a cachexia wasting syndrome due to enhanced activin signaling. Because activins signal through SMAD2 and SMAD3 in vitro and loss of SMAD3 attenuates ovarian tumor development in inhibin-deficient females, we sought to determine the role of SMAD2 in the development of ovarian tumors originating from the granulosa cell lineage.

Methods: Using an inhibin α null mouse model and a conditional knockout strategy, double conditional knockout mice of Smad2 and inhibin alpha were generated in the current study. The survival rate and development of gonadal tumors and the accompanying cachexia wasting syndrome were monitored.

Results: Nearly identical to the controls, the Smad2 and inhibin alpha double knockout mice succumbed to weight loss, aggressive tumor progression, and death. Furthermore, elevated activin levels and activin-induced pathologies in the liver and stomach characteristic of inhibin deficiency were also observed in these mice. Our results indicate that SMAD2 ablation does not protect inhibin-deficient females from the development of ovarian tumors or the cachexia wasting syndrome.

Conclusions: SMAD2 is not required for mediating tumorigenic signals of activin in ovarian tumor development caused by loss of inhibin.

Background

The transforming growth factor β (TGFβ) superfamily ligands including activins and inhibins play integral roles in a wide variety of developmental processes [1-3]. Inhibins are α and β subunit heterodimers (inhibin A: α, βA; inhibin B: α, βB) that oppose activin signaling by antagonizing activin receptors (ACVRs), whereas activins are homodimers (activin A, βA: βA; activin B, βB: βB) or heterodimers (activin AB, βA: βB) of the β subunits [4-6]. Activin signal transduction is initiated when the ligand binds to its type 2 serine/threonine kinase receptor which in turn phosphorylates the type 1 receptor [7-11]. The type 1 receptor then phosphorylates and activates receptor-regulated SMADs (R-SMADs; SMAD2 and SMAD3), which subsequently form complexes with the common SMAD, SMAD4. The R-SMADs/SMAD4 can translocate into the nucleus to regulate gene expression via recruitment of specific transcription factors, activators, and repressors [12-15].

Activins and inhibins are expressed in ovarian granulosa cells and were first described for their roles in FSH regulation [16,17]. However, subsequent studies demonstrated the involvement of these ligands in multiple developmental and pathological events including carcinogenesis [18-20]. Inhibin is a tumor suppressor [21], as inhibin α (Inha) null mice develop gonadal sex cord-stromal tumors originating from the granulosa/Sertoli cell lineages [21], presumably due to the loss of activin antagonism. The tumors secrete an excessive amount of activins that signal through activin receptor type 2 (ACVR2) in the stomach and liver, leading to a cachexia wasting syndrome.
wasting syndrome and pathological changes in these organs (depletion of parietal cells in the glandular stomach and hepatocellular death in the liver) [22,23]. Lethality in Inha null mice is primarily caused by the cachexia wasting syndrome characterized by weight loss, lethargy, and anemia [24]. Although the mechanisms of tumorigenesis in Inha null mice are not fully understood, activin, FSH, and estradiol may play pivotal roles in the development of gonadal tumors [25-28]. As absence of an α subunit precludes α:β dimer assembly, activin is highly elevated in Inha null mice due to the ability of the β subunits to only form β:β activin dimers [24]. While activin-deficient mice die after birth due to craniofacial defects [9], accumulating evidence suggest that activins play important roles in gonadal tumor development in inhibin-deficient mice. Expression of the activin βA subunit is elevated in the gonads of inhibin-deficient mice [29]. Moreover, tumorigenesis is attenuated in inhibin-deficient mice that transgenically express follistatin, an activin antagonist [30,31]. More recently, we demonstrated that administration of a chimeric ACVR2 ectodomain (ActRII-mFc), a known activin antagonist, delayed gonadal tumorigenesis in inhibin-deficient mice [32].

To dissect the activin downstream signaling components during ovarian tumorigenesis, we previously generated Inha/Smad3 double knockout mice in which females are substantially, but not completely, protected from the development of ovarian tumors and the accompanying cachexia syndrome [28]. Since SMAD2 and SMAD3 are activin signal-transducers in vitro and the gonadal somatic cells (granulosa cells and Sertoli cells) from which inhibin-deficient tumors are derived express both SMADs, we hypothesized that SMAD2 may partially compensate for the loss of SMAD3 in mediating ovarian activin signals in the Inha/Smad3 double knockout females. To circumvent the embryonic lethality of Smad2 ubiquitous knockout [33-35], we conditionally deleted Smad2 in ovarian granulosa cells null for Inha to determine the role of SMAD2 in gonadal tumor development.

Methods
Generation of Inha/Smad2 conditional knockout mice
Mice used in this study were maintained on a mixed C57BL/6/129S6/SvEv background and manipulated according to the NIH Guide for the Care and Use of Laboratory Animals. Generation of the Inha null mice and the Smad2 null allele was described previously [21,36]. The Smad2 conditional allele was constructed by flanking exons 9 and 10 with two loxP sites using the Cre-LoxP system as previously documented [37,38]. The Amhr2cre/+ mice were produced via insertion of a Cre-Neo cassette into the fifth exon of the anti-Mullerian hormone receptor type 2 (Amhr2) locus [39]. Generation of the Smad2flox/+/Inha+/+; Amhr2cre/+ mice (experimental group) and Smad2flox/+/Inha--/-- (control group) is depicted in Figure 1.

Genotyping analysis
Genotyping of the mice was performed by PCR using genomic tail DNA. Table 1 lists the primer sequences utilized in the PCR assays. The annealing temperatures for Inha, Amhr2cre/+, and Smad2 were 61°C, 62°C, and 60°C, respectively. The resultant PCR products were separated and visualized on 1% agarose gels.

Measurement of body weight and generation of survival curve
Body weights of animals were measured and recorded weekly from ages 4-26 weeks, and the mice were closely monitored for the development of the cachexia wasting syndrome (i.e., weight loss, kyphoscoliosis, and lethargy) [24]. Mice were sacrificed when their body weights fell below 15 grams or when other severe cachexia symptoms developed as described elsewhere [24,40]. All mice were sacrificed at the end of 26 weeks for a final analysis. To determine the potential effect of conditional deletion of Smad2 on ovarian tumor development at early stages, the Inha/Smad2 cKO mice were also examined at 4 to 9 weeks of age.

Histological analysis
Mice were anesthetized by isoflurane inhalation at the time of sacrifice. A small portion of the tails were cut and stored at -70 °C for subsequent genotype verification. Ovaries, stomachs, and livers were removed from the mice and fixed in 10% (vol/vol) neutral buffered formalin overnight. The fixed samples were washed with 70% ethanol prior to paraffin embedding. Ovaries were sectioned and stained with periodic acid-Schiff (PAS)-hematoxylin, whereas livers and stomachs were processed for hematoxylin and eosin (HE) staining. All staining procedures were conducted in the Pathology Core Services Facility at Baylor College of Medicine using standard protocols.

Immunohistochemistry
Expression of SMAD2 in Smad2flox/+/Inha+/+; Amhr2cre/+ and Smad2flox/+/Inha--/-- mice was determined by immunohistochemistry. Briefly, ovaries from 4-week-old mice were fixed in formalin and serially sectioned (5 µm). Antigen retrieval was performed by boiling the sections in 10 mM citrate buffer (pH 6.0). The sections were then blocked using 3% BSA/10% serum in PBS, and incubated with SMAD2 primary antibody (1:100 dilution; Cell Signaling). Subsequent procedures were performed using ABC and DAB kits (Vector Lab). The sections were counterstained with hematoxylin and mounted with Permount.
Activin A analysis

Blood samples were collected from anesthetized mice by cardiac puncture upon sacrifice, placed in serum separator tubes (Becton Dickinson, Franklin Lakes, NJ, USA), and allowed to clot at room temperature. Serum was then isolated from the blood samples by centrifugation and stored at -20°C until assayed. Total serum activin A levels were measured using a specific ELISA [41] according to the manufacturer’s instructions (Oxford Bio-Innovations, Oxfordshire, UK) with modifications [42]. The average intraplate coefficient of variation (CV) was 7.4% and the interplate CV was 10.8% (n = 2 plates). The limit of detection was 0.01 ng/ml.

Statistical analyses

Differences among groups (average ovary weight, liver weight, and serum activin A levels) were assessed using one-way ANOVA followed by a Kruskal-Wallis post-hoc test. The survival curve was analyzed using a logrank test. For all analyses, significance was defined at P < 0.05. Data are reported as mean standard error of the mean (SEM).

Results

Generation of Smad2<sup>floxed−/−</sup>; Inha<sup>−/−</sup> mice

To understand the roles of SMAD2 in ovarian tumor development in inhibin-deficient mice, we took advantage of a conditional knockout strategy to disrupt the Smad2 gene in mouse ovarian granulosa cells. To overcome the embryonic lethality phenotype resulting from Smad2 ubiquitous deletion, a Smad2 floxed allele was generated by flanking exons 9 and 10 of the Smad2 gene with 2 loxP sites [37]. The Amhr2<sup>cre/+</sup> knock-in mouse line validated in our previous studies to delete genes expressed in ovarian granulosa cells [38,43-47] was uti-
lized. Figure 1A depicts the breeding scheme used to generate the control (Smad2^{flox}+/Inha^{-/-}) and experimental Smad2^{flox}/Inha^{-/-}; Amhr2cre/+ (Inha/Smad2 cKO) female mice. Representative genotype analyses are presented in Figure 1B. We previously demonstrated that the Smad2 floxed allele can be recombined and deleted in mouse granulosa cells in Smad2 cKO mice [38]. As a further support that inhibin-deficient tumors originate from granulosa cells, the Smad2 recombined allele was readily detectable in the tumor tissues of Smad2^{flox}/Inha^{-/-}; Amhr2^cre/+ mice, but not in the controls lacking the Cre-recombinase (Figure 1C). Moreover, immunostaining revealed a dramatic reduction of SMAD2 protein levels in the granulosa cells of Smad2^{flox}/Inha^{-/-}; Amhr2^cre/+ mice vs. controls (Figure 1D and 1E).

### Conditional knockout of Smad2 in inhibin-deficient mice does not alter lethality

To evaluate the overall effects of Smad2 conditional deletion on the life span of inhibin-deficient mice, we generated and analyzed the survival curves of the Smad2^{flox}/Inha^{-/-} controls (n = 19) and the Smad2^{flox}/Inha^{-/-}; Amhr2^cre/+ (n = 16) experimental mice (Figure 2). The median survival was 13 weeks for both Smad2^{flox}/Inha^{-/-} and Smad2^{flox}/Inha^{-/-}; Amhr2^cre/+ females. Statistical analysis indicated that Smad2 deficiency did not alter the lifespan of Inha null mice (P > 0.05).

### Development of ovarian tumors and cachexia wasting syndrome in Smad2^{flox}/Inha^{-/-}; Amhr2^cre/+ mice

An early sign of tumor development in Inha null mice is severe body weight loss, a prominent feature of the cachexia wasting syndrome. To determine if Smad2 deficiency affects the development and progression of the cachexia syndrome, the body weights of Smad2^{flox}/Inha^{-/-} and Smad2^{flox}/Inha^{-/-}; Amhr2^cre/+ mice were measured weekly. The results showed that Smad2^{flox}/Inha^{-/-}; Amhr2^cre/+ mice suffered from weight loss similar to that observed in Smad2^{flox}/Inha^{-/-} mice (data not shown).

Because the cachexia syndrome is also characterized by distinct activin-induced pathological changes in the stomach and liver [22,23], these organs were collected and examined along with the gonads. The ovary and liver weights of the wild type (WT), Smad2^{flox}/Inha^{-/-}, and Smad2^{flox}/Inha^{-/-}; Amhr2^cre/+ mice were measured. Despite the marked changes of the weights of ovary and liver in both Smad2^{flox}/Inha^{-/-} (n = 11) and Smad2^{flox}/Inha^{-/-}; Amhr2^cre/+ mice (n = 5) compared to WT mice (n = 5; P < 0.01), no significant differences in these parameters were found between the Smad2^{flox}/Inha^{-/-}; Amhr2^cre/+ mice and the Smad2^{flox}/Inha^{-/-} controls at a similar stage of tumor progression (P > 0.05; Figure 3).
Histological analyses were performed on the ovaries and livers of WT, Smad2flox/cre/+; Inha-/- control (Con) and Smad2flox/cre/+; Inha-/-; Amhr2cre/+(S2/Inha cKO) experimental mice. Note the dramatic alteration of the weights of the ovary and liver of the Smad2flox/cre/+; Inha-/- (6-26 wk; n = 11) and Smad2flox/cre/+; Inha-/-; Amhr2cre/+(6-23 wk; n = 5) mice compared to adult WT mice (12 wk; n = 5) due to tumor development. However, no differences in the ovary and liver weights were observed between the control and S2/Inha cKO mice. All data are shown as mean ± SEM, and bars without a common letter are significantly different at \( P < 0.01 \).

Figure 3 Ovary and liver weights of WT, Smad2flox/cre/+; Inha-/- control (Con) and Smad2flox/cre/+; Inha-/-; Amhr2cre/+(S2/Inha cKO) experimental mice. Note the dramatic alteration of the weights of the ovary and liver of the Smad2flox/cre/+; Inha-/- (6-26 wk; n = 11) and Smad2flox/cre/+; Inha-/-; Amhr2cre/+(6-23 wk; n = 5) mice compared to adult WT mice (12 wk; n = 5) due to tumor development. However, no differences in the ovary and liver weights were observed between the control and S2/Inha cKO mice. All data are shown as mean ± SEM, and bars without a common letter are significantly different at \( P < 0.01 \).

Discussion

The aim of the current study was to define the role of SMAD2 in the development of ovarian tumors and activin-induced cancer cachexia syndrome. We demonstrated that conditional deletion of SMAD2 did not prevent the inhibin-deficient females from ovarian tumorigenesis and death; all Inha/Smad2 cKO mice developed sex cord-stromal tumors resembling those observed in Inha null mice. Furthermore, Inha/Smad2
cKO mice suffered from the cancer cachexia syndrome, as evidenced by the severe weight loss and pathological lesions in the stomach and liver (i.e., mucosal atrophy with depletion of parietal cells in the glandular stomach and hepatocellular necrosis around the central vein) [24]. These results indicate that SMAD2 is not required for transducing superphysiological activin signals in the context of gonadal tumor development due to loss of inhibin.

Activins play complex roles in carcinogenesis. In several extragonadal tissues, activin A has been reported to be an anti-tumorigenic factor. For example, activin prevents cell proliferation in breast cancer through SMAD2/3-dependent regulation of cell cycle arrest genes [48]. Similarly, activin A acts as a tumor suppressor in neuroblastoma cells via inhibition of angiogenesis, a key feature of tumorigenesis. Inhibition of endothelial cell proliferation can be achieved by active forms of SMAD2 and SMAD3, suggesting this inhibitory effect is SMAD2/3 dependent [49]. Moreover, activin A has also been reported to prevent the proliferation of tumor cells derived from the prostate and gall bladder [50,51].

Despite the above anti-tumorigenic effects of activins in extragonadal tissues, activins promote tumor development in the gonads [28,52]. The tumorigenic roles of activins have been suggested by the \textit{Inha} knockout mouse model [21], and the inhibin-deficient mouse model has been exploited to gain a deep understanding of the activin signaling pathway in gonadal tumor development. The \textit{Inha}/SMAD3 double knockout mice generated in our previous study highlights the importance of activins in...
gonadal tumorigenesis [28]. Since deletion of SMAD3 only delays ovarian tumor development in the Inha null mice [28], we were interested in determining the potential involvement of SMAD2 in mediating the potentiated activin signaling in ovarian tumors lacking inhibins.

SMAD2 and SMAD3 are functionally distinct proteins. Structural differences at the MH1 domain exist between SMAD2 and SMAD3. The extra amino acids (encoded by exon 3) in the SMAD2 MH1 domain prevents its direct binding to DNA, and specific transcription factors are required for SMAD2-DNA binding [53-55]. In contrast, SMAD3 has direct DNA-binding ability [56,57]. Additionally, SMAD3-SMAD4 signaling-dependent genes outnumber SMAD2-SMAD4 dependent genes by more than 4-fold, as identified in Hep3B cells in a recent microarray experiment [58]. Finally, distinct signaling outcomes have been identified in developing mouse Sertoli cells linked with developmentally regulated, differential use of SMAD2 and SMAD3 [59]. Despite these distinctive aspects, SMAD2 and SMAD3 share more than 90% identity in their amino acid sequences [60], and functional redundancy between these two proteins has been demonstrated in the ovary [58,38].

Our current findings, in combination with our previous results, indicate that SMAD2 and SMAD3 may function redundantly to mediate gonadal tumorigenesis in inhibin-deficient mice. In the case of conditional deletion of Smad2, SMAD3 compensates for the deficiency of SMAD2 and transduces essential signals contributing to ovarian tumor development; consequently, tumorigenesis is not altered. On the other hand, loss of SMAD3 in the Inha null mice attenuates but does not prevent ovarian tumor development, suggesting that SMAD2 may partially compensate for the loss of SMAD3. However, our model does not rule out the potential involvement of SMAD-independent signaling in inhibin-deficient ovarian tumor development or the possibility that SMAD2 may not be involved in gonadal tumor development.

Conclusions
SMAD2 is not required for mediating tumorigenic signals of activin in ovarian tumor development caused by loss of inhibin.

Abbreviations
Inha: inhibin α; cKO: conditional knockout; TGFβ: transforming growth factor β; ACVR: activin receptor; WT: wild type.

Competing interests
The authors declare that they have no competing interests.

Figure 5 Hypothetical working model for SMAD2/3 signaling in mediating gonadal tumorigenesis in inhibin-deficient female mice. (A) In the WT ovary, the signaling of activins is finely tuned by inhibins, which is important in the maintenance of normal granulosa cell function, follicular development, and fertility. (B) In the absence of inhibins, activin signaling is potentiated with increased production of activins by the gonads and tumors due to the loss of antagonism by inhibins. Superphysiological levels of activins can signal through both SMAD2 and SMAD3 in the ovary. As demonstrated by the Inha/Smad3 double knockout mouse model [28], ovarian tumor development is attenuated in Inha null mice lacking SMAD3, implying that the function of SMAD3 is not fully compensated by SMAD2. Complementarily, the Inha/Smad2 cKO mouse model generated in the current study suggests that SMAD3 can potentially mediate essential tumorigenic signals of activins in the Inha null mice (C). However, our model does not rule out the potential involvement of SMAD-independent signaling (dotted lines) in inhibin-deficient ovarian tumor development or the possibility that SMAD2 may not be involved in gonadal tumor development.
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References


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