

SMAD4 promotes somatic-germline contact during murine oocyte growth

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Abstract

Development of the mammalian oocyte requires physical contact with the surrounding granulosa cells of the follicle, which provide it with essential nutrients and regulatory signals. This contact is achieved through specialized filopodia, termed transzonal projections (TZPs), that extend from the granulosa cells to the oocyte surface. Transforming growth factor (TGF β) family ligands produced by the oocyte increase the number of TZPs, but how they do so is unknown. Using an inducible Cre recombinase strategy together with expression of green fluorescent protein to verify Cre activity in individual granulosa cells, we examined the effect of depleting the canonical TGF β mediator, SMAD4. We observed a 20-50% decrease in the total number of TZPs in SMAD4-depleted granulosa cell-oocyte complexes, and a 50% decrease in the number of newly generated TZPs when the granulosa cells were reaggregated with granulosa cell-free wild-type oocytes. Three-dimensional image analysis revealed that TZPs of SMAD4-depleted cells were also longer than controls and more frequently oriented towards the oocyte. Strikingly, the transmembrane proteins, N-cadherin and Notch2, were reduced by 50% in these cells. SMAD4 may thus modulate a network of cell adhesion proteins that stabilize the attachment of TZPs to the oocyte, thereby amplifying signalling between the two cell types.

Impact statement

Genetic deletion of SMAD4 in the granulosa cells of growing ovarian follicles reduces the number of transzonal projections that contact the oocyte, implicating TGF β -family signaling in maintaining soma-germ line communication.

eLife assessment

This study reports an **important** mechanism through which the TGF-beta signaling pathway promotes contacts between oocytes and their surrounding somatic cells by regulating the number of transzonal projections (TZPs) in mice. **Convincing** data support the conclusions. The work will be of interest to biomedical researchers who work on ovarian biology and female fertility.

Introduction

The development of a healthy oocyte within the ovarian follicle requires continuing contact between the germ cell and the somatic granulosa cells that surround it (El-Hayek and Clarke, 2016 [↗](#), Clarke, 2018 [↗](#), Richani et al., 2021 [↗](#), Robert, 2021 [↗](#), Li and Albertini, 2013 [↗](#), Doherty et al., 2022 [↗](#), Xie et al., 2023 [↗](#), Marchais et al., 2022 [↗](#)). Signals from the granulosa cells initiate the increase in volume of the oocyte when primordial (non-growing) follicles are activated to enter the growth phase (Zhang et al., 2014 [↗](#), Saatcioglu et al., 2016 [↗](#), Nagamatsu et al., 2019 [↗](#)). Subsequently, the granulosa cells provide nucleotides, amino acids and pyruvate to the growing oocyte via gap junctions that connect the two cell types (Xie et al., 2023 [↗](#), Clarke, 2018 [↗](#), Marchais et al., 2022 [↗](#)). When gap junctional communication between the two cell types is genetically disrupted, oocytes do not develop normally and cannot be fertilized (Gittens and Kidder, 2005 [↗](#), Simon et al., 1997 [↗](#), Winterhager and Kidder, 2015 [↗](#), Carabatsos et al., 2000 [↗](#)). Once the oocyte reaches full-size, it acquires the ability to undergo the final stage of development, termed meiotic maturation, but is prevented from precociously doing so by cyclic GMP that is produced and transferred to it by the granulosa cells, also via gap junctions (Jaffe and Egbert, 2016 [↗](#), Wigglesworth et al., 2013 [↗](#), Norris et al., 2009 [↗](#), Zhang et al., 2010 [↗](#)). In vitro, oocytes can develop normally when enclosed by granulosa cells but not when the granulosa cells are removed and provided in co-culture, further emphasizing the crucial importance of physical contact between the two cell types (O'Brien et al., 2003 [↗](#), Eppig, 1979 [↗](#), Herta et al., 2021 [↗](#)).

In primordial follicles, a single layer of squamous granulosa cells surrounds the oocyte and contact it via adherens junctions (Mora et al., 2012 [↗](#), Jorgensen, 2013 [↗](#)). Following activation of growth, a glycoprotein-rich coat termed the zona pellucida becomes established around the oocyte (Litscher and Wassarman, 2020 [↗](#), Wassarman and Litscher, 2018 [↗](#)). The zona pellucida steadily thickens as growth continues, reaching a final thickness of about 7 μm in mice and 15 μm in humans. Despite the barrier imposed by the zona pellucida, the granulosa cells and oocyte maintain direct contact by a dense network of thin filopodia-like projections, termed transzonal projections (TZPs), that grow out from the granulosa cells through the zona pellucida (Clarke, 2022 [↗](#), Macaulay et al., 2014 [↗](#), Baena and Terasaki, 2019 [↗](#), Motta et al., 1994 [↗](#), Zhang et al., 2021 [↗](#), Albertini and Rider, 1994 [↗](#)). The tips of the TZPs establish contact with the oocyte plasma membrane through heterotypic adherens junctions comprising N-cadherin (granulosa cell) and E-cadherin (oocyte) and harbour the gap junctions that link the two cell types (Mora et al., 2012 [↗](#)) and likely also through focal adhesions (McGinnis and Kinsey, 2015 [↗](#)). New TZPs are generated throughout follicular growth, thereby increasing intercellular molecular trafficking (El-Hayek et al., 2018a [↗](#)). A genetically imposed decrease in the number of TZPs leads to defects in oocyte gene expression and to abnormal meiotic maturation and embryonic development (Crozet et al., 2023 [↗](#)). Despite the essential role of TZPs during oocyte development in mammals, as well as the presence of analogous structures in non-mammalian species (Kessel et al., 1985 [↗](#), Schroeder, 1981 [↗](#)), the molecular mechanisms that govern their generation and stability are not yet understood.

The TGF β signalling pathway regulates the functions and interactions of a wide range of cell types under various physiological and pathological conditions (Derynck and Budi, 2019 [↗](#), Peng et al., 2022 [↗](#)). Binding of the ligand to a complex of type I and type II receptors located at the cell surface activates signalling within the cell, most commonly through the members of the SMAD family of transcription factors, although SMAD-independent signaling can also occur (Aashaq et al., 2022 [↗](#), Derynck and Budi, 2019 [↗](#)). Upon phosphorylation of the tetrameric type II and type I receptors and subsequent phosphorylation of receptor-activated SMAD proteins (R-SMADs; SMAD1, 2, 3, 5 and 8), a heterotrimeric complex with SMAD4 is formed which enters the nucleus and regulates gene expression by binding to specific SMAD-binding DNA elements. Although R-SMADs can signal in the absence of SMAD4, evidence indicates that the presence of SMAD4 in the

heterotrimer amplifies TGF β cell signalling by stabilizing the complex and by favouring the binding of other transcriptional co-repressors or co-activators of target genes (Aragón et al., 2019 [↗](#), Guglielmi et al., 2021 [↗](#)).

Genetic, pharmacological and in vitro studies have revealed multiple roles for TGF β /SMAD signaling during follicular growth and development (Patton et al., 2021 [↗](#), Pangas and Rajkovic, 2015 [↗](#)). TGF β signalling via SMAD3 in granulosa cells acts initially in primordial follicles to maintain their quiescence (Granados-Aparici et al., 2019; Wang et al., 2014) and later, following their activation, becomes excluded from the nucleus in association with rapid granulosa cell growth and proliferation (Hardy et al., 2018). The TGF β superfamily members, growth-differentiation factor (GDF) 9, which signals through SMAD2/3, and bone morphogenetic protein (BMP) 15, which signals through SMAD1/5/8, are produced by the oocyte (Dong et al., 1996 [↗](#), Yan et al., 2001 [↗](#)). Female mice lacking GDF9 rarely generate more than a single layer of granulosa cells (Elvin et al., 1999a [↗](#), Elvin et al., 1999b [↗](#), Dong et al., 1996 [↗](#)). This defect can be rescued by preventing the production of inhibin, and the doubly mutant follicles can develop at least to the pre-antral stage (Wu et al., 2004 [↗](#)). Genetic inactivation of SMAD4 in the granulosa cells from growing follicles leads to a reduction in the number of antral follicles and ovulated eggs, as well as the appearance of luteinized follicles containing entrapped oocytes (Pangas et al., 2006 [↗](#)). Similar phenotypes are observed when both SMAD2 and SMAD3 are inactivated in the granulosa cells (Li et al., 2008 [↗](#)). These effects are likely due at least in part to a failure of the cumulus layer to expand in response to the ovulatory surge of LH (Pangas et al., 2006 [↗](#), Li et al., 2008 [↗](#)), as expansion is known to require SMAD signaling and to be required for ovulation (Dragovic et al., 2007 [↗](#), Hao et al., 2022 [↗](#), Mottershead et al., 2015 [↗](#), Peng et al., 2013 [↗](#), Yu et al., 2013 [↗](#)).

Two lines of evidence point to a potential role for SMAD signaling in the generation or maintenance of TZPs. First, deletion of GDF9 leads to both a decrease in the number of TZPs and a failure of a proportion of the TZPs to correctly orient towards the oocyte (Carabatsos et al., 1998 [↗](#)). Second, TZP number is reduced in follicles grown in vitro following RNAi-mediated depletion of GDF9 in the oocyte (El-Hayek et al., 2018a [↗](#)). To test the role of SMAD4 specifically, we used a Cre-lox strategy to inactivate *Smad4* in granulosa cells, through expression of the recombinase under the control of the *Amhr2* (anti-Müllerian hormone receptor 2) promoter, as previously described (Jorgez et al., 2004 [↗](#), Pangas et al., 2006 [↗](#)). Using image analysis tools and three-dimensional reconstructions, we show that depletion of SMAD4 in the granulosa cells decreases the number of TZPs that project from individual granulosa cells in both intact granulosa cell-oocyte complexes (GOCs) and in reaggregated complexes. Morphometric parameters including length and orientation are also altered. The decrease in TZP number in intact GOCs is associated with a reduction in the amount of N-cadherin and Notch2 in the granulosa cells, suggesting that the composition of the granulosa cell surface is perturbed in the absence of SMAD4. We propose that SMAD4, through its activity to increase expression of target genes, stabilizes TZPs by promoting their adhesion to the oocyte surface, thereby helping to increase the number of TZPs and communication between the growing oocyte and its follicular microenvironment.

Results

We began by verifying the expression of SMAD4 during folliculogenesis. As shown in **Fig. 1A** [↗](#) and consistent with prior reports, SMAD4 was detectable in primordial and growing follicles as well as in the granulosa cells collected from pre-antral follicles. SMAD2 and SMAD3 were also present, with SMAD2 being the major species detected in oocytes. Two strategies were tested to deplete SMAD4 from the granulosa cells of growing follicles. First, mice were generated carrying two floxed *Smad4* alleles and a transgene encoding Cre recombinase under the control of the *Amhr2* promoter (**Fig. 1B** [↗](#)). These mice also carried a transgene (*mTmG*, see Methods) constructed such that Tomato fluorescent protein is expressed in the absence of Cre activity, whereas Cre-mediated recombination switches expression to green fluorescent protein (GFP), thus

allowing us to identify cells where Cre had been active. Upon immunostaining of ovarian sections or whole-mount intact follicles, only a small number of cells expressed GFP (**Fig. 1C**, left). Consistent with this observation, less than 10% of FACS-sorted granulosa cells were GFP-positive (**Fig. 1C**, right). Thus, in our system, *Amhr2-Cre* did not mediate efficient recombination of floxed DNA in granulosa cells. This is consistent with results described in previous reports (Pangas et al., 2006, Li et al., 2008).

We therefore turned to mice that expressed Cre under the control of a tamoxifen-inducible form of the estrogen receptor (ER) gene promoter (*ER-Cre*). Because ER is expressed in pituitary gonadotropes and SMAD4 regulates the transcription of the *Fshb* gene encoding the β -subunit of follicle-stimulating hormone (Li et al., 2018), this model could not be used in vivo. Granulosa cell-oocyte complexes (GOCs) from mid-growth stage follicles (diameter 100-120 μ m) were therefore isolated, exposed to tamoxifen for one day and then incubated in tamoxifen-free medium for another five days. Under these conditions, a substantial fraction of the granulosa cells underwent Cre-mediated recombination, as indicated by the expression of GFP in immunostained GOCs and by FACS analysis (**Fig. 1E**). This in vitro system was used for the experiments described here.

To examine the efficiency of Cre-mediated *Smad4* recombination, the mRNA level was assessed in intact GOCs using the RNAscope technique and in purified granulosa cells using qRT-PCR. Following tamoxifen treatment and culture, there was an approximately 60% reduction in the number of foci in the granulosa cells from 5.78 $\pm$ 0.36 (mean, s.e.m., n=21) in *ER-Cre⁻; Smad4^{fl/fl}* mice to 2.16 $\pm$ 0.26 (n=26) in *ER-Cre⁺; Smad4^{fl/fl}* mice ($p < 0.0001$), and a 70% reduction in the amount of PCR product from granulosa cells (n=5, $p = 0.0001$) (**Fig. 1F**). Immunoblotting of granulosa cells revealed a corresponding 70% reduction in SMAD4 protein in the granulosa cells of *ER-Cre⁺; Smad4^{fl/fl}* mice compared to *ER-Cre⁻; Smad4^{fl/fl}* controls (n=11, $p < 0.0001$) (**Fig. 1G**). Thus, the in vitro ER-Cre strategy substantially reduced although did not eliminate SMAD4 from the granulosa cells.

To examine the effect of SMAD4 depletion on the TZPs, GOCs were isolated and cultured as above, fixed and stained using anti-GFP to label cells where Cre had been active and phalloidin to label the TZPs, and imaged them using confocal microscopy. First, the TZPs were analyzed as a population. Because TZPs arise from both the innermost and more distal granulosa cell layers (Baena and Terasaki, 2019), it was not possible to reliably determine how many granulosa cells contributed the GFP-positive TZPs in a GOC. The total number of phalloidin-stained TZPs – irrespective of whether they were GFP-positive – that projected to the oocyte in equatorial optical sections of GOCs from control (*Smad4^{wt/wt}*) and experimental (*Smad4^{fl/fl}*) mice were therefore counted (**Fig. 2A, B**). TZP-number was normalized to the diameter of the corresponding oocyte, although the mean oocyte diameter did not differ between the groups (**Fig. 2B**). We observed a 20.4 $\pm$ 0.05% reduction in the number of TZPs in the GOCs from the *Smad4^{fl/fl}* mice (n=63 [control], 59 [experimental], $p = 0.002$).

A limitation of quantifying TZPs in intact GOCs is that the granulosa cells, depending on their number and spatial distribution, can variably impair their resolution and discrimination even using confocal microscopy. Therefore, the number of TZPs was also counted after mechanically removing the granulosa cells from the GOCs. This analysis revealed a striking 51.1 $\pm$ 0.04% reduction in the normalized number of TZPs in the GOCs from the *Smad4^{fl/fl}* mice (n=44 [control], 47 [experimental], $p < 0.0001$) (**Fig. 2C, D**). As observed using intact GOCs, the mean oocyte diameter did not differ (**Fig. 2D**). To verify the difference in TZP-number, we also compared *ER-Cre⁺; Smad4^{fl/fl}* to a different control genotype, *ER-Cre⁻; Smad4^{fl/fl}* mice. A quantitatively similar 43.6 $\pm$ 0.04% decrease in TZPs within the *ER-Cre⁺* population (n=164 [control], 172 [experimental], $p < 0.0001$) (**Fig. 2D**) was observed following tamoxifen treatment and culture. Together, these results indicate that depletion of SMAD4 in the granulosa cells is associated with a decrease in the number of TZPs.

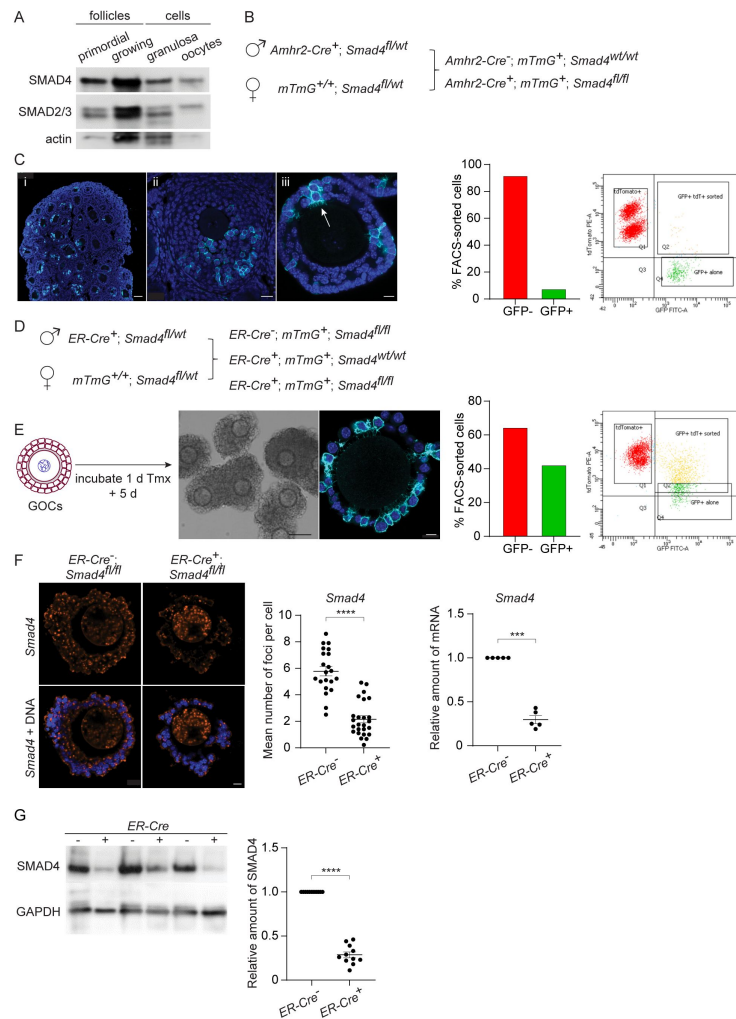


Figure 1.

SMAD4 is efficiently depleted in granulosa cells of GOCs in vitro using tamoxifen-induced Cre recombinase

A. Immunoblot of primordial follicles, growing follicles, and granulosa cells and oocytes isolated from antral follicles, probed using anti-SMAD4 and anti-SMAD2/3.

B. Breeding scheme to delete *Smad4* in the granulosa cells using *Amhr2-Cre*. The *mTmG* cassette enables Cre recombinase activity to be verified through expression of EGFP.

C. Left: Confocal images of histological sections of paraffin-embedded 12-d ovary (i, ii) and an isolated follicle (iii) stained using an anti-GFP antibody and DAPI to counterstain DNA. Right: Histogram and chart showing the percentage of FACS-sorted GFP+ and GFP-granulosa cells from growing follicles. Granulosa cells expressing both td tomato and GFP and GFP alone were scored as GFP+.

D. Breeding scheme to delete *Smad4* in vitro using generate tamoxifen-inducible *ER-Cre*. The *mTmG* cassette enables Cre recombinase activity to be verified through expression of EGFP.

E. Left: Schematic illustrating time-line of GOC exposure to tamoxifen and subsequent incubation. Center: Bright-field image of GOCs following incubation and confocal image of a representative GOC stained using anti-GFP antibody and DAPI to counterstain DNA. Right: Bar chart showing the percentage of FACS-sorted GFP+ and GFP-granulosa cells from GOCs. Granulosa cells expressing td tomato and GFP and GFP alone are counted as GFP+.

F. Left: Confocal image of representative GOCs stained with single-molecule RNA FISH probe for *Smad4* exon 8 and DAPI to counterstain DNA. Right: Mean and SEM of the average number of *Smad4* mRNA foci per cell (left) and relative mRNA amounts of *Smad4* quantified using qRT-PCR (right) in granulosa cells five days after tamoxifen treatment.

G. Immunoblot and plot of mean and SEM of SMAD4 protein content in granulosa cells from GOCs five days after tamoxifen treatment.

Statistical analysis using t-test (F, left) and one-sample t-test (F, right and G), ***P<0.001; ****P<0.0001.

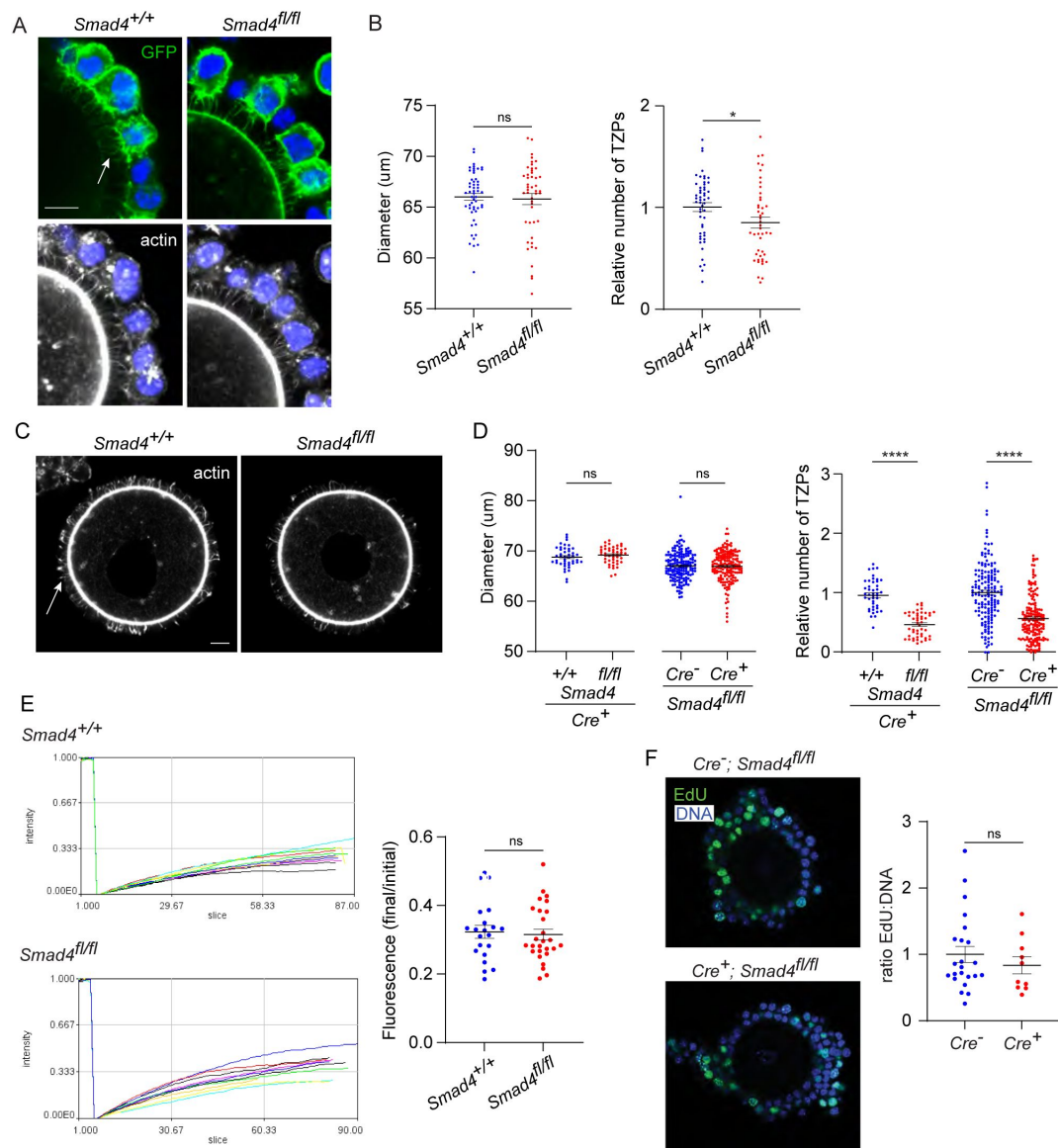


Figure 2.

Depletion of SMAD4 reduces TZP-number but does not affect granulosa cell communication with the oocyte or proliferation.

A. Confocal images of GOCs five days after tamoxifen treatment stained with anti-GFP, phalloidin to visualize F-actin and DAPI to counterstain DNA. Arrow shows GFP-labelled TZPs. Scale bar = 20 μm.

B. Mean and SEM of oocyte diameter and TZP number five days after tamoxifen treatment. TZP number in each GOC is normalized to the oocyte diameter.

C. Confocal image of a denuded GOC five days after tamoxifen treatment and removal of the granulosa cells, stained with anti-GFP, phalloidin to visualize F-actin and DAPI to counterstain DNA. Arrow shows phalloidin-labelled TZPs that remain embedded in the zona pellucida. Scale bar = 20 μm.

D. Mean and SEM of oocyte diameter and TZP number five days after tamoxifen treatment in denuded GOCs. TZP number for each specimen is normalized to the oocyte diameter.

E. Left: Time course of fluorescence recovery in oocyte of GOCs following bleaching. Each line represents an individual GOC. Right: Mean and SEM of the ratio of the final to pre-bleach fluorescence in each GOC.

F. Left: Confocal image of a GOC after tamoxifen treatment followed by treatment with EdU and visualization. DNA stained using DAPI. Scale bar = 20 μm. Right: Mean and SEM of the ratio of Edu-labelled cells to the total number of cells.

Statistical analysis using t-test, * $P < 0.05$; **** $P < 0.0001$; ns, not significant.

TZPs provide the physical platform that enables essential gap junctional communication between the granulosa cells and the oocyte. To test whether the reduction in TZP number in SMAD4-depleted granulosa cells impaired this communication, we used a fluorescence recovery after photobleaching (FRAP) assay. Control and experimental GOCs were treated with tamoxifen and incubated as above. After five days, they were exposed to calcein-AM, a fluorescent dye that can pass through gap junctions. The fluorescence in the oocyte of each GOC was bleached using the laser of a confocal microscope and its recovery was then recorded. As shown in **Fig. 2E**, which plots the fluorescence in the oocyte as a function of time after photobleaching and at the end of the recording period normalized to the initial fluorescence, we observed no difference between groups in the rate of transfer of fluorescence from the granulosa cells to the oocyte. Thus, despite the decreased number of TZPs, depletion of SMAD4 did not detectably impair gap junctional communication between the granulosa cells and the oocyte.

As TZPs project not only from the cells immediately adjacent to the zona pellucida but also from those in more distal layers, it was possible that the reduced number of TZPs in *ER-Cre⁺; Smad4^{fl/fl}* complexes reflected an impaired ability of the granulosa cells to proliferate. *ER-Cre⁺; Smad4^{+/+}* and *ER-Cre⁺; Smad4^{fl/fl}* GOCs were incubated for 5 hr in the presence of the thymidine analogue, EdU, added five days after the tamoxifen exposure. No difference between the groups in the fraction of EdU-positive cells was observed (**Fig. 2F**), however, implying that the reduced number of TZPs was not due to impaired proliferation of the granulosa cells.

We then focused our analysis on individual granulosa cells. GOCs where individual GFP-positive cells could be readily identified and the TZPs were easily attributable to a particular cell (**Fig. 3A**) were selected, serial optical sections were obtained using confocal microscopy, and 3-dimensional images were reconstructed using Imaris software. **Fig. 3B** shows one such reconstruction, where GFP-positive granulosa cells are pseudo-coloured in green. We observed a 25% decrease in the mean volume of *Smad4^{fl/fl}* granulosa cells compared to wild-type cells (323.2 $\pm$ 11.2 vs 242.6 $\pm$ 10.0; $n=293, 322$; $p<0.0001$), but no difference between genotypes in the sphericity of the cells (**Fig. 3C**). The TZPs in a subset of the GFP-positive granulosa cells were then analyzed (**Fig. 3D**). In this subset, there was a 15% difference in mean cell volume (392.2 $\pm$ 20.8 vs 334.0 $\pm$ 21.2; $n=55, 63$) which did not reach statistical significance ($p=0.054$). However, whereas wild-type cells harbored a mean of 15.8 $\pm$ 0.87 ($n=55$) TZPs per cell, this number was reduced by 35% in *Smad4^{fl/fl}* cells to 10.5 $\pm$ 0.71 ($n=65$, $p<0.0001$). As compared to those of wild-type cells, the TZPs of the *Smad4^{fl/fl}* cells were about 10% longer (4.64 $\pm$ 0.08 μm vs 5.19 $\pm$ 0.09 μm ; $n=893, 720$; $p<0.0001$) and very slightly narrower (0.40 $\pm$ 0.003 μm vs 0.38 $\pm$ 0.003 μm ; $n=870, 719$; $p<0.0001$) (**Fig. 3E**). We did not detect other differences between the genotypes in TZP morphology. These results obtained using reconstructed 3-dimensional images of GFP-positive cells establish that the number of TZPs is reduced in individual SMAD4-depleted granulosa cells.

A limitation of analyzing TZPs in GFP-positive cells within GOCs is that this strategy cannot distinguish TZPs that were generated in a low-SMAD4 environment (i.e., after the Cre recombinase was expressed) from those generated earlier in a normal-SMAD4 environment. To ensure that we examined only TZPs that were generated after Cre had been expressed, we used our previously published reaggregation assay (El-Hayek et al., 2018a) (**Fig. 4A**). GOCs obtained from mid-growth stage follicles of *ER-Cre; mTmG* mice carrying the appropriate *Smad4* alleles were exposed to tamoxifen for one day and then incubated for an additional four days. The granulosa cells were then separated from the oocyte and combined with an oocyte that did not carry the *mTmG* gene. These reaggregated GOCs were incubated for an additional 6 days, then fixed and stained using anti-GFP (**Fig. 4B**).

When the entire population of granulosa cells in the reaggregated GOCs was analyzed, there was a ~30% reduction in mean volume in the *Smad4^{fl/fl}* cells as compared to *Smad4^{+/+}* cells (394.5 $\pm$ 18.8 vs 279.8 $\pm$ 16.6; $n=185, 159$; $p<0.0001$) and no difference in cell sphericity (**Fig. 4C**), as observed using intact GOCs.

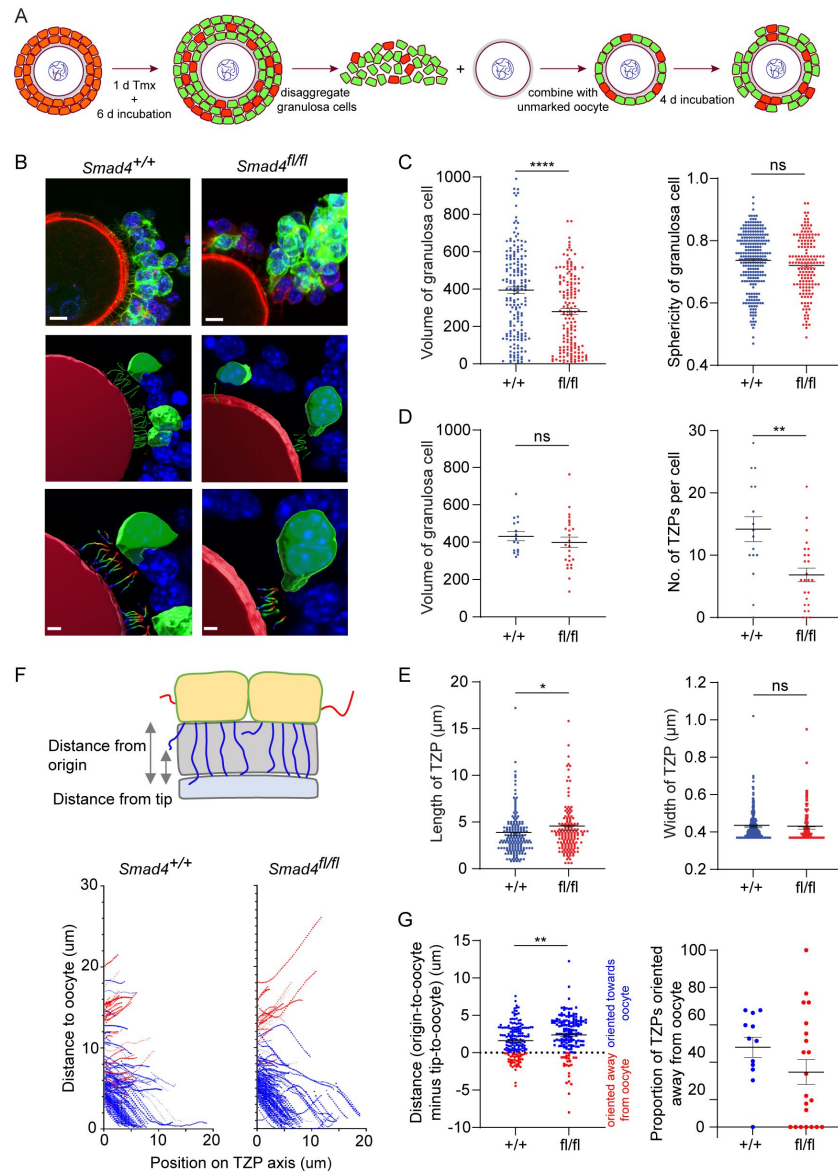


Figure 4.

Depletion of SMAD4 in reagggregates reduces the number of newly generated TZPs and alters their morphometric parameters.

A. Schematic illustrating the reaggregation procedure.

B. Three-dimensional reconstruction of a reaggregate, showing granulosa cell bodies, TZPs and oocyte cortex. Serial optical confocal sections were processed using Imaris software. GOCs were stained using anti-GFP (green), phalloidin (red) and DAPI (blue). Scale bar = 10 μ m (upper), 3 μ m (lower).

C. Mean and SEM of volumes and sphericities of individual granulosa cells.

D, E Mean and SEM of volumes of individual granulosa cells, number of TZPs per cell, TZP length and TZP width.

F. Upper: Schematic illustrating how the distance from the origin and the tip, respectively, of TZPs (blue) and non-TZP projections (red) to the oocyte surface was determined. Lower: TZP trajectories of 3D-reconstructed TZPs, representing the distance from origin relative to the position on the TZP axis.

G. Left: Mean and SEM of the difference between the distance from the origin and the tip of individual TZPs to the oocyte surface. Blue symbols (>0) represent TZPs oriented towards the oocyte; red symbols (<0) represent non-TZPs oriented away from the oocyte. Right: Mean and SEM of the proportion of TZPs in individual cells that were oriented away from the oocyte. Statistical analysis using t-test, *P < 0.05; **P < 0.01; ****P < 0.0001; ns, non-significant.

There was no difference between genotypes, however, in mean volume among the cells whose TZPs were characterized (**Fig. 4D**). Within this group, the mean number of TZPs per cell was reduced by ~50%, from 14.2 $\pm$ 2.0 (n=16) in the *Smad4*^{+/+} cells to 6.84 $\pm$ 1.1 (n=25) in the *Smad4*^{fl/fl} cells (**Fig. 4D**, $p=0.001$). A ~20% increase in the mean length of the TZPs of *Smad4*^{fl/fl} cells (3.71 $\pm$ 0.17 vs 4.40 $\pm$ 0.28; n=174, 154; $p=0.03$) was also detected, as in intact GOCs, but no difference in the mean width (**Fig. 4E**).

The increased mean length of the TZPs of *Smad4*^{fl/fl} cells compared to *Smad4*^{+/+} cells prompted us to analyze the trajectory of individual TZPs. We traced their path from their point of origin to their tip, then measured the distance between the point of origin and the oocyte and subtracted from this the distance between the tip and the oocyte. A large positive difference indicates that the tip of the TZP is relatively close to the oocyte; conversely, a small positive or a negative difference indicates that that tip is relatively far from the oocyte (**Fig. 4F**). This analysis revealed that the mean difference was 50% greater in the *Smad4*^{fl/fl} cells compared to *Smad4*^{+/+} cells (1.61 $\pm$ 0.16 vs 2.40 $\pm$ 0.21; n=174, 157; $p=0.003$) (**Fig. 4G**). This indicates that the tips of the TZPs of the *Smad4*^{fl/fl} cells were, on average, closer to the oocyte than those of the *Smad4*^{+/+} cells. We also found that the proportion of TZPs that were oriented away from the oocyte trended towards being greater in the *Smad4*^{+/+} cells, although the difference did not reach statistical significance (**Fig. 4G**). Taken together, the trajectory analyses provide additional evidence that the TZPs of *Smad4*^{fl/fl} cells differ from those of *Smad4*^{+/+} cells.

The observation that TZP-number was modestly reduced when intact GOCs were analyzed, yet markedly reduced when oocytes from which the granulosa cells had been stripped were analyzed, suggested that the attachment between the TZPs and oocyte might be weakened when SMAD4 was depleted. To evaluate this, the amount of N-cadherin, which is the principal cell adhesion molecule that has been identified in granulosa cells, was compared between *Smad4*^{+/+} and *Smad4*^{fl/fl} granulosa cells. Consistent with the previous results (see **Fig. 1E**), SMAD4 was reduced by 73.5 $\pm$ 0.05% in the *Smad4*^{fl/fl} granulosa cells (**Fig. 5A**, $g_n=6$, $p<0.0001$). Strikingly, N-cadherin was reduced by 52.7 $\pm$ 0.04 % (n = 6, $p=0.0004$) in the *Smad4*^{fl/fl} granulosa cells (**Fig. 5A**). Moreover, Notch2, a transmembrane receptor expressed in granulosa cells that associates with membrane-bound Jagged1 expressed on the oocyte, was reduced by 52.6 $\pm$ 0.05% (n=5; $p=0.0003$) in these cells (**Fig. 5B**). In contrast to the protein, however, there was no detectable reduction in the amount of encoding mRNA in either case as assayed using qRT-PCR, although *Smad4* was reduced in the same samples (**Fig. 5C**). Thus, a reduction in SMAD4 in granulosa cells is associated with a reduction in certain transmembrane proteins, including those responsible for cell adhesion.

As we previously reported that depletion of GDF9, which is secreted by the oocyte and signals in the granulosa cells through SMAD4, was associated with a reduction in mRNAs encoding structural components of TZPs – *Myo10*, *Fscn1*, and *Daam1* (El-Hayek et al., 2018a), we measured the quantity of these mRNAs using qRT-PCR. We found that they were not depleted in the *Smad4*^{fl/fl} granulosa cells compared to the *Smad4*^{+/+} cells (**Fig. 5D**). This implies either that the SMAD4 which remained was sufficient to induce expression of these genes or the GDF9 regulates their expression through a SMAD4-independent pathway.

Discussion

We have examined the role of SMAD4 establishing the TZPs that connect the granulosa cells to the oocyte. Using a tamoxifen-inducible Cre recombinase strategy, we were able to reduce SMAD4 in the granulosa cells to about 30% of its wild-type level. This reduction was associated with a decrease ranging from 20-50% in the number of TZPs in the granulosa cells assessed as a population (i) in intact GOCs and (ii) after removal of the granulosa cell bodies to increase optical resolution, and as individual cells (iii) in intact GOCs and (iv) in reconstructed complexes. Previous studies where SMAD4, or both SMAD2 and SMAD3, was depleted from granulosa cells described a

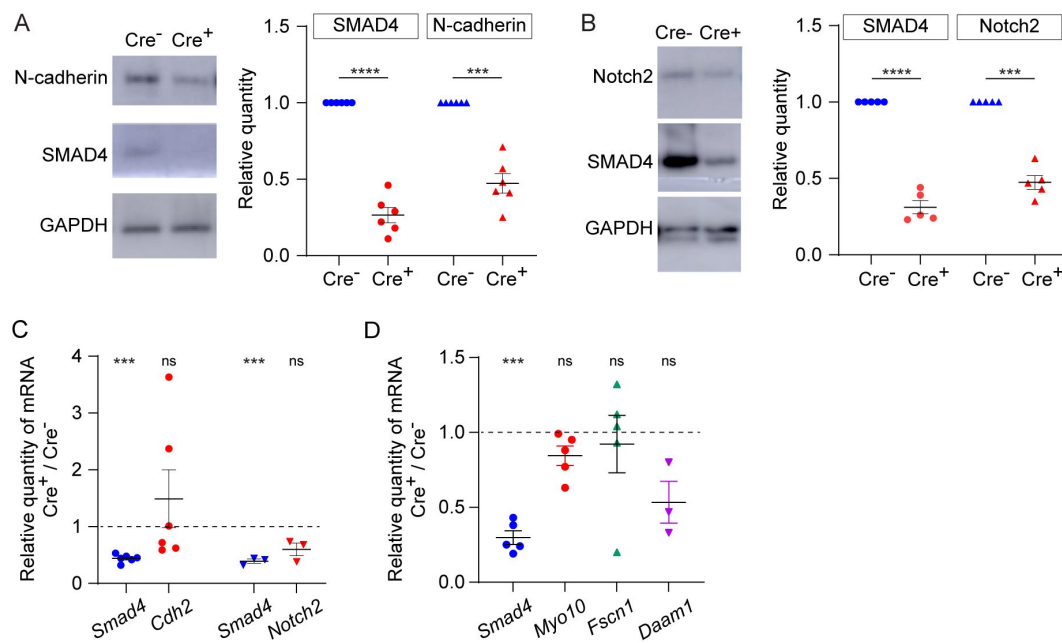


Figure 5.

Expression of transmembrane N-cadherin and Notch proteins is reduced in SMAD4-depleted granulosa cells.

A. Immunoblot and graph showing mean and SEM of SMAD4 and N-cadherin in granulosa cells. Protein quantities are normalized to amounts in ER-Cre⁻ cells.

B. As in A, showing Notch2.

C. Mean and SEM of relative mRNA amounts of *Smad4*, *Cdh2* (encoding N-cadherin), and *Notch2* in granulosa cells purified from GOCs five days after tamoxifen treatment. mRNA quantities are normalized to amounts in ER-Cre⁻ cells.

D. As in C, showing *Smad4*, *Myo10*, *Fscn1* and *Daam1*.

Statistical analysis using one-sample t-test, ***P < 0.001; ****P < 0.0001; ns non-significant.

loose or loss association of the cumulus cells with the oocyte in some follicles (Pangas et al., 2006 [↗](#), Li et al., 2008 [↗](#)). Moreover, as described in the Introduction, blocking production of GDF9 by the oocyte leads to a decrease in the number of TZPs (Carabatsos et al., 1998 [↗](#), El-Hayek et al., 2018a [↗](#)). Together these observations suggest that SMAD4 regulates the size of the population of TZPs in growing follicles.

The population of TZPs presumably reflects the net result of the generation of new TZPs and the retraction of existing TZPs, as is the case for filopodia (Gallop, 2020 [↗](#), Heckman and Plummer, 2013 [↗](#), Mattila and Lappalainen, 2008 [↗](#)). SMAD4 could thus promote either or both processes. The observation that GFP-expressing granulosa cells in reaggregated complexes generated new TZPs implies that SMAD4 is not required for this process, although it does not rule out a role in increasing the rate of generation. A clue to the function of SMAD4 may lie in the observation that the decrease in the number of TZPs in SMAD4-depleted cells was quantitatively greater when the granulosa cell bodies were removed prior to analysis. This suggests that the process of removing the cell bodies also removed the TZPs more easily in the SMAD4-depleted cells than in wild-type cells.

In this context, it is intriguing that N-cadherin, which is thought to stabilize attachment of the TZP tips to the oocyte surface, was depleted in the mutant cells. We propose that, by increasing the quantity of N-cadherin in the granulosa cells, SMAD4 increases the strength of adhesion of the TZPs to the oocyte surface (Fig. 6 [↗](#)). This in turn would decrease the probability of retraction, thereby increasing the steady-state number of TZPs. This model is consistent with the observation that the non-attached (free-ended) TZPs of SMAD4-depleted cells were on average longer and closer to the oocyte than those of wild-type cells. Specifically, if the ability of the TZPs to establish a stable contact with the oocyte were impaired in SMAD4-depleted cells, a relatively large proportion might continue to ‘search’ for an attachment and to lengthen during the searching process.

Mechanistically, some reports have linked SMAD4 to transcription of the *Cdh2*, which encodes N-cadherin (Kang et al., 2014 [↗](#), Yang et al., 2015 [↗](#)), but we did not detect a difference in mRNA quantity between normal and SMAD4-depleted granulosa cells. In view of the reduced quantity in the latter of Notch2, also a membrane-associated protein, it may be speculated that SMAD4 might act more broadly to regulate the expression of genes whose products maintain the normal configuration of the granulosa cell plasma membrane. An alternative explanation is raised by observations that granulosa-cell depletion of SMAD4 is associated with the presence of follicles containing luteinized granulosa cells enclosing an entrapped oocyte and increased expression of markers of luteinization, including *Cyp11a1*, *Sfrp4*, *Star* and *Ptgfr* (Pangas et al., 2006 [↗](#)). This suggests that the SMAD4-depleted granulosa cells in the GOCs studied here might have become luteinized, and that luteinization in turn caused a reduction in the number of TZPs. We did not detect increased expression of the luteinization markers (unpublished data), suggesting that luteinization did not occur under the conditions of our experiments. This difference may arise because we examined GOCs that had been maintained in vitro, whereas the previous study examined follicles in intact ovaries of prepuberal mice following injection of an FSH analogue.

Regardless of the mechanism linking SMAD4 to TZP number, it is noteworthy that the number of TZPs that project from an individual granulosa cells – about 15 in this study and about 40 in a previous study where more advanced (antral) follicles were examined (Baena and Terasaki, 2019 [↗](#)) – considerably exceeds the number of filopodia that are present on individual blastomeres of mouse embryos (5-6) (Fierro-Gonzalez et al., 2013 [↗](#)) or on chick embryonic cells (~4) (Sagar et al., 2015 [↗](#)). SMAD4 may therefore act in granulosa cells to amplify an innate ability to generate filopodia, which become TZPs and increase the efficiency of communication between the growing germ cell and its somatic microenvironment.

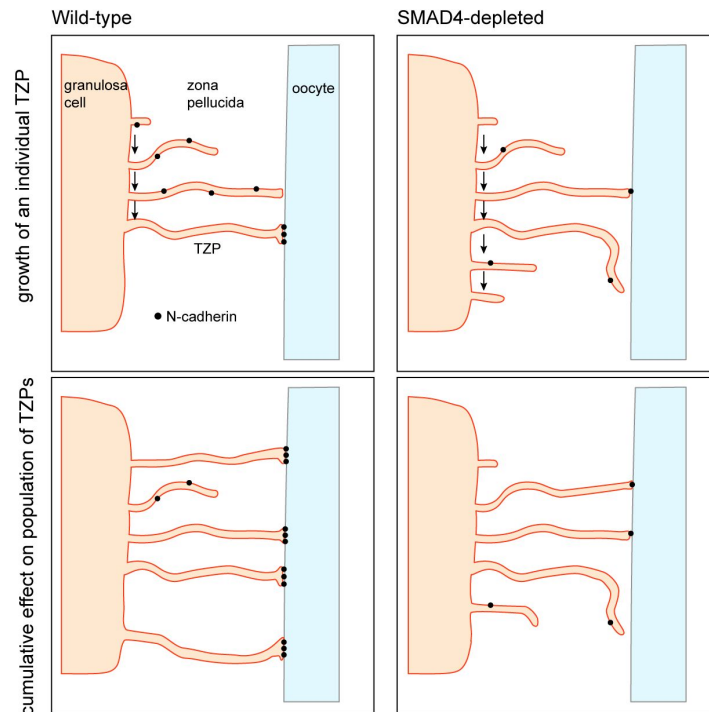


Figure 6.

Model for regulation of the size of the TZP population.

Upper: Dynamics of an individual TZP. When growing TZP contacts the surface of the oocyte, it becomes stably attached if there is sufficient N-cadherin, as in wild-type cells. In SMAD4-depleted cells, there is insufficient N-cadherin to stabilize the attachment. The TZP may continue to elongate but eventually becomes retracted. Lower: Dynamics of the population of TZPs. Owing to increased stability of attachment of individual TZPs to the oocyte surface, the total number of TZPs becomes larger in wild-type cells than in SMAD4-depleted cells.

Methods

Key Resources Table				
Reagent type	Designation	Source or reference	Identifiers	Additional information
gene (mouse)	<i>Smad4</i>	GenBank	Gene ID: 17128	
Strain (mouse)	<i>Smad4</i> ^{tm2.1Cxd} /J	Jackson Laboratories	strain 017462	Supplied by Dr. Daniel. J. Bernard, McGill University
Strain (mouse)	<i>Amhr2</i> ^{tm3(cre)Bhr}	MMRRC	strain 014245-UNC	Supplied by Dr. Makoto Nagano, McGill University
Strain (mouse)	B6.129- <i>Gt(ROSA)26Sor</i> ^{tm1(cre/ERT2)Tyj} /J	Jackson Laboratories	strain 008463	
Strain (mouse)	B6.129(Cg)- <i>Gt(ROSA)26Sor</i> ^{tm4(ACTB-tdTomato,-EGFP)Luo} /J	Jackson Laboratories	strain 007676	
RNA in situ hybridization probe	<i>Smad4</i> , exon 8	Advanced Cell Diagnostics	857571	
RNA in situ hybridization probe	<i>Bacillus subtilis DapB</i>	Advanced Cell Diagnostics	310043	
Primary antibody	SMAD2/3	Cell Signaling Technology	8685	

Primary antibody	SMAD4	Sigma	HPA019154	
Primary antibody	N-cad	BD Biosciences	B610920	
Primary antibody	Notch	Abcam	Ab8926	
Primary antibody	GFP	Invitrogen	A11122	
Secondary antibody	Goat anti-rabbit IgG-HRP	Promega	W4011	
Secondary antibody	Goat anti-mouse IgG-HRP	Promega	W4021	
Secondary antibody	Donkey anti-rabbit IgG- Alexa 488	Invitrogen	A21206	
Reagent	Tamoxifen	Sigma	T176	
Reagent	Phalloidin	Thermo Fisher	A12380	
Reagent	EdU	Thermo Fisher	C10337	

Primers				
Target	application	5-prime	3-prime	Predicted size
Smad4 -wt	Genotype	GGGCAGCGTAGCATATAAGA	GACCCAAACGTCACCTTCAG	390
Smad4 - flox	Genotype	GGGCAGCGTAGCATATAAGA	GACCCAAACGTCACCTTCAG	450
Smad4 - recombined	Genotype	TAAGAGCCACAGGGTCAAGC	GACCCAAACGTCACCTTCAG	450
Amhr2-Cre	Genotype	CTCTGGTGTAGCTGATGATC	TAATCGCCATCTTCCAGCAG	380
ER-Cre	Genotype	CGTGATCTGCAACTCAGTC	AGGCAAATTTTGGTGTACGG	150

Smad4	mRNA	TCACAATGAGCTTGCAATTCC	CCATCCACAGTCACAACAGG	143
Cdh2 (N-Cad)	mRNA	CCAGGAAAAGTGGCAGGTAG	CACTGTGCTTGGCAAGTTGT	121
Notch2	mRNA	GACCTATCCTACCCTCTAGTG	AGCAGGATGAAGAACAGGATG	103
Myo10	mRNA	TCCAGACAGACTATGGGCAGG	GGAAGCCATGTCGTCCACG	109
Fscn1	mRNA	AGAACGCCAGCTGCTACTTT	CGAGGAATCACTACCCACCG	331
Daam1	mRNA	GCGGCTGCTCAGAGTATAGAAA	AAACATGGCTTCCCTGTGTTTG	273
RPL-19	mRNA	GAAATCGCCAATGCCAACTC	CTTCCCTATGCCCATATGCC	147

Mouse strains

All experiments were performed in compliance with the regulations and policies of the Canadian Council on Animal Care and were approved by the Animal Care Committee of the Research Institute of the McGill University Health Centre (RI-MUHC, protocol 7783). CD-1 mice were obtained from Charles River (St-Constant, QC). The following transgenic strains were obtained from commercial suppliers: B6.129- *Gt(ROSA)26Sor^{tm1(cre/ERT2)Tyj}/J* (hereafter *ER-Cre*) carrying a tamoxifen-inducible sequence encoding Cre recombinase under the control of the promoter of the estrogen receptor 2 gene (Jackson Laboratories, Bar Harbor, ME, strain 008463); B6.129(Cg)- *Gt(ROSA)26Sor^{tm4(ACTB-tdTomato,-EGFP)Luo}/J* (Jackson, strain 007676; hereafter *mTmG*) carrying sequences encoding a membrane-targeted tdTomato and membrane-targeted EGFP; Cre-mediated recombination of *loxP* sites switches expression from RFP to EGFP. *Smad4^{tm2.1Cxd}/J* (hereafter *Smad4^{fl}*) carrying *loxP* sites flanking exon 8 (Jackson, strain 017462) was kindly supplied by Dr. Daniel Bernard (McGill University). *Amhr2^{tm3(cre)Bhr}* (hereafter *Amhr2-Cre*) carrying a sequence encoding Cre recombinase under the control of the promoter of the Anti-Müllerian hormone receptor 2 gene was kindly supplied by Dr. Makoto Nagano (McGill University).

To generate *Amhr2-Cre⁺*; *Smad4^{fl/fl}* females carrying an *mTmG* allele, *Smad4^{fl/wt}*; *mTmG^{+/-}* females were crossed with *Amhr2-Cre⁺*; *Smad4^{fl/wt}* males. To generate *ER-Cre⁺*; *Smad4^{fl/fl}* females carrying an *mTmG* allele, *Smad4^{fl/wt}*; *mTmG^{+/-}* females were crossed with *ER-Cre⁺*; *Smad4^{fl/wt}* males. *ER-Cre⁻*; *mTmG⁺*; *Smad4^{fl/fl}* females and *ER-Cre⁺*; *mTmG⁺*; *Smad4^{wt/wt}* females served as controls. Genotyping of the offspring was done using the EZ Tissue/Tail DNA Isolation Plus PCR kit (EZ BioResearch). After DNA extraction from tail snips, PCR was used to detect wild type, floxed and recombined *Smad4* and the presence of *ER-Cre* and *Amhr2-Cre* alleles. Primer sequences are given in Supplemental Table 1.

Tissue embedding and immunohistochemistry

Sections of paraffin-embedded ovaries were deparaffinized using xylene, rehydrated in a decreasing ethanol series (100, 90, 70%) for 3 min each, and then placed in Milli-Q water for 5 min. Slides were heated intermittently in a microwave during 20 min in 0.1 M citrate buffer solution at pH 6.0, washed in PBS for 10 min, and blocked with CAS universal blocking solution (Thermo Fisher, 008120) for 30 min at room temperature to reduce nonspecific binding. After overnight incubation with primary antibody, slides were washed three times in PBS at room temperature and then incubated for 1 h at room temperature with secondary antibody with DAPI. Slides were washed twice in PBS for 5 min and mounted on a drop of VECTASHIELD solution (Vector Laboratories, H-1200-10). Images were acquired using a Zeiss LSM 880 confocal microscope.

Collection, culture and tamoxifen treatment of granulosa cell-oocyte complexes (GOCs)

GOCs were obtained as previously described (El-Hayek et al., 2018b [DOI](#), O'Brien et al., 2003 [DOI](#)). Briefly, ovaries of 10- to 12-day-old mice were dissected from the ovarian bursa. Follicles were mechanically isolated by carefully tearing the ovaries apart and using fine needles to separate the follicles from each other in HEPES-buffered minimal essential medium (MEM, pH 7.2, Life Technologies, Burlington, ON) (MEM-H). A Pipetman P1000 was used to carefully pick up and transfer the individual follicles to dishes containing fresh medium. GOCs were obtained by incubating follicles in the presence of collagenase (10 µg/ml; Cedarlane, Burlington, ON) and DNase I (10 µg/ml; Sigma) for 5 min at 37°C in air. GOCs were collected and briefly washed in NaHCO₃-buffered MEM (MEM-C) and cultured in 4-well plates containing 500 µl of MEM-C supplemented with ITS (10 µg/mL insulin, 5.5 µg/mL transferrin and 5 ng/mL selenium; Sigma), FSH (10 mIU/ml; EMD Serono, Mississauga, ON), and estradiol (10 nM, Sigma, E2758) in an

atmosphere of 5% CO₂ in air. To induce Cre-mediated recombination of the *Smad4* allele, GOCs were incubated with 4-hydroxytamoxifen (1 µg/ml, Sigma, T176) in the above medium for 24 hours. They were then transferred to medium without tamoxifen.

Immunoblotting

Cell samples were lysed in 10 µl of Laemmli buffer. After denaturation at 95°C for 10 min, proteins were separated using 4–15% precast polyacrylamide gels (BioRad, 4568085) and transferred onto a polyvinylidene fluoride membrane (Amersham, Oakville, ON, Canada) under constant voltage. The membrane was subsequently blocked using 5% BSA, in 0.1% Tween-PBS. The membrane was washed three times in Tween-PBS and incubated with primary antibody overnight at 4°C. After washing for 45 min, the membrane was incubated in secondary antibody conjugated to horseradish peroxidase (Promega) at a dilution of 1:5000 for 1h at room temperature. After washing for 30 min, bound antibody was revealed using ECL+ (Amersham).

RNA purification and quantitative real-time PCR

RNA was extracted using a Picopure RNA isolation kit (Life Technologies) following the manufacturer's instructions and eluted in 10 µL of the provided elution buffer, as described (El-Hayek *et al*, 2018a [\[1\]](#)). Briefly, SuperScript II Reverse Transcription kit (Life Technologies) was used to generate cDNA. PCR amplification was performed using a Corbett Rotorgene 6000 (Montreal Biotech, Montreal, QC). Each reaction contained 4 µL of EvaGreen Mix (Montreal Biotech), 13 µL of UltraPure DNase/RNase-free distilled water (Life Technologies), 1 µL of 10 mM primers and 2 µL of cDNA (diluted 1:20 from original stock). Primers were designed using Primer-BLAST (National Institutes of Health) and obtained from Sigma. For each primer pair, a standard curve was generated using serial dilutions of cDNA prepared from ovarian RNA and used to determine the efficiency of amplification. Melt-curve analysis and electrophoresis of amplified products confirmed that only a single product of the expected size was generated. Data was analyzed using software provided by the manufacturer. Relative quantities of amplified product were calculated according to 2-ΔΔCT method, using *Rpl19* for normalization.

Immunofluorescence

GOCs and denuded oocytes were fixed for 15 min in freshly prepared 2% (w/v) para-formaldehyde (Fisher Scientific 04042) in PBS (pH 7.2) containing 0.1% Triton X-100 (ACROS 9002-93-1) (PBST), then washed in PBST. The specimens were incubated overnight in primary antibody in PBST at 4 °C with gentle agitation, washed twice in PBST, then incubated for 1 h at room temperature in the secondary antibody as well as phalloidin and DAPI in PBST, then washed in PBST. GOCs or denuded oocytes were placed in a 2 µl drop of PBS containing 0.3% polyvinylpyrrolidone (Sigma, PVP360) on a glass bottom dish covered with mineral oil. Images were acquired using a Zeiss LSM880 confocal microscope. Images were obtained as 16-bit RGB stack files. GOCs were imaged with a Plan Apo 63x/1.4 oil immersion objective and the following lasers and filters (in parentheses): 488 nm (491–553 nm), 561 nm (571–642) and 405 nm (410–484). Laser strength ranged between 1.5 and 2% and digital gain was adjusted until the first saturated pixels were observed for each channel.

EdU incorporation and detection

EdU (5-ethynyl-2'-deoxyuridine) detection was performed on GOCs according to the Click-iT EdU Alexa Fluor 488 Imaging Kit manufacturer's instructions (Molecular Probes, C10337). Briefly, cells were incubated in MEM containing 100 µM EdU for 5 hrs to allow for incorporation into newly synthesized DNA, then fixed, permeabilized, and incubated in Click-iT cocktail reaction for 30 minutes. EdU labeling was visualized by confocal microscopy and quantified with ImageJ (NIH).

In situ hybridization

GOCs were fixed in freshly prepared 2% paraformaldehyde in phosphate-buffered saline (PBS, pH 7) for 15 min at room temperature, dehydrated by passing through a gradient of ice-cold methanol (50%, 75%, 100%) for 3 minutes each, then sealed and stored at 4°. The next day, the GOCs were rehydrated using the reverse methanol gradient (75%, 50%, PBS) for 3 minutes each. Up to 10 GOCs were placed in the middle of a glass microscope slide, encircled by a hydrophobic barrier created using an Immedge pen, and incubated with protease solution (ACD, 322337) for 20 min. Slides were rinsed 3 times with PBS and processed as indicated by the manufacturer for the BaseScope Assay (Advanced Cell Diagnostics (ACD), 323971). Briefly, slides were incubated with the *Smad4* probe specific for the exon8 allele (ACD, 857571) or negative control probe against *Bacillus subtilis DapB* (ACD, 310043) at 40°C for 2 h in a HybEZ oven. Subsequent amplification and detection steps were performed as recommended by the manufacturer and slides were taken for image acquisition using a Zeiss LSM 880 confocal microscope.

Fluorescence-activated cell sorting

GOCs were dissected from the ovaries of 12-15 d-old *Amhr2-Cre⁺; mTmG⁺; Smad4^{fl/fl}* mice or were collected after tamoxifen treatment and culture in the case of *ER-Cre⁺; mTmG⁺; Smad4^{fl/fl}* mice. GOCs were dissected from *Amhr2-Cre⁻* and *ER-Cre⁻* littermates were used as negative controls of EGFP expression. They were transferred to a 4-well dish containing Ca-free MEM (Thermo Fisher, 21068028) supplement with 0.25% trypsin (Thermo Fisher, 25200056), 1mM EDTA (Sigma) and DNase I for several minutes. After pipetting up and down for a few times to dissociate the cells, fetal bovine serum was added (4% final concentration) to inactivate the trypsin. Cells were centrifuged at 1500xg for 5 minutes and supernatant was carefully removed under the dissecting microscope to better visualise the pellet. FACs buffer containing PBS, 1%FBS, 1mM EDTA and 1mg/ml of DAPI was used to resuspend the pellet. Cells were resuspended and passed through a Flowmi® 40-µm strainer (Merck,BAH136800040) to remove oocytes and cell clumps, and then kept on ice for sorting. Resulting granulosa cells were analyzed on a BD FACSaria Fusion 8.0.2 sorter at the Immunophenotyping Facility of the RI-MUHC. All events were collected for each sample. A 488-nm excitation laser was used for both Tomato and EGFP excitation since the wavelength of 488 nm falls within the peak excitation of EGFP but only at 25% of peak excitation for tdTomato. Data were analyzed using FlowJo FACS analysis software. Cells were gated for singlets (using forward scatter vs. side scatter area), viability (DAPI scatter vs. side scatter area), and fluorescence (FITC and PE) without compensation.

Measurement of granulosa cell-oocyte communication using fluorescence recovery after photobleaching (FRAP)

A protocol for FRAP was adapted from our previous study (El-Hayek & Clarke, 2015). GOCs were incubated for 15 min in MEM supplemented with calcein-AM and then for 60 min in calcein-free MEM to allow transfer of calcein from the granulosa cells into oocyte. A circular region of interest of fixed diameter (10 µm, to ensure the bleaching did not extend to the surrounding granulosa cells) was set in the center of the enclosed oocyte and subjected to 60 repetitive bleaches of 50 iterations (1 min total duration) using the argon laser at 50% transmission strength. The intensity of fluorescence in the oocyte and the layer of granulosa cells immediately surrounding it was recorded before bleaching and after the 10th, 20th, 30th, 40th, 50th, and 60th bleaching. Fluorescence was calculated as intensity of fluorescence in granulosa cell layer at each time point divided by the pre-bleach intensity of the layer. As oocytes of all experimental groups were subjected to the same bleaching procedure within the fixed ROI, the loss in fluorescence was further divided by initial pre-bleach intensity of the oocyte normalized to intensity of fluorescence in oocyte at the given time point, in order to correct for differences in bleaching effect in oocytes of different sizes.

Generation of granulosa cell-oocyte reagggregates

GOCs were obtained from 12-day *ER/Cre⁺; mTmG⁺; Smad4^{+/+}* and *ER/Cre⁺; mTmG⁺; Smad4^{fl/fl}* mice, exposed to tamoxifen for 24 hr and incubated for an additional five days as described above. They were then incubated in Ca-Mg-free PBS and drawn in and out of a mouth-controlled micropipette to generate a suspension of denuded oocytes surrounded by the zona pellucida and individual or small clumps of granulosa cells. Briefly, the granulosa cells were deposited together with oocytes from CD-1 mice into the base of a 200- μ l microfuge tube and PBS was added to a total volume of 100 μ l. The tube was spun at top speed in a microcentrifuge for one minute, then rotated 180° and centrifuged for one minute. This step was repeated twice more. The tube was then cut near the base and the cell-pellet was carefully scooped out and incubated for 2 days in 4-well plates in MEM supplemented with FSH, estradiol and ITS. At the end of the incubation period, complexes of oocytes surrounded by granulosa cells were fixed and processed for analysis.

Image analysis

To quantify the number of F-actin-stained-TZPs, a confocal optical section was obtained at the equatorial plane of the oocyte. Using Fiji software (National Institutes of Health, Bethesda, MD), a segmented circle was drawn around the oocyte in the middle of the zona pellucida, and the fluorescence intensity at each point on the line was obtained. Each point whose value was above the background value of the oocyte cytoplasm and higher than each of its immediately neighboring points was counted as a TZP.

To quantify EdU incorporation in granulosa cells, Fiji was used to calculate the ratio of Edu-labelled cells per GOC by dividing the number of Edu-positive cells by the total number of cells counterstained with DAPI.

To individually segment granulosa cells and their corresponding TZPs, Imaris 9.7.2 Software (Bitplane) was used. First, z-stacks of 0.2 μ m thickness covering the middle region of a follicle were imaged to obtain a conventional 2D Maximum Intensity Projection (MIP). Then, a 3D rendered segmentation of GFP-positive granulosa cells was created with the “Cell detection” tool where cell boundaries were detected based on GFP membrane staining. Smallest diameter was set to 100 μ m, membrane detail of 0.2 μ m and the threshold was adjusted based on local contrast. Segmented cells with a volume less than 100 μ m² were deleted and fusing or fragmented cells were edited using the “merge” or “split” tools to match the original image. The oocyte was also segmented using the phalloidin red channel. After edition, cells were converted to surface objects with the tool “Convert to surfaces” to record their measurements. To render individual TZPs, the “Filament tracer” tool and semi-automated tracing with the tool “Autopath” was used to draw the path of each TZP from the granulosa cell body (start) to the oocyte surface (end) (Supplemental Figure 1A). TZP diameter (thickness) was calculated automatically with a minimum diameter of 0.1 μ m. To determine the distance of the TZP tip to the oocyte surface, the segmented TZP was automatically divided into discrete spots using the extension “Filament to Spots”. This allowed the calculation of the distance from each spot to the oocyte surface. All measurements were exported using the “distance to oocyte surface” from each TZP spot. To determine the TZP orientation (away or towards the oocyte), the distance of the spot located at the TZP start was subtracted from the distance of the spot located at the TZP tip. A positive difference indicates that the TZP tip is closer to the oocyte and a negative difference indicates that the TZP tip is farther from the oocyte (Supplemental Figure 1B).

Statistical analysis

Data are presented as mean $\pm$ standard error of mean and were analyzed and represented using GraphPad Prism 8.4.2 software. Statistical significance was evaluated using one- or two-sample two-tailed t-tests.

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Supervision: S.G.A. and H.C.

Writing – original draft: S.G.A. and H.C.

Writing – review and editing: All authors have read and agreed to the final version of the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

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Reviewer #1 (Public Review):

Granados-Aparici et al., investigate somatic-germline interactions in female mice. Mammalian oocytes are nurtured in multi-cellular ovarian follicles and communication with surrounding somatic cells is critical for oocyte development. This study focused on transzonal projections (TZP) extending from granulosa cells to the surface of oocytes and document the importance of SMAD4, a TGF- β mediator, in regulating the TZPs. They propose a model in which individual TZPs contact the surface of the oocyte and stably attaches if there is sufficient N-cadherin. In SMAD4-depleted cells, there is insufficient N-cadherin to stabilize the attachment. The TZP continues to elongate but eventually retracts. Their model is well supported by their experimental evidence and the manuscript is both well-formulated and written.

Comments on revised version:

The authors have addressed the issues raised in the original review.

<https://doi.org/10.7554/eLife.91798.2.sa1>

Reviewer #2 (Public Review):

Summary:

This study proposed a new mechanism by which TGF-beta signaling pathway promotes contacts between oocyte and the surrounding somatic cells in mouse, by regulating the numbers of transzonal projections (TZPs).

Strengths:

The conditional Smad4 knockout and three-dimensional observation of transzonal projections are solid and sufficiently support the major conclusions.

Comments on revised version:

The authors have adequately addressed the reviewers' questions and comments.

<https://doi.org/10.7554/eLife.91798.2.sa0>

Author response:

The following is the authors' response to the original reviews.

Reviewer #1 (Public Review):

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Reviewer #2 (Public Review):

Summary:

This study proposed a new mechanism by which the TGF-beta signaling pathway promotes contacts between oocytes and the surrounding somatic cells in mice, by regulating the numbers of transzonal projections (TZPs).

Strengths:

The conditional Smad4 knockout and three-dimensional observation of transzonal projections are solid and sufficiently support the major conclusions.

Weaknesses:

The physiological significance of SMAD4-dependent formation of transzonal projection networks is not assessed in this study.

Previous studies have shown that physical contact and gap junctional communication with the granulosa cells is essential for normal oocyte development. A recent study has also shown that depleting Myo10 in granulosa cells reduces the number of TZPs and leads to abnormalities in oocyte and embryo development. Thus, the importance of TZPs is well-established. These findings, which were insufficiently brought out in the Introduction of the original manuscript, have now been made more clearly (Introduction, 2nd paragraph). We recognize that these reports do not directly test a role for SMAD4-dependent TZPs. Unfortunately, it is beyond our technical capacity to obtain embryos following meiotic maturation and fertilization of oocytes that have grown in vitro, which would be necessary for us to fully test the physiological role of SMAD4-dependent TZPs.

Recommendations for the authors:

Reviewer #1 (Recommendations for the authors):

(1) The authors switch from Amhr2-cre to ER-cre to increase the number of GFP-positive granulosa cells in 12 d/o ovaries. To avoid disruption of FSH secretion by SMAD4, they use an in vitro model that requires 6 days in GEO culture (1 d tamoxifen + 5 d). Could it be that Amhr2-cre didn't work because most follicles would not have reached the atretic preantral stage in 12 d/o ovaries? Did the authors consider 6 days in vitro GEO culture to determine if Amhr2-cre would be efficient and avoid exposure to tamoxifen?

Please see below.

When is Amhr2 expressed?

Previous studies (Jorgez et al, 2004; Pangas et al, 2006) report that Amhr2 is expressed in growing follicles that have progressed beyond a single layer of granulosa cells (often defined as secondary and primary follicles, respectively). As shown in Fig. 1C, we did not observe evidence of widespread Cre activity in multilayer follicles. At least two factors may contribute why we observed relatively weak Cre activity. One possibility is that, on the genetic background our mice, Amhr2 is expressed relatively late during follicular growth. Thus, we might have observed more GFP-positive granulosa cells in antral or pre-ovulatory follicles. Because the granulosa cells of these late-stage follicles would already have produced many TZPs, the number of new TZPs generated in wild-type but not SMAD4-depleted cells after Amhr2 activation would be a relatively small proportion of the total population. This would make it more difficult to detect a reduction in TZP number in the absence of SMAD4.

A second point is that we used pre-puberal mice whereas Jorgez et al examined Amhr2 expression in ovaries of adult mice. Pangas et al evaluated both prepuberal and adult females. It may be that Amhr2 is expressed earlier or more strongly in granulosa cells of adult mice. Regarding the suggestion to culture complexes obtained from mice on the Amhr2-Cre background, as this might allow widespread expression of Cre without the need for tamoxifen, this is an excellent idea. If there is considerable heterogeneity among cells in the timing of Amhr2-Cre activity, though, this may further cloud efforts to uncover the role of SMAD4 in the production or stability of TZPs, as noted above.

(2) Did most of the GEO cultured in vitro reach the antral follicle stage after 6 days?

Since GOCs were treated with collagenase, the thecal layer was removed. Therefore, development of an antrum does not occur. We observed that, in some cases, the oocyte was

extruded from the granulosa cell mass. These abnormal complexes were discarded.

(3). Was the development/diameter of the oocyte in the GEO comparable to the oocyte growing in vivo?

We did not compare the diameter of the oocytes grown in vitro to those grown in vivo. Thus, we cannot say whether the oocytes grown in vitro reached the same size as those grown in vivo. We did, however, compare the diameter of the oocytes in the wt and ko groups and observed no difference (Figure 2). This indicates that depletion of SMAD4 in the granulosa cells does not impair oocyte growth. Importantly for our studies, it excludes the possibility that the reduction in TZP-number is simply due to a smaller surface area of the oocyte.

(4) SMAD4 depletion in granulosa cells disrupts steroidogenesis leading to increased progesterone levels and precocious luteinization of granulosa cells (Pangas et al., 2006). Did the authors determine the expression level of luteal markers of granulosa cells in the in vitro GEO culture Smad4 knockout model? Are their observations direct effects of the absence of SMAD4?

This is an excellent point. We checked our previously performed RNA-seq analysis of the wild-type and knockout granulosa cells, but found no difference in the quantities of Cyp11a1, Sfrp4, Star or Ptgr. This is now described in the Discussion (4th paragraph). One potentially important difference between our study and that of Pangas et al (2006) is that they observed premature luteinization when prepuberal (3-week old) mice were injected with the FSH analogue, equine serum gonadotropin, whereas we studied granulosa-oocyte complexes cultured in vitro. This could underlie the apparent differences with respect to luteinization.

(5) Could the reduced number of TZPs in ER-cre+; Smad4^{fl/fl} GOCs be explained by luteinization?

This interesting and logical possibility is related to the previous point. In other words, luteinization could be considered as a default pathway of differentiation that is suppressed by SMAD signaling. It is possible that luteinized cells are unable to generate or maintain TZPs. This model offers a potential mechanistic basis for our observation, and we now raise it in the Discussion (3rd paragraph).

Reviewer #3 (Recommendations For The Authors):

The expression and localization of N-cadherin should be observed in Smad4 and control granulosa cell-oocyte complexes.

We agree that this would be an excellent approach to confirm the decreased expression of N-cadherin in the granulosa cells that was observed by immunoblotting. We were confronted by two challenges, however. First, we were unable to consistently obtain strong staining of granulosa cell membranes in the inner layers of multilayer granulosa-oocyte complexes. Other antibodies are able to stain structures at the oocyte surface, indicating that antibodies are not physically blocked from penetrating the complex. More likely, the anti-N-cadherin does not bind its target strongly enough to generate a robust signal that can be detected through multiple overlying layers of cells. Second, whereas for immunoblotting we collect all granulosa cells from culture complexes, for immunofluorescence we are only able to examine those that remain in the complex. This means that, for immunofluorescence, we essentially but unavoidably select against cells that are only loosely attached – as would be expected for N-cadherin-deficient cells – to their neighbours. Given these challenges, we believe that the immunoblotting approach, which produced highly reproducible results over six biological replicates (Fig. 6), is the most reliable.