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ORIGINAL ARTICLE

A novel mouse model of testicular granulosa cell tumors

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STUDY QUESTION: What is the role of dysregulated transforming growth factor beta (TGFB) signaling in the development of sex cord-stromal tumors in the testis?

SUMMARY ANSWER: Overactivation of TGFB signaling results in the development of testicular tumors resembling granulosa cell tumors (GrCTs).

WHAT IS KNOWN ALREADY: In an earlier study, we demonstrated that constitutively active TGFB receptor 1 (TGFBR1) in ovarian somatic cells promotes the development of ovarian GrCTs. However, the consequence of dysregulation of TGFB signaling in the pathobiology of the testis, remains poorly defined.

STUDY DESIGN, SIZE, DURATION: To identify the impact of dysregulation of TGFB signaling on the testis, we generated mice with constitutive activation of TGFBR1 using anti-Mullerian hormone receptor type 2 (*Amhr2*)-Cre recombinase. The effect of constitutively active TGFBR1 on testis development and the timeline of testicular tumor formation were examined. We further investigated the molecular features of testicular tumors and determined the expression of beta-catenin (CTNNB1) known to be involved in testicular GrCT development.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Male mice with constitutive activation of TGFBR1 were examined at various developmental stages (i.e. from 1 week up to 6 months) along with controls. Testis samples were collected and processed for histological and molecular analyses, including haematoxylin and eosin (H and E) staining, real-time PCR, immunohistochemistry, immunofluorescence and western blotting. Immunostaining/immunoblotting and real-time PCR experiments were performed using at least three animals per genotype. Data are presented as mean ± SEM. Statistical significance was determined using unpaired two-tail *t*-test and reported when *P* value is <0.05.

MAIN RESULTS AND THE ROLE OF CHANCE: Mice harboring constitutively active TGFBR1 in the testes developed tumors resembling testicular GrCTs, a rare type of tumors in the testis. The formation of testicular tumors led to altered cell proliferation, loss of germ cells and defective spermatogenesis. Immunohistochemically, these tumors were positive for inhibin alpha (INHA), forkhead box O1 (FOXO1), and more importantly, forkhead box L2 (FOXL2), a protein specifically expressed in the ovary and required for normal granulosa cell differentiation and function. Consistent with the immunohistochemical findings, FOXL2 proteins were only detectable in testes of TGFBR1-CA^{Acre} mice but not those of controls by western blotting, suggesting potential alteration of Sertoli cell fate. To explore mechanisms underlying the tumor-promoting effect of TGFBR1 overactivation, we examined the expression of CTNNB1. The results revealed increased expression of CTNNB1 in testicular tumors in TGFBR1-CA^{Acre} mice. Collectively, this study uncovered tumorigenic function of enhanced TGFB signaling in the testis.

LARGE-SCALE DATA: N/A.

LIMITATIONS, REASONS FOR CAUTION: This study was performed using mice, and the direct relevance of the experimental paradigm and findings to human testicular GrCTs awaits further investigation. Of note, constitutive activation of TGFBR1 was employed to enhance TGFB/SMAD signaling activity and may not be interpreted as the genetic cause of the disease.

WIDER IMPLICATIONS OF THE FINDINGS: This mouse model may prove to be a useful addition to the mouse genetics toolkit for GrCT research. Our finding that dysregulation of TGFB signaling results in the development of testicular GrCTs supports a common origin

between Sertoli cells and granulosa cells, and highlights the paramount importance of balanced TGFB signaling in reproduction and development.

STUDY FUNDING/COMPETING INTEREST(S): This research was supported by the National Institutes of Health grant R03HD082416 from the Eunice Kennedy Shriver National Institute of Child Health & Human Development and the New Faculty Start-up Funds from Texas A&M University awarded to Q.L. The authors declare no competing interest.

Key words: testicular granulosa cell tumor / gonad / TGFBR1 / SMAD2/3 / mouse model

Introduction

Transforming growth factor beta (TGFB) superfamily members, which are structurally related proteins, regulate a broad range of cellular properties, including, but not limited to growth, differentiation, migration and apoptosis (Massague, 1998; Massague *et al.*, 2000). Because of their fundamental roles in these cellular events, TGFB superfamily members exhibit diverse physiological and pathological functions in multiple organ systems. In the reproductive system, recent studies, particularly those using genetics and functional genomics approaches uncovered many previously unrecognized roles of this growth factor family in reproductive development and cancer (Massague *et al.*, 2000; Padua and Massague, 2009; Li, 2014; Fang *et al.*, 2016). TGFB signaling is known to be a double-edged sword in tumor development, exhibiting tumorigenic or tumor suppressive effects at different tumor stages (Massague, 2008). To initiate signal transduction, TGFB ligands recruit type II and type I receptor complexes and activate canonical and/or non-canonical pathways depending on the signaling context (Akhurst and Hata, 2012). In general, TGFB ligands signal through TGFBR1/TGFBR2-SMAD2/3 axis, whereas bone morphogenetic proteins (BMPs) activate BMP type II and type I receptors and the SMAD1/5/9 signaling branch. SMAD4 is a common SMAD utilized by both TGFB and BMP signaling to access gene regulatory apparatus within the nuclear compartment (Fang *et al.*, 2016).

Approximately 95% of malignant testicular tumors belong to germ cell tumors, comprising seminomas and nonseminomas (Motzer *et al.*, 2012). Sex cord-stromal tumors (3–5%) of the testis include Leydig cell tumor, Sertoli cell tumor, granulosa cell tumor (GrCT), mixed sex cord-stromal tumor and unclassified sex cord-stromal tumor (Moch *et al.*, 2016). Testicular GrCTs are rare tumors (Mohapatra *et al.*, 2016). The formation of testicular GrCTs has been reported in several mouse models (Matzuk *et al.*, 1992; Boyer *et al.*, 2009; Richards *et al.*, 2012). The pathogenesis of GrCTs is poorly understood in both genders, due in part to the limited access to human GrCT specimens. A breakthrough has been recently made toward understanding the etiology of ovarian GrCTs. Forkhead box L2 (FOXL2) is an ovary-expressed gene essential for granulosa cell differentiation (Uhlenhaut *et al.*, 2009). It was discovered that the vast majority of adult type GrCTs in the ovary bear a missense mutation of *FOXL2* (402 C→G; C134W) (Shah *et al.*, 2009). This mutation was also found in testicular GrCTs of males (Lima *et al.*, 2012), indicating the importance of *FOXL2* mutation in the pathogenesis of GrCTs. However, how *FOXL2* mutation activates tumorigenic cascade within granulosa cells is not known. Interestingly, experimental evidence supports a potential link between *FOXL2* mutation and alteration of TGFB/activin signaling in GrCT development (Rosario *et al.*, 2012; Cheng *et al.*, 2014; Fang *et al.*, 2016).

TGFB superfamily signaling is involved in the development of GrCTs (Middlebrook *et al.*, 2009). In an earlier study, we created a mouse model harboring constitutively active TGFB receptor I (TGFBR1) using anti-Mullerian hormone receptor type 2 (*Amhr2*)-Cre recombinase (Gao *et al.*, 2016). *Amhr2*-Cre has been widely used to target genes in granulosa and theca cells of the ovary, myometrial and endometrial cells of the uterus (Li *et al.*, 2008, 2011; Ren *et al.*, 2009; Wang *et al.*, 2016), and Sertoli cells and Leydig cells of the testis (Boyer *et al.*, 2008). Expression of *Amhr2*-Cre has been detected as early as 11.5 days post-coitum in the urogenital ridges of both males and females, and the Cre activity is present in the somatic cells of gonads and the mesenchyme of Mullerian ducts (Jamin *et al.*, 2002). To generate constitutively active TGFBR1, three missense mutations in the GS domain were made to activate the TGFBR1 kinase (T204D) and block the binding of the TGFBR1 inhibitor, FKBP12 (L193A/P194A) (Bartholin *et al.*, 2008). A LoxP-flanked stop sequence was engineered to direct the expression of constitutively active TGFBR1 to Cre-expressing cells/tissues. Overactivation of TGFBR1 in the ovary results in formation of ovarian tumors reminiscent of GrCTs (Gao *et al.*, 2016). Here, we report that male mice with constitutive activation of TGFBR1 in the testes developed tumors resembling testicular GrCTs. This study developed a useful mouse model for testicular GrCTs.

Materials and Methods

Ethics statement

Animal use protocol for this study was approved by the Institutional Animal Care and Use Committee (IACUC) at Texas A&M University. All animals were handled in accordance with the guidelines by the IACUC of Texas A&M University.

Animals and tissue collection

Mice harboring a latent constitutively active TGFBR1 (*TGFBR1*^{CA}) were created as described (Bartholin *et al.*, 2008; Vincent *et al.*, 2010). The *TGFBR1*$^{CA\ flox/flox}$ mice were crossed to *Amhr2-Cre* line (Jamin *et al.*, 2002) to generate mice containing constitutively active TGFBR1 in the testes (*TGFBR1*$^{CA\ flox/+}$; *Amhr2*-Cre). These mice were termed TGFBR1-CA^{Acre}. Mice were on a mixed C57BL/6/129 background and maintained by controlled light/dark cycles with free access to food and water. Testis samples were collected from both control and TGFBR1-CA^{Acre} males during developmental stages for histological and immunohistochemical analyses described below. Genotyping was performed using DNA extracted from mouse tails described previously (Bartholin *et al.*, 2008; Gao *et al.*, 2016). PCR products were separated on 1% agarose gels containing ethidium bromide and digital images captured using a VWR Gel Imager.

Histology

Testis samples from control and experimental mice were fixed in 10% (v/v) neutral buffered formalin and/or Bouin's solution for histology and immunostaining. Tissue samples were processed and embedded using the histology core lab in the Department of Veterinary Integrative Biosciences at Texas A&M University. Haematoxylin and eosin (H and E) staining was performed using standard procedures.

Immunohistochemistry and immunofluorescence

Immunohistochemistry (IHC) and Immunofluorescence (IF) were performed as described elsewhere (Gao *et al.*, 2015, 2016). In brief, paraffin sections (5 μm) were deparaffinized in xylene, rehydrated in graded alcohol and boiled in 10 mM citrate buffer (pH 6.0) for 20 min to retrieve the antigen. After antigen retrieval, sections were treated with 0.3% H_2O_2 for 30 min to quench endogenous peroxidase activity. This step was only needed for IHC. Then, sections were sequentially incubated with non-immune serum (for IHC) or bovine serum albumin (for IF), primary antibodies with indicated dilutions (Table I) overnight at 4°C and secondary antibodies conjugated with horseradish peroxidase (HRP; 1:300; for IHC) or Alexa Fluor 488/594 (1:400; for IF). Intervening washes were performed using Tris-buffered saline (TBS; pH 7.4). Following secondary antibody incubation, an avidin-biotin complex (ABC; Vector Laboratories, USA) was applied to amplify immunoreactive signals, which were developed using NovaRed substrate (Vector Laboratories; USA; for IHC). The sections were counterstained using haematoxylin, mounted with Permount and examined under BX43F microscope (Olympus; USA). For IF, sections were directly mounted with ProLong Gold Slowfade media containing 4′, 6-diamidino-2-phenylindole (DAPI; Catalog number: P36931; Invitrogen, USA) after incubation with secondary antibody. Fluorescence signals were visualized using a IX73 microscope (Olympus, USA) interfaced with an XM10 CCD camera and cellSens Software. Isotype-matched IgG controls were included to determine the staining background of the experiment.

Western blotting

Western blotting was conducted as described (Li *et al.*, 2008). Protein lysates from mouse testis tissues were prepared using radioimmunoprecipitation assay buffer containing both proteinase (Catalog number: 04 693 116 001; MilliporeSigma, USA) and phosphatase inhibitors (Catalog number: 4 906 837 001; MilliporeSigma, USA). Protein quantification was performed using bicinchoninic acid reagent purchased from Thermo Scientific (USA). Approximately 30 μg of proteins were loaded on each lane and separated by electrophoresis using 12% Mini-PROTEAN TGX Precast Gels (Bio-Rad Laboratories, USA). After electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories; USA), which were subsequently incubated with primary antibodies at 4°C overnight (Table I). After being washed thoroughly, membranes were further incubated with HRP-conjugated anti-rabbit/rat/goat secondary antibodies (1:20 000; Jackson ImmunoResearch, USA). Immunoreactive signals were developed using Immobilon Western Chemiluminescent HRP Substrate (EMD Millipore, USA). Signals were visualized and captured using Kodak Image Station 4000 mm PRO digital scanner or Bio-Rad ChemiDoc MP Imaging System. Membranes were stripped and reprobed with antibody directed to beta-actin (ACTB) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table I).

Table I Primary antibodies for immunostaining and western blotting analyses.

Name	Manufacturer	Catalog no.	Host	IHC	IF	WB
ACTA2	Abcam, USA	Ab76549	mouse		1:2000	
YBX2	Abcam, USA	Ab33164	rabbit	1:500		
Ki67	Abcam, USA	Ab16667	rabbit	1:200		
Ki67	BD Biosciences	550 609	mouse		1:20	
DMRT1	Obtained from Dr David Zarkower	N/A	rabbit		1:400	
GJA1	Cell Signaling Technology, USA	3512	rabbit	1:100		
SOX9	EMD Millipore, USA	Ab5535	rabbit	1:2000	1:2000	
WT1	Abcam, USA	Ab89901	rabbit	1:200		
INHA	Bio-Rad Laboratories, USA	MCA951ST	mouse	1:300		
FOXL2	Abcam, USA	Ab5096	goat	1:1500		1:1000
FOXO1	Cell Signaling Technology, USA	2880	rabbit	1:800		
CTNNB1	Cell Signaling Technology, USA	8480	rabbit	1:100		1:1000
CTNNB1	BD, USA	610 153	Mouse	1:100		
CYP17A1	Santa Cruz Biotechnology, USA	SC-46 081	Goat	1:1500		
pSMAD2	Cell Signaling Technology, USA	3101s	rabbit			1:1000
pSMAD2	EMD Millipore, USA	Ab38491	rabbit			1:1000
pSMAD3	Abcam, USA	Ab52903	rabbit			1:1000
SMAD2	Cell Signaling Technology, USA	5339	rabbit			1:1000
SMAD3	Abcam, USA	Ab28379	rabbit			1:1000
HA	Roche, Germany	12 013 819 001	rat			1:500
GAPDH	Cell Signaling Technology, USA	2118	Rabbit			1:1000
ACTB	Sigma-Aldrich, USA	A3854	mouse			1:100 000

Quantification of western blotting results was performed using NIH image J (version 1.51e).

RNA preparation and reverse transcription-real-time PCR

RNA samples were isolated from testes of control and TGFBR1-CA^{Acre} males using RNeasy Mini Kit (Qiagen, USA) based on instructions provided by the supplier. Potential genomic DNA contamination was eliminated by inclusion of an additional on-column DNase digestion step. Concentrations of the RNA preparations were determined by using NanoDrop Spectrophotometer ND 1000 (NanoDrop Technologies, USA). RNA samples were then subjected to reverse transcription for cDNA synthesis (Gao *et al.*, 2014). Real-time PCR was performed using a CFX Connect Real-time PCR Detection System (Bio-Rad Laboratories; USA). The 10 µl reaction system contained *iTaq* Universal SYBR Green Supermix (Bio-Rad Laboratories; USA) or TaqMan Universal PCR Master Mix, cDNA and oligo primers (Gao *et al.*, 2016) or the Taqman probe. Assays were performed in duplicates for each sample and a mean cycle threshold (CT) value was calculated. Primers for *Ccnd2* were 5′-GAGTGGGAACTGGTAGTGTTG-3′ (forward) and 5′-CGCACAGAGCGATGAAGGT-3′ (reverse) (PrimerBank ID 6 753 310a1) (Spandidos *et al.*, 2008). *Gja1* expression was analyzed using Taqman probe (Assay ID: Mm01179639_s1). Ribosomal protein L19 (*Rpl19*) was used as an internal control to normalize gene expression (Livak and Schmittgen, 2001; Gao *et al.*, 2014). A Taqman probe for *Rpl19* (Assay ID: Mm02601633_g1) was included in Taqman gene expression assays.

Statistical analysis

Comparisons of means between two groups were made using unpaired two-tail *t*-test. Data are presented as mean ± SEM. Statistical significance was defined when *P* value is <0.05 and marked as $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

Results

Generation of mice with constitutively active TGFBR1 in testes using *Amhr2*-Cre recombinase

We previously reported that constitutive activation of TGFBR1 in the ovary using *Amhr2*-Cre recombinase leads to the development of ovarian sex cord-stromal tumors reminiscent of GrCTs (Gao *et al.*, 2016). Because *Amhr2*-Cre is also expressed in Sertoli and Leydig cells of the testis (Boyer *et al.*, 2008), we examined potential effects of overactivation of TGFBR1 on the development and function of the testis by generating a mouse model harboring constitutively active TGFBR1 in the testis (Fig. 1A). As expected, TGFBR1^{CA} proteins were readily detectable in the testes of TGFBR1-CA^{Acre} males but not controls by western blotting using anti-hemagglutinin (HA) antibody (Fig. 1B). These findings support that *Amhr2*-Cre drives the expression of TGFBR1^{CA} in mouse testes. TGFBR1 phosphorylates and activates SMAD2/3 signal transducers. To verify activation of TGFB signaling in TGFBR1-CA^{Acre} testes, we performed western blotting to determine the levels of phospho-SMAD2/3 in the testes of TGFBR1-CA^{Acre} males. Results showed that the levels of both phospho-SMAD2 and phospho-SMAD3 were elevated in TGFBR1-CA^{Acre} testes compared with controls (Fig. 1B). SMAD2, SMAD3 and beta-actin (ACTB) were included as controls (Fig. 1B).

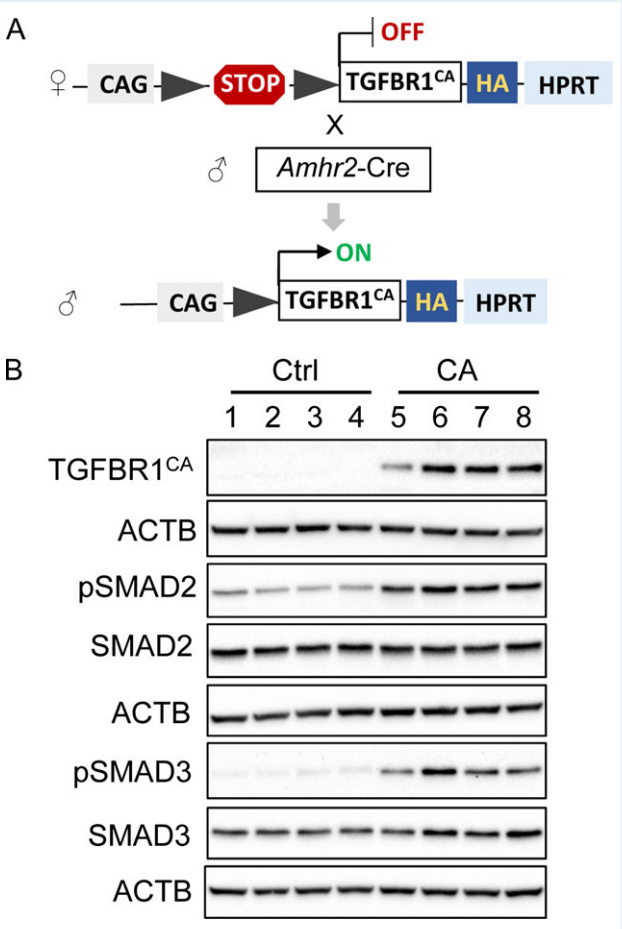

Figure 1 Constitutive activation of TGFBR1 in mouse testes. (**A**) A diagram depicting the generation of mice with constitutive activation of TGFBR1 using *Amhr2*-Cre recombinase. (**B**) Western blotting analysis of hemagglutinin (HA)-tagged TGFBR1^{CA}, phospho-SMAD2 and phospho-SMAD3 in the testes of control and TGFBR1-CA male mice at the age of 2 weeks. Each lane represents an independent testis sample. $n = 4$. SMAD2, SMAD3 and ACTB were included as controls.

TGFBR1-CA^{Acre} males develop testicular tumors with impaired spermatogenesis

Sterility was observed in TGFBR1-CA^{Acre} male mice when they were used as breeders. To determine the cause of sterility, we performed macroscopic and histological analyses of testes and epididymides of control and TGFBR1-CA^{Acre} males. Gross testicular tumors with hemorrhagic foci were observed in TGFBR1-CA^{Acre} mice around 2 months of age with full penetrance (Fig. 2A). In contrast, no tumors were observed in control mice (Fig. 2A). Testicular tumors progressed with age (Fig. 2B). Histological analysis of 2-month-old testes showed that while control mice had organized seminiferous tubules (Fig. 2C and E), TGFBR1-CA^{Acre} males developed testicular tumors resembling GrCTs (Fig. 2D and F), which were observed in the ovary of TGFBR1-CA^{Acre} female mice (Gao *et al.*, 2016). Microscopically, seminiferous tubules

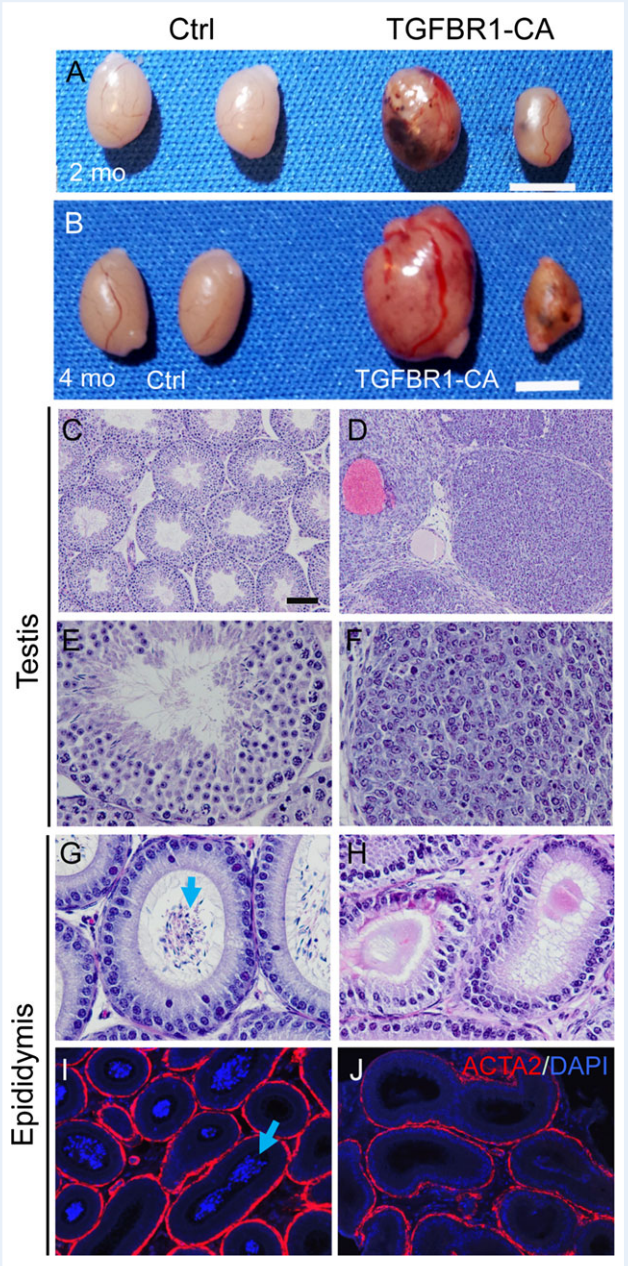

Figure 2 Sustained activation of TGFBR1 in the testis leads to sex cord-stromal tumor formation. (**A**, **B**) Development of testicular tumors in TGFBR1-CA^{Acre} males at the age of 2 and 4 months. Scale bars in panels (A and B) equal 500 μm. Images of paired testes from two controls and two TGFBR1-CA^{Acre} mice are shown. Testes from control and TGFBR1-CA^{Acre} mice around 2 months of age were examined for testicular tumor development. Macroscopic and microscopic tumors were found in all TGFBR1-CA^{Acre} males ($n = 9$) but not controls ($n = 10$). (**C**–**F**) H and E staining of testes from control and TGFBR1-CA^{Acre} males at 2 months of age. Panels (E and F) represent higher magnification images for panels (C and D), respectively. (**G**–**J**) Loss of sperm in the epididymis of TGFBR1-CA^{Acre} mice revealed by haematoxylin and eosin staining and immunofluorescence. Antibody directed to ACTA2 (red) was used to label seminiferous tubule structures. Note that controls had abundant sperm (G) and DNA (I) within the epididymis (blue arrows), while TGFBR1-CA^{Acre} males showed a lack of sperm/DNA in the epididymis (H and J). Scale bar is representatively shown in (C) and equals 25 μm (E–H), 50 μm (I and J) and 100 μm (C and D).

were displaced by circumscribed tumor nests containing poorly differentiated cells or granulosa-like cells (Fig. 2D and F and Supplementary Fig. 1B and D). Remnant seminiferous tubules lacking mature germ cells which were observed in control testes could be found near the edge of TGFBR1-CA^{Acre} testes (Supplementary Fig. 1A–D). Both histological results and IF analyses using antibody directed to smooth muscle actin alpha (ACTA2) to mark the structure of epididymis demonstrated lack of sperm in the epididymis of TGFBR1-CA^{Acre} males (Fig. 2H and J), and this was in sharp contrast to wild type controls where abundant sperm/sperm DNA could be found within the epididymis (Fig. 2G and I). Analysis of testes from 5–6-month-old TGFBR1-CA^{Acre} males demonstrated development of more hemorrhagic tumors (Supplementary Fig. 1F and H), in contrast to control testes containing morphologically normal seminiferous tubules (Supplementary Fig. 1E and G). Thus, testicular tumors resulting from constitutive activation of TGFBR1 are detrimental to normal testis development and spermatogenesis.

Disruption of testis development in postnatal TGFBR1-CA^{Acre} males

To define a timeline of testicular tumor development in the TGFBR1-CA^{Acre} males, we first performed histological analyses using testes from both control and TGFBR1-CA^{Acre} mice at the age of 1 week, 2 weeks and 1 month. No histological difference was observed in the testes of TGFBR1-CA^{Acre} mice versus controls at 1 week of age (Supplementary Fig. 2A and B). Although the basic histological structure of seminiferous tubules appeared to be maintained in the TGFBR1-CA^{Acre} testes by 2 weeks of age (Supplementary Fig. 2C and D), microscopic lesions were evident within the testes of TGFBR1-CA^{Acre} mice at 1 month of age (Supplementary Fig. 2F and H). Compared with control testes consisting of highly organized seminiferous tubules (Supplementary Fig. 2E and G), loss of germ cells occurred within the seminiferous tubules of TGFBR1-CA^{Acre} testes (Supplementary Fig. 2F and H). Enrichment of Sertoli-like cells was observed in abnormal tubules of TGFBR1-CA^{Acre} mice (Supplementary Fig. 2F). Tumor nodules were readily discernible in TGFBR1-CA^{Acre} testes at this stage (Supplementary Fig. 2H). The histological findings were confirmed by immunohistochemical staining of Y box protein 2 (YBX2, also known as MSY2), a marker for germ cells (Gu *et al.*, 1998) and Ki67 (a marker for cell proliferation). YBX2 was abundantly expressed in germ cells within seminiferous tubules in control testes (Fig. 3A). However, loss of YBX2-positive cells was pronounced in the seminiferous tubules of TGFBR1-CA^{Acre} males at 1 month of age (Fig. 3B). The adverse effect of constitutive activation of TGFBR1 on germ cell production could be observed at 2 weeks (Supplementary Fig. 2I and J). Moreover, the neoplastic tumor foci contained proliferative cells, evidenced by intense staining of Ki67 versus controls (Fig. 3C and D). Enhanced cell proliferation of TGFBR1-CA^{Acre} testes was further supported by upregulation of cyclin D2 (CCND2) protein and transcripts (Fig. 3E and F). Interestingly, double IF demonstrated colocalization of Ki67 and SOX9, a Sertoli cell marker, in 1-month-old TGFBR1-CA^{Acre} testes (yellow/orange labeling; Fig. 3J–L), in sharp contrast to control testes where SOX9 and Ki67 signals did not overlap (Fig. 3G–I). A similar result supporting Sertoli cell proliferation in TGFBR1-CA^{Acre} testes was found using 2-week-old mice (Supplementary Fig. 3). Since Sertoli cells stop proliferating around 2 weeks after birth in mice (Vergouwen

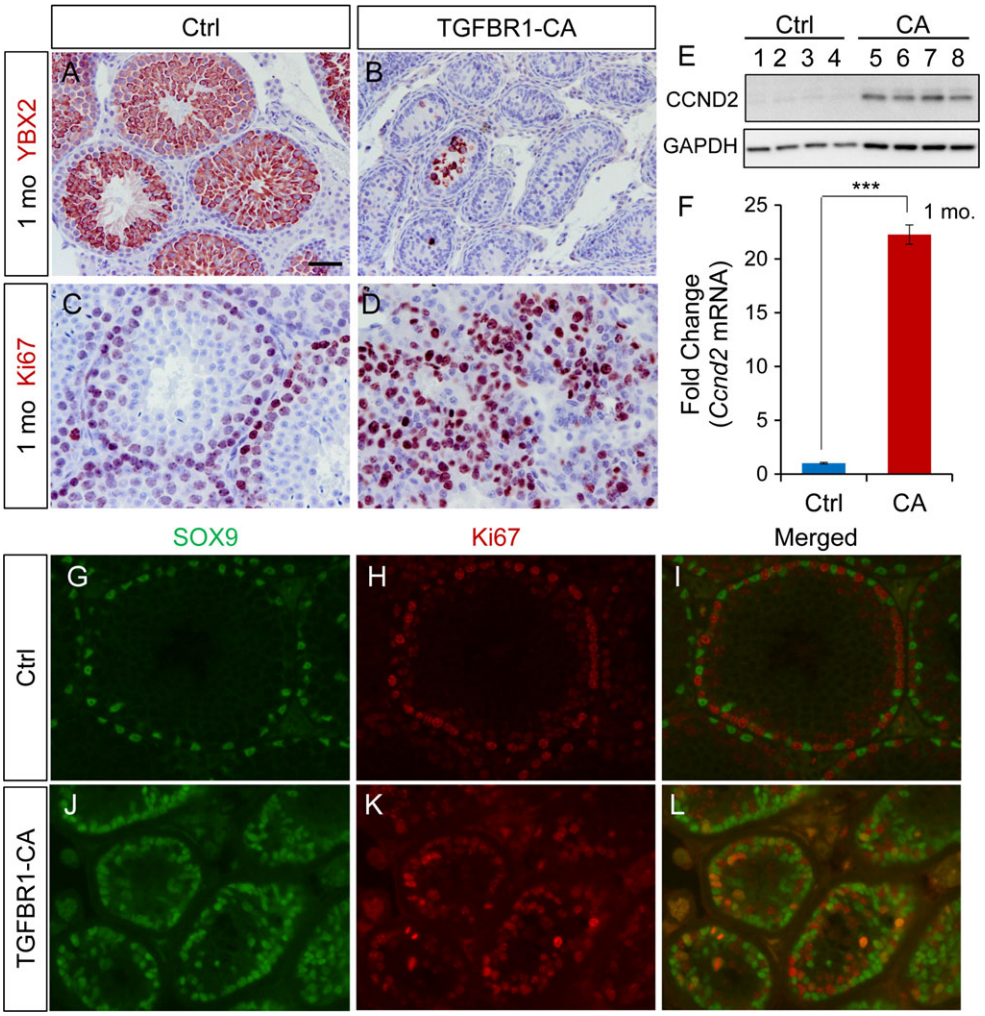

Figure 3 Depletion of germ cells and alteration of cell proliferation in the testes of TGFBR1-CA^{Acre} males. (**A**–**D**) Immunostaining of YBX2 and Ki67 in the testes of control and TGFBR1-CA^{Acre} mice at 1 month of age. Note the presence of abundant YBX2-positive germ cells within the seminiferous tubules in controls (A). However, loss of YBX2-positive cells was observed in TGFBR1-CA^{Acre} testes (B). Meanwhile, tumor foci were positively stained for Ki67 (D), indicative of cell proliferation. Three mice per group were examined. (**E**) Western blotting analysis of CCND2 expression in testes of 1-month-old control and TGFBR1-CA^{Acre} males. Each lane represents an independent testis sample. $n = 4$. (**F**) Increased expression of *Ccnd2* transcripts in TGFBR1-CA^{Acre} testes versus controls using real-time PCR. Data are presented as mean ± SEM. $n = 5$. ***$P < 0.001$. (**G**–**L**) Immunofluorescence staining of SOX9 and Ki67 using testes from control and TGFBR1-CA^{Acre} males at 1 month of age. Note the presence of double positive cells (yellow/orange) in TGFBR1-CA^{Acre} testes (L) but not controls (I). Scale bar is representatively shown in (A) and equals 25 μm (C, D and G–L) and 50 μm (A and B).

et al., 1991), our results indicate that sustained activation of TGFBR1 extends the proliferation of Sertoli cells. As gap junction protein alpha 1 (GJA1), also known as Connexin 43, is implicated in Sertoli cell development and conditional loss of GJA1 in Sertoli cells impedes the differentiation but extends the proliferation of Sertoli cells (Sridharan et al., 2007), we examined the expression of GJA1 in TGFBR1-CA^{Acre} testes to determine whether altered Sertoli cell proliferation was associated with reduced expression of GJA1. A significant change of *Gja1* mRNA abundance was not found in the testes of TGFBR1-CA^{Acre} mice at 2 weeks of age (Supplementary Fig. 4A), although localization of GJA1 proteins appeared to be increased in cells within the basal region of the seminiferous tubules (Supplementary Fig. 4B and C). At 1 month of age, mRNA levels of *Gja1* were markedly elevated and abundant immunoreactive signals of GJA1 detected in TGFBR1-CA^{Acre} testes compared with controls (Supplementary Fig. 4D–F). These results are in concordance with reports that TGFB upregulates GJA1 expression in human granulosa cells and trophoblast cells (Chen et al., 2015; Cheng et al., 2015) and suggest that altered Sertoli cell proliferation in TGFBR1-CA^{Acre} mice is not a result of deficient production of GJA1.

To further probe the cellular and molecular alterations resulting from constitutive activation of TGFBR1 in the testis, we performed IF using antibodies directed to SOX9 and ACTA2 (Fig. 4). Results showed that ACTA2-labeled seminiferous tubules (green) in the testes of TGFBR1-CA^{Acre} were structurally comparable to those of controls at 2 weeks of age, except for disorganized arrangement of Sertoli cells labeled by SOX9 (red; Fig. 4A–F). In accordance with the histological observation of enrichment of Sertoli-like cells in the dysplastic seminiferous tubules in 1-month-

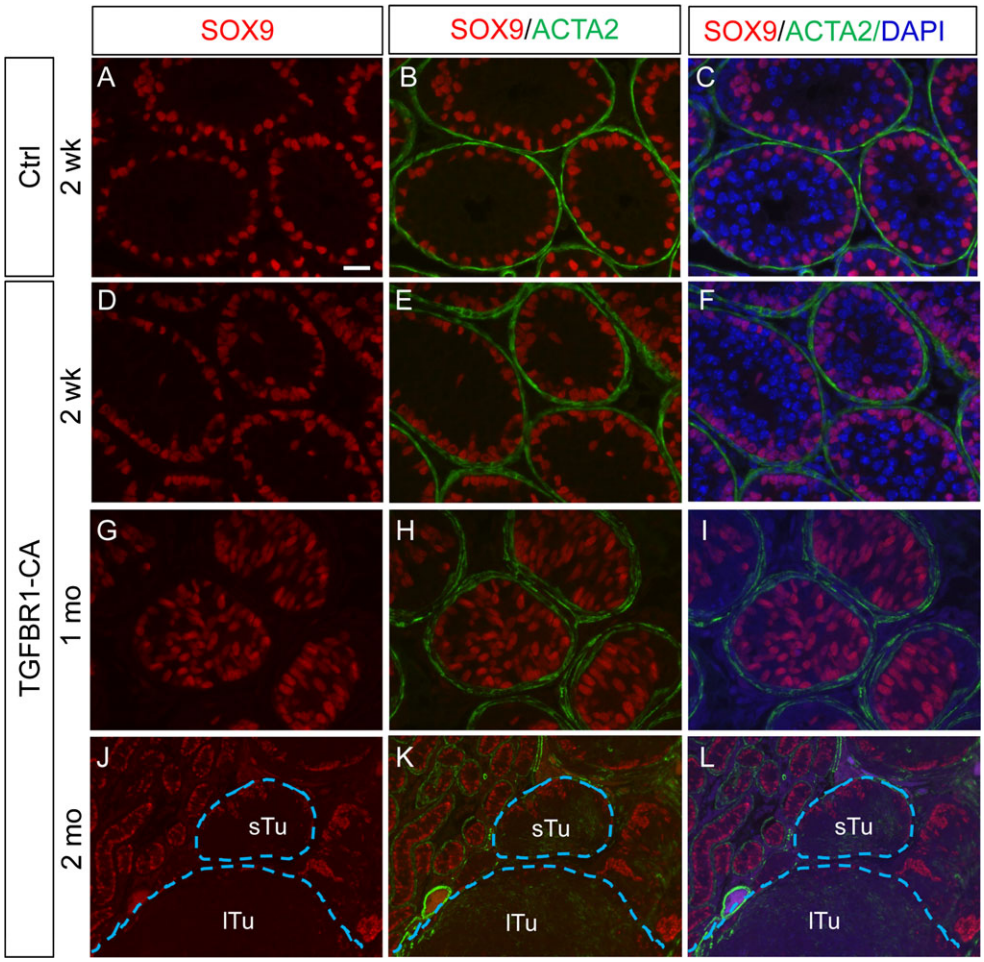

Figure 4 Immunofluorescence analysis of testicular tumor development in TGFBR1-CA^{Acre} males. (**A**–**L**) Immunofluorescence staining of SOX9 and ACTA2 using testes from control and TGFBR1-CA^{Acre} males at the age of 2 weeks, 1 month and 2 months. Note that seminiferous tubules in the testes of TGFBR1-CA^{Acre} males were structurally comparable to those of controls at 2 weeks of age (A–F), except for disorganized arrangement of Sertoli cells. However, at 1 month of age, abnormal seminiferous tubules containing dramatically disorganized arrangement of SOX9-positive cells were found in TGFBR1-CA^{Acre} testes (G–I). Minimal expression of SOX9 was observed in testicular tumor foci/nodules of TGFBR1-CA^{Acre} mice, in sharp contrast to the adjacent abnormal tubules consisting of SOX9-positive cells. Dotted blue lines mark small and large tumor foci/nodules (sTu/lTu). Results of immunofluorescence were verified by immunohistochemical staining of SOX9, in which three independent samples per group were used. Scale bar is representatively shown in (A) and equals 20 μm (A–I) and 80 μm (J–L).

old TGFBR1-CA^{Acre} males (Supplementary Fig. 2F), markedly disorganized arrangement of SOX9-positive cells was found within these abnormal tubules (Fig. 4G–I). This result also provided indirect support for the aforementioned observation of germ cell depletion in TGFBR1-CA^{Acre} testes. In addition, SOX9 staining was low to undetectable in tumor foci/nodules, in contrast to the strong immunoreactive signals of SOX9 observed in adjacent dysplastic tubules (Fig. 4J–L). Therefore, these studies suggest potential loss of SOX9 expression in tubule-like structures/neoplastic nodules during tumor development and progression.

Testicular tumors in TGFBR1-CA^{Acre} males express markers of GrCTs

As testicular tumors in TGFBR1-CA^{Acre} mice histologically resembled ovarian GrCTs, we examined the expression of several granulosa cell markers that are expressed by GrCTs including inhibin alpha (INHA), forkhead box O1 (FOXO1) and FOXL2 within the testes of TGFBR1-CA^{Acre} males. IHC revealed that testicular tumor nodules in 2-month-old TGFBR1-CA^{Acre} mice were positively stained for INHA (Fig. 5B and C), FOXO1 (Fig. 5E and F) and FOXL2 (Fig. 5H and I) compared with controls (Fig. 5A, D and G). Notably, FOXL2 is a granulosa cell lineage marker expressed in the ovary but not the testis (Wilhelm *et al.*, 2009). To determine the temporal expression of FOXL2 in TGFBR1-CA^{Acre} testes, we examined mice at 1 month of age, when microscopic tumor foci were detectable (Supplementary Fig. 2H). Interestingly, FOXL2 staining was found in some abnormal seminiferous tubules or tumor nodules in TGFBR1-CA^{Acre} testes (Fig. 5K and L), in stark contrast to control testes which did not express FOXL2 (Fig. 5J). Representative negative controls using isotype-matched IgGs are depicted in Supplementary Fig. 5. Supporting the IHC findings,

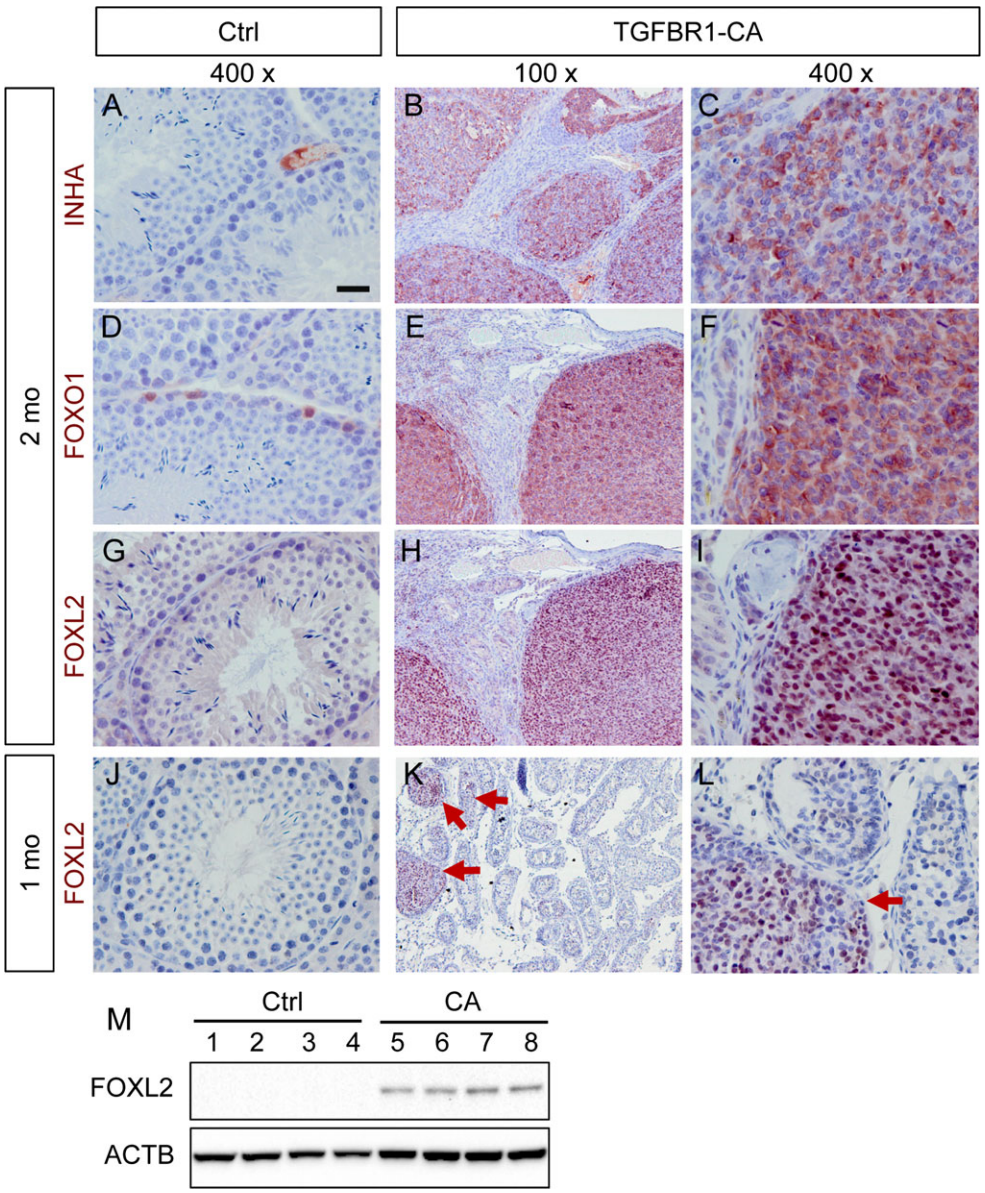

Figure 5 Testicular tumors of TGFBR1-CA^{Acre} males express granulosa cell tumor markers. (**A**–**I**) Immunostaining of INHA, FOXO1 and FOXL2 in control and TGFBR1-CA^{Acre} testes at 2 months of age. Note that intensive immunoreactive signals for INHA, FOXO1 and FOXL2 were detected in testicular tumors of TGFBR1-CA^{Acre} males compared with controls (A–I). (**J**–**L**) Immunostaining of FOXL2 in control and TGFBR1-CA^{Acre} testes at 1 month of age. Note that FOXL2 was not expressed in control testis (J) but localized to some tumor foci (K and L; red arrows). Panels (C, F, I and L) represent higher magnification images for corresponding panels (B, E, H and K). Three mice per group were examined. Scale bar is representatively shown in (A), and equals 25 μm (A, C, D, F, G, I, J and L) and 100 μm (B, E, H and K). (**M**) Western blotting analysis of FOXL2 in 1-month-old control and TGFBR1-CA^{Acre} testes. Note that the FOXL2 proteins were only detected in the testes of TGFBR1-CA^{Acre} males. Each lane represents an independent testis sample. $n = 4$.

protein bands of FOXL2 were only detected in the testes of TGFBR1-CA^{Acre} mice but not those of controls by western blotting (Fig. 5M). These results indicate that constitutive activation of TGFBR1 in the testis may alter Sertoli cell fate.

It has been well established that doublesex and mab-3 related transcription factor 1 (DMRT1) maintains testis identity; and Sertoli cells could be reprogramed to granulosa cells in the testis upon deletion of *Dmrt1* due to activation of *Foxl2* and other female-promoting genes (Raymond *et al.*, 2000; Matson *et al.*, 2011). In postnatal mouse testis, DMRT1 is expressed in mitotic spermatogonia and Sertoli cells (Matson *et al.*, 2011). To explore whether the aberrant expression of FOXL2 in our mouse model was associated with loss of DMRT1 expression, we performed immunohistochemical analysis of DMRT1 and IF of DMRT1 and FOXL2 using testes from control and TGFBR1-CA^{Acre} mice. DMRT1 was expressed in the testes from both control and TGFBR1-CA^{Acre} mice at 2 weeks of age (Supplementary Fig. 6). At 1 month of

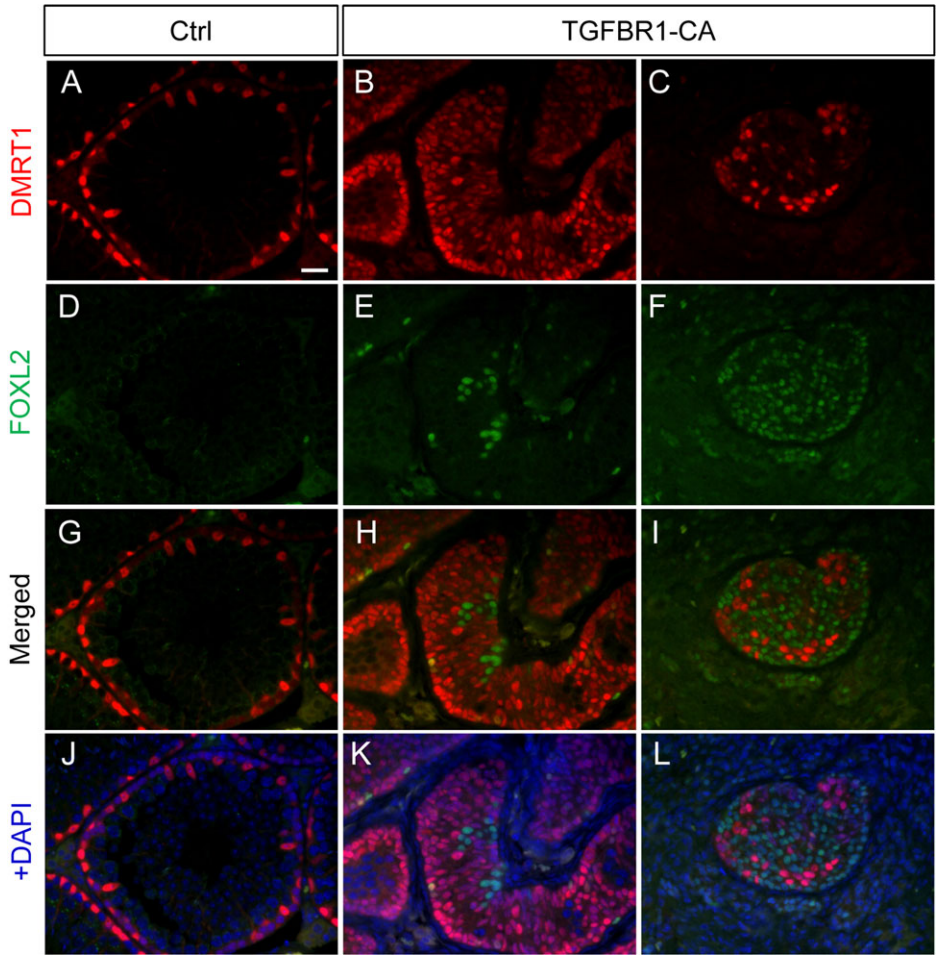

Figure 6 Immunofluorescence analysis of DMRT1 and FOXL2 distribution during testicular tumor development. **(A–L)** Immunofluorescence staining of DMRT1 and FOXL2 using testes from control and TGFBR1-CA^{Acre} males at the age of 1 month. Note that while DMRT1 was readily detectable in control testes, FOXL2 specific signals were not observed as expected (A, D, G and J). Scattered FOXL2-postive cells were found within some abnormal tubules in TGFBR1-CA^{Acre} testes (B, E, H and K). Panels (C, F, I and L) show a small tumor nodule in which many tumor cells were positive for FOXL2 but lacked DMRT1 expression. Three independent samples per group were used. Scale bar is representatively shown in (A) and equals 20 μm (A–L).

age, expression of FOXL2 in TGFBR1-CA^{Acre} testes could be detected in a subset of DMRT1-negative cells within disorganized tubules (Fig. 6B, E, H and K) or in majority of cells within some tumor nodules containing scattered DMRT1-expressing cells (Fig. 6C, F, I and L). In tumor foci where FOXL2-positive cells prevailed, no DMRT1 was detected (data not shown). In contrast, immunoreactive signals for FOXL2 were not detected in control testes (Fig. 6A, D, G and J). These results provide circumstantial evidence supporting a potential link between loss of DMRT1 and altered Sertoli cell fate in TGFBR1-CA^{Acre} testes.

As mentioned above, tumor nodules in TGFBR1-CA^{Acre} testes expressed minimal SOX9. However, loss of SOX9 may also occur during the development of Sertoli cell tumors (Chang *et al.*, 2009). To further determine whether tumor cells expressed other Sertoli cell markers, we examined Wilms tumor 1 (WT1), a Sertoli cell expressed protein that is detectable in mouse Sertoli cell tumors (Chang *et al.*, 2009). The results showed that WT1 was expressed in Sertoli cells of control testes and abnormal seminiferous tubules (Fig. 7A-D), but not the parenchyma of the tumors at 2 months of age (Fig. 7C and D). In addition, to elucidate whether the tumors expressed Leydig cell markers, we analyzed the expression of CYP17A1 (Liu *et al.*, 2016). Results showed that CYP17A1 was localized to Leydig cells in control testes (Fig. 7E and F). However, only scattered CYP17A1-postive cells were observed in testicular tumors of TGFBR1-CA^{Acre} mice (Fig. 7G and H), supporting the finding that tumors were not derived from Leydig cells. These results collectively indicate that constitutive activation of TGFBR1 in the testis leads to development of sex cord-stromal tumors resembling GrCTs.

WNT/CTNNB1 signaling is active in GrCTs resulting from TGFBR1 overactivation

Misregulation of WNT/beta-catenin (CTNNB1) signaling is implicated in tumorigenesis of GrCTs in multiple species including humans, horses

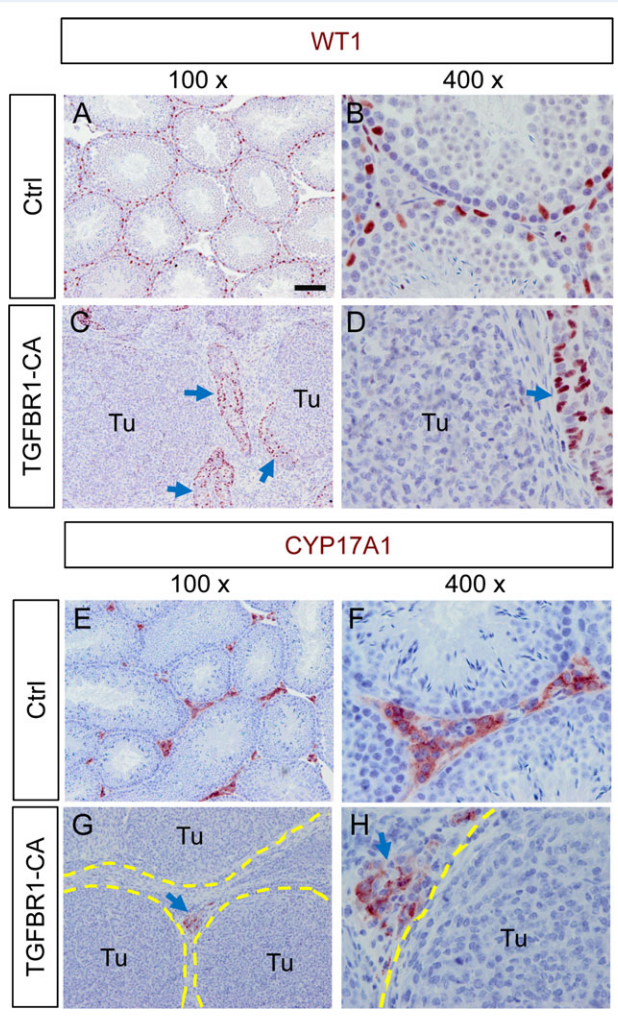

Figure 7 Analysis of Sertoli cell and Leydig cell markers in tumors from TGFBR1-CA^{Acre} males. (**A**–**H**) Immunostaining of WT1 (A–D) and CYP17A1 (E–H) in control and TGFBR1-CA^{Acre} testes at 2 months of age. Panels (B, D, F and H) represent higher magnification images for corresponding panels (A, C, E and G). Note that tumor nodules (Tu) were not major sites for the expression of WT1 (C and D) or CYP17A1 (G and H) in TGFBR1-CA^{Acre} mice. Strong immunoreactive signals of WT1 were observed in Sertoli cells of controls (A and B) and abnormal seminiferous tubules of TGFBR1-CA^{Acre} mice (C and D; blue arrows). In contrast, expression of CYP17A1 was mainly found in Leydig cells of control testes (E and F) and internodular areas of testicular tumors (blue arrows; G and H). Dotted yellow lines in panels (G and H) mark the borders of tumor nodules. Testis samples from three mice per group were examined. Scale bar is representatively shown in (A) and equals 25 μm (B, D, F and H) and 100 μm (A, C, E and G).

and mice (Boerboom *et al.*, 2005; Boyer *et al.*, 2009). To understand how constitutive activation of TGFBR1 promoted the development of testicular tumors in TGFBR1-CA^{Acre} mice, we examined the expression of CTNNB1 known to be involved in GrCT development (Boerboom *et al.*, 2005; Boyer *et al.*, 2009; Richards *et al.*, 2012). Herein, we demonstrated that the levels of CTNNB1 were increased in testicular tumor tissues of TGFBR1-CA^{Acre} mice at 1 month of age compared with controls (Fig. 8A and B). IHC confirmed the results of western blotting by revealing extensive immunoreactive signals of CTNNB1 in testicular tumors of TGFBR1-CA^{Acre} males versus controls (Fig. 8C–E). Moreover, variable degrees of nuclear/cytoplasmic staining of CTNNB1 were observed in testicular tumor tissues (Fig. 8E). These results suggest that dysregulation of WNT/CTNNB1 signaling is potentially linked to GrCT development in TGFBR1-CA^{Acre} mice.

Discussion

GrCTs may arise from the testis with low incidence (Jimenez-Quintero *et al.*, 1993; Wang *et al.*, 2002). The adult type GrCTs of the testis are extremely rare and occur after puberty, whereas the juvenile type GrCTs account for approximately 1–4% of total prepubertal testicular tumors and the majority of them develop within the first 6 months (Lawrence *et al.*, 1985; Shukla *et al.*, 2004; Dudani *et al.*, 2008). While adult GrCTs are associated with somatic mutation (Lima *et al.*, 2012), the juvenile type GrCTs may be associated with chromosomal abnormalities and gonadal dysgenesis (Young *et al.*, 1985; Kos *et al.*, 2005). Animal models are useful to investigate the pathogenesis of this disease. Several mouse models are available for sex cord-stromal tumors, including but not limited to, mice with targeted deletion of *Inha* (Matzuk *et al.*, 1992), *Smad1/5* (Pangas *et al.*, 2008) and BMP type I receptors (Edson *et al.*, 2010), mice with overexpression of R-spondin 1 (*Rspo1*) (De Cian *et al.*, 2017), mice containing KRAS activation and dominant-stable CTNNB1 (Richards *et al.*, 2012), and mice with dominant-stable CTNNB1 or both dominant-stable CTNNB1 and *Pten* inactivation (Boerboom *et al.*, 2005; Boyer *et al.*, 2009; Richards *et al.*, 2012). Although it is challenging to faithfully model human GrCTs, mouse models have helped to gain significant insights into the pathobiology and signaling cascades of GrCT development.

We previously reported that overactivation of TGFBR1 using *Amhr2*-Cre to target mouse granulosa cells provokes the development of ovarian GrCTs (Gao *et al.*, 2016). Since *Amhr2*-Cre has been successfully used to conditionally target/overexpress genes in somatic cells of the gonad in both sexes (Jamin *et al.*, 2002; Jeyasuria *et al.*, 2004; Boyer *et al.*, 2008, 2009; Pangas *et al.*, 2008; Papaioannou *et al.*, 2009; Tanwar *et al.*, 2010; Kyronlahti *et al.*, 2011), we determined the phenotypic consequence of constitutive activation of TGFBR1 using *Amhr2*-Cre in the testis. Formation of microscopic abnormalities observed in testicular tubules of TGFBR1-CA^{Acre} males before puberty suggests balanced TGFB signaling is essential for testis development. Development of testicular tumors was accompanied by increased cell proliferation and depletion of germ cells. It was reported that TGFB superfamily signaling is active in the mouse testis, and the dosage of activin A maintains balanced numbers of Sertoli cells and germ cells (Mendis *et al.*, 2011). As activin and TGFB signaling shares the same downstream SMADs, SMAD2/3, it appears possible that loss of germ cells in TGFBR1-CA^{Acre} testes may be associated with altered SMAD2/3 signaling activity.

To strengthen the conclusion that testicular tumors resembled GrCTs, we examined the spatiotemporal localization of several protein markers including SOX9, CYP17A1 and FOXL2 for the respective Sertoli cells, Leydig cells and granulosa cells. During the development

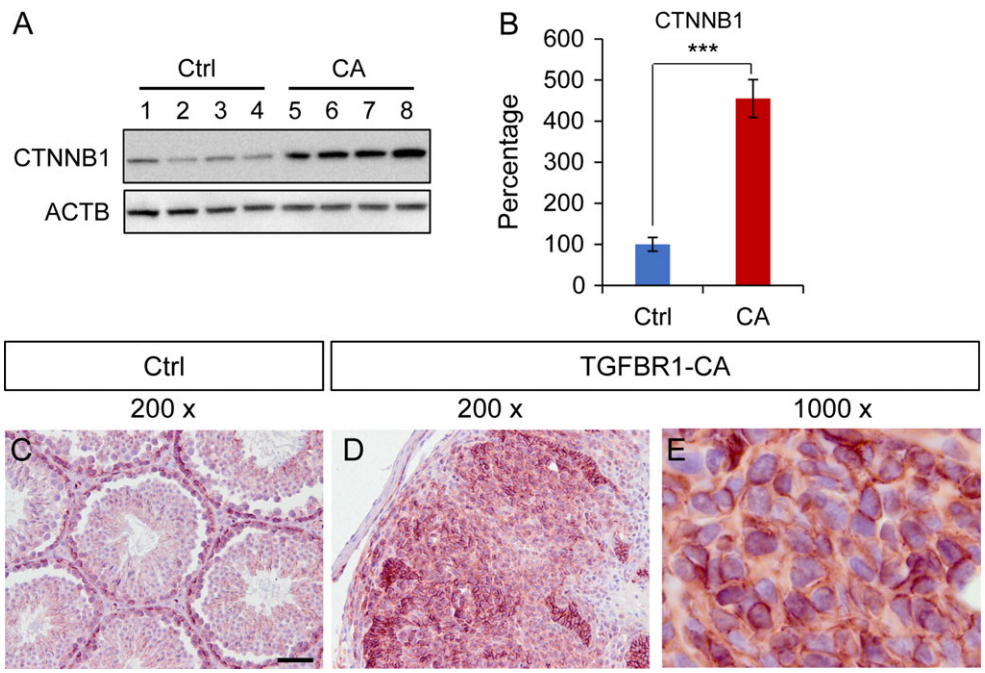

Figure 8 WNT/CTNNB1 signaling is active in GrCTs resulting from overactivation of TGFBR1. (**A**) Western blotting analysis of CTNNB1 expression in control and TGFBR1-CA^{Acre} testes of 1-month-old males. ACTB was included as an internal control. $n = 4$. (**B**) Quantification of the western blotting result shown in panel (A). Image J was used to calculate the ratio of CTNNB1 to ACTB for each sample. Results are presented as percentage, in which relative protein levels of control group are set to 100%. $n = 4$. Data are presented as mean ± SEM. $^{***}P < 0.001$. (**C**–**E**) Immunostaining of CTNNB1 in tumor nodules of testes from TGFBR1-CA^{Acre} mice along with controls. Panel (E) represents a higher magnification image of panel (D). Three mice per group were examined. Scale bar is representatively shown in (C), and equals 10 μm (E) and 50 μm (C and D).

of testicular GrCTs, we observed accumulation of Sertoli cells within seminiferous tubules, expression of Ki67 in Sertoli cells and reduction of SOX9 expression but gain of FOXL2 expression in tumor nodules. The origin of testicular GrCT is poorly defined. However, based on the aforementioned findings and the observation that testicular GrCTs arose within seminiferous tubules, it is tempting to postulate that Sertoli cells might transdifferentiate into granulosa tumor cells. Moreover, neither the histological feature nor the expression pattern of CYP17A1 supported that these tumors were Leydig cell tumors. Of note, the most common cell type of Leydig cell tumors is medium-sized hexagonal cell that has eosinophilic/vacuolar cytoplasm, irregular round/oval shaped nucleus and recognizable cell border (Mostofi, 1973). It is unclear whether gain of FOXL2 expression in TGFBR1-CA^{Acre} testes is a cause or consequence of testicular GrCT development. It has been suggested that both granulosa cells and Sertoli cells share the same progenitors (Albrecht and Eicher, 2001), and granulosa cells can transdifferentiate into Sertoli cells in adult female mice lacking FOXL2 (Uhlenhaut *et al.*, 2009). Conversely, it is logic to speculate that the fate of Sertoli cells may be altered to resemble granulosa cells when FOXL2 is misregulated. Indeed, Sertoli cells could be reprogramed to granulosa cells upon conditional loss of DMRT1 due to activation of *Foxl2* and other female-promoting genes (Matson *et al.*, 2011). Another study showed that constitutive activation of CTNNB1 in Sertoli cells promotes FOXL2 expression ectopically in the testis, resulting in the transformation of Sertoli cells into granulosa-like cells (Li *et al.*, 2017). Moreover, upregulation of FOXL2 in testicular GrCTs was found in an independent mouse model, where CTNNB1 and AKT signaling was genetically enhanced (Boyer *et al.*, 2009). Our result is also in agreement with the discovery that aberrant expression of FOXL2 links to the development of human juvenile GrCTs of the testis (Kalfa *et al.*, 2008). The finding of gain of FOXL2-expressing cell population but loss of cells positive for DMRT1, a key protein that maintains Sertoli cell identify (Raymond *et al.*, 2000; Matson *et al.*, 2011) in testicular tubules of TGFBR1-CA^{Acre} males during tumor development highlights the importance of TGFB pathway in maintaining the balance of 'male-female' characteristics in the gonad. In view of the early onset of tumorigenesis, the potential of TGFBR1-CA^{Acre} mice as a model for the juvenile GrCTs should be further explored.

Our current findings support an equally important role of TGFB signaling in the development of GrCTs in both male and female gonads (Gao *et al.*, 2016). Somatic mutation of *FOXL2* has been identified as a hallmark of ovarian adult GrCTs (Shah *et al.*, 2009). *FOXL2* mutation was also revealed in testicular GrCTs (Lima *et al.*, 2012). Moreover, activation of TGFB signaling appears to be associated with GrCT development (Pangas *et al.*, 2008; Middlebrook *et al.*, 2009; Edson *et al.*, 2010; Liu *et al.*, 2015; Kim *et al.*, 2016). Interestingly, *FOXL2* mutation may impact activin/TGFB signaling (Rosario *et al.*, 2012; Cheng *et al.*, 2014; Fang *et al.*, 2016). Previous studies indicated that unopposed activin signaling resulting from loss of inhibin promotes testicular tumor development partially through SMAD3, a critical regulator of sex cord-stromal tumor development (Li *et al.*, 2007). Thus, SMAD mediated signaling likely contributes to testicular tumor

development in our model system, as is supported by increased phospho-SMAD2/3 levels in the testes of TGFBR1-CA^{Acre} males.

TGFB signaling is complex and known to interact with other pathways. WNT pathway plays fundamental roles in normal cell functions and the development of multiple types of cancers (Giles *et al.*, 2003). Activation of the WNT/CTNNB1 pathway is involved in the pathogenesis of GrCTs; mice expressing a dominant-stable CTNNB1 mutant in ovarian granulosa cells develop GrCTs (Boerboom *et al.*, 2005). Simultaneous dysregulation of CTNNB1 and phosphatidylinositol 3-kinase (PI3K)/AKT pathways causes testicular GrCT formation (Boyer *et al.*, 2009). Increased expression of phospho-AKT was found in both ovarian tumors (Gao *et al.*, 2016) and testicular tumors (unpublished observation) from TGFBR1-CA^{Acre} mice. Moreover, the R-Spondin family proteins activate WNT signaling pathway (Kim *et al.*, 2008). Recent studies discovered that amplification of RSPO1 signaling leads to GrCT formation through altering the fate and properties of granulosa cells (De Cian *et al.*, 2017). These studies collectively indicate that enhanced WNT signaling promotes GrCT development. Supporting interactions between TGFB and WNT signaling (Akhmetshina *et al.*, 2012), we demonstrated that CTNNB1 expression was upregulated in testicular tumors. Moreover, WNT signaling appears to be active in gonadal tumors resulting from constitutive activation of TGFBR1. Of note, CTNNB1 signaling is suppressed during normal testis development, and WT1 negatively regulates CTNNB1 signaling (Chang *et al.*, 2008). Thus, lack of WT1 in tumor nodules of TGFBR1-CA^{Acre} testes may facilitate the expression of CTNNB1. While our studies uncovered a role of TGFBR1 activation in GrCT development, a potential regulatory mechanism of SMAD2/3-CTNNB1 activation in the pathogenesis of human GrCTs warrants further investigation. Illuminating the signaling network that underpins GrCT development will provide opportunities for designing new therapeutic interventions.

In summary, the current study created a new mouse model resembling testicular GrCTs. This mouse model may prove to be a useful addition to the mouse genetics toolkit for testicular GrCT research.

Supplementary data

Supplementary data are available at *Molecular Human Reproduction* Online.

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Authors' roles

X.F., N.N. and Y.G. conducted the experiments, collected the data and analyzed the results. D.F.V. and L.B. created and provided mice containing the latent constitutively active TGFBR1. Q.L. contributed to study conception and supervised the project. X.F. and Q.L. drafted the manuscript. All authors critically revised and/or commented on the manuscript and approved the final version of the manuscript and its submission.

Conflict of interest

None declared.

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