FGF9, activin and TGF β promote testicular characteristics in an XX gonad organ culture model

Sonja E Gustin¹, Jessica M Stringer¹, Kirsten Hogg¹, Andrew H Sinclair^{2,*} and Patrick S Western^{1,*}

¹Centre for Genetic Diseases, Hudson Institute of Medical Research and Department of Molecular and Translational Science, Monash University, Clayton, Victoria, Australia and ²Murdoch Children's Research Institute and Department of Paediatrics, University of Melbourne, Royal Children's Hospital, Parkville, Victoria, Australia

Correspondence should be addressed to P S Western; Email: patrick.western@hudson.org.au

*(A H Sinclair and P S Western contributed equally to this work)

Abstract

Testis development is dependent on the key sex-determining factors SRY and SOX9, which activate the essential ligand FGF9. Although FGF9 plays a central role in testis development, it is unable to induce testis formation on its own. However, other growth factors, including activins and TGF β s, also present testis during testis formation. In this study, we investigated the potential of FGF9 combined with activin and TGF β to induce testis development in cultured XX gonads. Our data demonstrated differing individual and combined abilities of FGF9, activin and TGF β to promote supporting cell proliferation, Sertoli cell development and male germ line differentiation in cultured XX gonads. FGF9 promoted proliferation of supporting cells in XX foetal gonads at rates similar to those observed *in vivo* during testis cord formation in XY gonads but was insufficient to initiate testis development. However, when FGF9, activin and TGF β were combined, aspects of testicular development were induced, including the expression of Sox9, morphological reorganisation of the gonad and deposition of laminin around germ cells. Enhancing β -catenin activity diminished the testis-promoting activities of the combined growth factors. The male promoting activity of FGF9 and the combined growth factors directly or indirectly extended to the germ line, in which a mixed phenotype was observed. FGF9 and the combined growth factors promoted male germ line development, including mitotic arrest, but expression of pluripotency genes was maintained, rather than being repressed. Together, our data provide evidence that combined signalling by FGF9, activin and TGF β can induce testicular characteristics in XX gonads.

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Introduction

Male or female development is initiated in the developing foetus when the bipotential gonadal primordium is committed to form a testis or an ovary. In mice, testis development is initiated by expression of the Y-chromosome-linked testis-determining gene *Sry* (sex-determining region of chromosome Y). *SRY* promotes pre-Sertoli cell proliferation and expression of the dominant male sex-determining transcription factor *Sox9* (*Sry*-box containing gene 9), leading to commitment of bipotential pre-supporting cells to Sertoli cell development and testis differentiation (Lin & Capel 2015). As Sertoli cells proliferate, they organise into laminin-delineated testis cords that surround germ cells, thereby defining the interstitial space where steroidogenic Leydig cells differentiate (Lin & Capel 2015).

SOX9 directly or indirectly promotes the expression of a number of downstream genes, including *Fgf9*

(fibroblast growth factor 9), AMH (anti-Müllerian hormone) and CYP26B1 (cytochrome P450, family 26, subfamily B, polypeptide 1), which are involved in Müllerian duct regression and germ cell differentiation respectively (Behringer *et al.* 1994, Colvin *et al.* 2001, Bowles *et al.* 2006). FGF9 promotes the maintenance of *Sox9* expression and the proliferation and differentiation of Sertoli cells, but FGF9 is insufficient to initiate testis development (Colvin *et al.* 2001, Schmahl *et al.* 2004, Kim *et al.* 2006).

In addition to *Fgf9*, *Inhba*, *Inhbb* and *Tgfb1–3* are expressed in XY foetal gonads as testis cords form (Mendis *et al.* 2011, Miles *et al.* 2013). These genes encode activin A and B and transforming growth factor beta 1–3. Blocking activin/TGF β signalling at E11.5 or E12.5 significantly reduces Sertoli cell proliferation and disrupts testis cord formation (Miles *et al.* 2013), indicating that activin-TGF β signalling promotes testis formation in the critical sex-determining window at

E11.5. However, testes develop in mice that lack *Inhba*, *Inhbb* or individual *Tgfb* genes, demonstrating that they are individually dispensable for male sex determination (Shull *et al.* 1992, Memon *et al.* 2008, Archambeault & Yao 2010, Moreno *et al.* 2010, Mendis *et al.* 2011), although the possibility that these factors compensate for one another's function in testis development has not been addressed.

Ovarian development is promoted by complementation of the transcription factor FOXL2 (forkhead box L2) and RSPO1 (R-spondin homolog 1)/WNT4 (wingless-related MMTV integration site 4)/ β -catenin signalling in GATA4-expressing supporting cells (Chassot *et al.* 2014, Tevosian 2014). During early ovarian development, proliferative GATA4-expressing cells activate expression of the cell cycle inhibitor p27KIP1 (p27) and FOXL2 as they form non-proliferative granulosa cells (Mork *et al.* 2012, Rastetter *et al.* 2014, Gustin *et al.* 2016). Although loss of FOXL2 function does not result in female-to-male sex reversal in humans, individuals carrying FOXL2 mutations are susceptible to primary ovarian insufficiency (Crisponi *et al.* 2001). Consistent with this, morphologically normal foetal ovaries form in mice that lack *Foxl2*, but ovarian gene expression programmes are disrupted and follicles fail to properly activate at postnatal stages (Schmidt *et al.* 2004, Uda *et al.* 2004, Garcia-Ortiz *et al.* 2009).

WNT4 and RSPO1 signal through β -catenin, which plays a key role in repressing male developmental pathways in the XX foetal gonad (Chassot *et al.* 2014). WNT/ β -catenin signalling is regulated through glycogen synthase kinase (GSK), which phosphorylates β -catenin, targeting it for proteasomal degradation (Bennett *et al.* 2002). Conversely, inhibition of GSK stabilises β -catenin, promoting ovarian development (Bernard *et al.* 2012). Excess β -catenin function antagonises Sox9 expression and results in partial male-to-female sex reversal, while loss of RSPO1 or β -catenin function results in partial female-to-male sex reversal (Chassot *et al.* 2008, Maatouk *et al.* 2008, Tomizuka *et al.* 2008). Loss of WNT4 function does not result in sex reversal, but permits development of the testis-specific coelomic vessel in XX individuals, expression of male steroidogenic enzymes and disruption of germ cell development (Vainio *et al.* 1999, Chassot *et al.* 2008, Tomizuka *et al.* 2008). Although ovaries form in XX fetuses that lack WNT4 signalling, structures similar to testis cords arise in late-stage XX gonads. Taken together, these findings demonstrate that activity of WNT4 and RSPO1 promotes sufficient β -catenin activity to promote ovary development (Chassot *et al.* 2014).

Somatic cells in the testis and ovary also produce signals that commit primordial germ cells to the spermatogenic or oogenic pathways respectively (Kocer *et al.* 2009). In mice, XY germ cells commit to the spermatogenic pathway at embryonic day (E)12.5 and enter mitotic arrest between E12.5 and E14.5,

which involves regulation of the G₁-S phase checkpoint (Adams & McLaren 2002, Western *et al.* 2008, 2011, Spiller *et al.* 2010). Male germ line development is characterised by upregulation of *Nanos2*, *Piwi2* and *Dnmt3l*; strong nuclear localisation of DPPA4 protein (developmental pluripotency associated 4); and repression of pluripotency factors NANOG, SOX2 and OCT4 (Suzuki & Saga 2008, Barrios *et al.* 2010, Bowles *et al.* 2010, Western *et al.* 2010, 2011). In the developing ovary, germ cells express *Stra8* (stimulated by retinoic acid gene 8) and enter meiosis between E13.5 and E15.5, which is marked by distinctive nuclear localisation of phosphorylated H2AX (γ H2AX) (Baltus *et al.* 2006, Bowles *et al.* 2006, Koubova *et al.* 2006, Miles *et al.* 2010). *Cyp26b1* is expressed by somatic cells of the developing testis and is considered essential for metabolising retinoic acid (RA), thereby preventing *Stra8* expression and entry of germ cells into meiosis (Bowles *et al.* 2006, Koubova *et al.* 2006). In addition, FGF9 represses *Stra8* and germ cell entry into meiosis, while promoting male germ line development (Suzuki & Saga 2008, Barrios *et al.* 2010, Bowles *et al.* 2010). Although *Nanog* and *Sox2* are transcriptionally repressed in both XX and XY germ cells, NODAL is thought to maintain pluripotency slightly longer in XY germ cells (Spiller *et al.* 2012, Miles *et al.* 2013).

FGF9 is indispensable for testis development, but is not sufficient to initiate it. In addition to *Fgf9*, *Inhba*, *Inhbb* and *Tgfb1-3* are all expressed in the developing testis and blocking activin-TGF β signalling in E11.5 and E12.5 developing testes disrupts Sertoli cell proliferation and testis cord formation. Based on these observations, we proposed that the combined actions of FGF9, activin and TGF β signalling might promote testis development in XX gonads. As expected, FGF9 promoted supporting cell proliferation but was insufficient to induce testis development in XX gonads. However, FGF9 combined with activin and TGF β induced testicular characteristics in cultured XX gonads. This activity directly or indirectly extended to the germ line, in which characteristic male developmental processes were induced, although pluripotency was maintained. Moreover, the testis-promoting activity of these ligands was inhibited by facilitating β -catenin signalling. Combined, these data indicate that FGF9, activin and TGF β cooperatively promote testis development in the critical sex-determining window in mice.

Materials and methods

Animals

Mice used in all experiments were derived from pure 129T2SvJ OG2 (Szabo *et al.* 2002) (*Oct4*-GFP; octamer-binding transcription factor 4, also known as *Pou5f1*) transgenic male mice x Swiss female matings. The presence of a vaginal plug in the morning was used to indicate mating and was recorded

as E0.5. All animal procedures were carried out under Monash University Animal Ethics Committee and Murdoch Childrens Research Institute Animal Ethics Committee approvals.

Organ culture

E11.5 gonad/mesonephros and E12.5 gonad/mesonephros samples were collected from *Oct4*-GFP transgenic embryos and sexed using PCR as described previously (McFarlane *et al.* 2013, Miles *et al.* 2013). XX or XY gonad/mesonephros tissues were placed onto 25 mm Millicell Biopore 0.4 µm membranes (Millipore) in six-well plates containing 1200 µL organ culture media (15 mM Hepes, 1X non-essential amino acids, 1 mg/mL *N*-acetylcysteine, 55 µM β-mercaptoethanol, 1X penicillin/streptomycin and 10% FCS in DMEM/F12 with Glutamax). Phosphate-buffered saline (PBS) was included between wells and in empty wells to maintain a humid environment. FGF9 (50 ng/mL), TGFβ1 (25 ng/mL), activin A/activin B (25 ng/mL of each) (R&D Systems) or Chir99021 (GSKi, 3 µM; Cayman Chemicals, Ann Arbor, MI, USA) (Bennett *et al.* 2002, Ying *et al.* 2008) were added to the culture medium at the outset of the experiment. These concentrations of growth factors are similar to those used in a range of studies of gonad, germ cell and pluripotent cell development (DiNapoli *et al.* 2006, Rosairo *et al.* 2008, Vallier *et al.* 2009, Bowles *et al.* 2010, Kjartansdottir *et al.* 2015, Tepekoy & Akkoyunlu 2016), whereas Chir99021 robustly induces β-catenin signalling (Naujok *et al.* 2014). Tissues were cultured for 24, 72, 96 or 120 h in 5.0% CO₂ at 37°C with organ culture media changed daily. All organ cultures were replicated at least two times using two to four individual gonads per treatment. Gonads were photographed under bright field and fluorescence optics and were carefully removed from culture membranes. Gonads were dissociated into single cells using trypsin and fixed for flow cytometric analysis (Wakeling *et al.* 2013), or fixed whole for immunofluorescence (IF) in 4% paraformaldehyde (PFA) or snap frozen for RNA preparation.

RNA isolation, reverse transcription and quantitative PCR

For qRT-PCR experiments, RNA was extracted from three to four cultured gonads pooled together per sample using Qiagen micro-RNeasy columns, DNase treated and integrity confirmed using a Bioanalyzer 2100 (Agilent), and quantified using the Qubit RNA assay (Life Technologies). RNA samples used had RIN values between 7.0 and 10.0. Reverse transcription was performed using Superscript III First-Strand cDNA synthesis kit (Life Technologies) according to the manufacturer's instructions. qRT-PCR was performed as described previously (Western *et al.* 2008, 2010, van den Bergen *et al.* 2009) using SYBR green chemistry and validated primers (Supplementary Table 1, see section on supplementary data given at the end of this article) and an ABI7900 (Life Technologies) instrument. qRT-PCR results were analysed using manufacturer's software. Relative expression levels were determined by normalisation against verified reference genes *Mapk1*, *Sdha* and *Canx* (van den Bergen *et al.* 2009) or against *Ddx4* to correct for germ cell number. Data represent one to three experiments each performed on biological

triplicate samples, with PCRs performed in technical triplicate. Relative gene expression was calculated using the 2^{-ΔΔCT} method (Livak & Schmittgen 2001). Fold change was calculated based on the highest expression value and expression was normalised relative to the XX control sample set at 1.0. Data were analysed using ANOVA with Tukey's *post hoc* *t*-tests and with Bartlett's test to compare variances. When variances were unequal, Kruskal–Wallis ANOVA (non-parametric) test with Dunn's multiple comparisons test was used. All statistical analyses/calculations were performed in Prism 6 (version 6.0e; GraphPad Software). A *P*-value less than 0.05 was considered significant.

Flow cytometry

For *in vivo* analysis of gonadal cell proliferation, pregnant mothers were injected intraperitoneally (i.p.) with 20 mg/kg EdU (5-ethynyl-2-deoxyuridine). After 2 h of the i.p. injection, the mothers were killed, fetuses removed, gonads dissected, dissociated and the cells fixed for 15 min at RT in Click-iT Fixative (Life Technologies) for flow cytometry. For analyses of cell proliferation in cultured gonads, EdU was added to the organ culture media at a final concentration of 20 µM for the final 2 h of culture, after which gonads were collected, dissociated into single cells and fixed with 4% PFA in PBS. Flow cytometry was carried out essentially as described (Wakeling *et al.* 2013, Gustin *et al.* 2016) using cells from 0.5 to 2 gonads/sample, depending on age. For example, *in vivo* analysis of Sertoli cell proliferation (Fig. 1A) was performed using the following approach: fixed cells were co-stained using a rabbit-raised SOX9 antibody to identify Sertoli cells and goat-raised MVH antibody to identify germ cells as the EdU staining buffers destroy eGFP fluorescence. The SOX9 and MVH antibodies were detected using anti-rabbit-Biotin-Pacific Blue Streptavidin and anti-goat-Alexa-488 secondary antibodies respectively. DNA synthesis was measured via incorporation of EdU and detection with click-iT 647 azide. After staining, the cells were resuspended in 300 µL PBS containing 20 µg/mL propidium iodide to stain DNA, allowing individual cell DNA content to be determined. Cells were analysed on a BD FACS Canto flow cytometry instrument. The outputs represent the relative proliferation (EdU incorporation) of FOXL2+ cells in XX gonads at E12.5 or SOX9+ in XY gonads at E11.5, E12.5, E13.5 and E14.5 days of development.

Staining protocols for other flow cytometry experiments were similar with the exception that antibodies specific for GATA4, SOX9, p27, STRA8, DPPA4 or γH2AX were used in conjunction with germ cell markers *Oct4*-GFP or goat- or rabbit-raised anti-MVH (antibodies are listed in the Supplementary Information). Average individual cell staining intensities were collected for p27, STRA8, DPPA4 and γH2AX, allowing quantification of the relative levels of these proteins in somatic and germ cells. Where data were combined from independent flow cytometry experiments, the control-treated samples were used to normalise staining intensity between runs, with all experimental samples then compared with control. Primary antibodies used are listed in Supplementary Table 2. Secondary antibodies used were 1/300 donkey anti-goat or rabbit Alexa-647, 1/300 donkey anti-goat or rabbit biotin 1/500, followed by 1/500

Figure 1 FGF9 and TGFβ1 acted as mitogens in cultured foetal gonads. (A) *In vivo* proliferation rates of SOX9 expressing cells in XY gonads at E11.5, E12.5, E13.5 and E14.5 compared with FOXL2 expressing cells at E12.5 in XX gonads assessed using flow cytometry. *a*, *b* and *c* are significantly different. (ANOVA with Tukey's multiple comparison test; $P < 0.05$. Values are expressed as mean $\pm$ S.E.M.; $n \geq 3$.) (B) Flow analysis in E12.5 XX gonads cultured for 24 h with control (C), FGF9, activin, TGFβ or combined (FAT) (i) Proliferation in GATA4+ cells; (ii) Percentage of p27+ cells in the GATA4+ population; (iii) p27 staining intensity in the p27+ population. *a*, *b*, *c* and *d* are statistically different from each other. (ANOVA with Tukey's multiple comparison test.) Values are expressed as mean $\pm$ S.E.M.; $n \geq 3$.

Streptavidin-Pacific Blue (405 nm) and 1/300 donkey anti-goat or rabbit Alexa-488 (Life Technologies). Gating for each primary antibody (Supplementary Fig. 1) was performed against negative control tissues that did not express that protein or was not treated with EdU: mesonephros or tail (p27 (CDKN1B), STRA8, DPPA4, γH2AX, GATA4 and EdU) or gonad of the opposite sex (SOX9, AMH and FOXL2), depending on the antibody used. Cell cycle analysis was performed using propidium iodide to determine DNA content and EdU incorporation during S-phase to determine proliferation (Wakeling *et al.* 2013, Gustin *et al.* 2016). Mean individual germ cell staining intensities were acquired using area under the curve in either Pacific Blue 405 nm (405-A) or Alexa-fluor 647 nm (647-A) (Life Technologies) channels for the whole germ cell population isolated using Oct4-GFP fluorescence or MVH staining in the 488 nm channel. All flow cytometry data was analysed using FlowJo v.X.0.7. software. Data represent three to eight biologically distinct samples and statistical significance was determined using ANOVA with Tukey's multiple comparison test for experimental groups >3 , or unpaired *t*-test, with *F*-test to compare variances for groups of two. Where variances were unequal, a non-parametric test was used (Mann-Whitney). A *P*-value less than 0.05 was considered significant.

Tissue fixation and IF

Cultured gonads were fixed in PBS containing 4% PFA for 20–45 min (depending on age) at RT and mounted in optimal cutting temperature. Cryosections were cut at 10 μm and IF was performed as described previously (Western *et al.* 2008, 2011, Miles *et al.* 2010). Details of primary antibodies are provided in Supplementary Table 2. Secondary antibodies were used at 1/300 dilution and included: Donkey anti-goat or rabbit Alexa-647 and donkey anti-goat or rabbit Alexa-594 (Life Technologies), whereas eGFP fluorescence was detected directly in the 488 nm channel. Where the 647 and 594 red channels were used together, one colour is shown in pseudo-coloured cyan. All IF experiments were replicated at least two times using two to four individual gonads per replicate.

Results

Testis formation is characterised by two phases of high *in vivo* Sertoli cell proliferation

A key characteristic of male sex determination includes high supporting cell proliferation, but the relative proliferation of Sertoli cells during sex determination and early testis development has not been quantitatively documented. To determine relative rates of *in vivo* Sertoli cell proliferation in developing testes, we exposed mice to EdU for 2 h at E11.5, E12.5, E13.5 and E14.5 of pregnancy. Foetal gonads were collected 2 h after EdU injection and Sertoli cell proliferation quantified relative to XX granulosa cell proliferation using SOX9 (XY) or FOXL2 (XX, E12.5 only) antibody staining, EdU labelling and flow cytometry. At E12.5, FOXL2-positive cells in XX gonads were not proliferative as less than 1% progressed through S-phase in the 2 h EdU labelling period. Sertoli cell proliferation was relatively low at E11.5 XY gonads despite high levels of SOX9 expression in these cells (Fig. 1A). However, Sertoli cell proliferation rose between E11.5 and E12.5 coincident with testis cord formation and declined again at E13.5 (Fig. 1A). Sertoli cell proliferation then increased at E14.5 in a second wave of high proliferation coincident with testis cord expansion (Fig. 1A).

FGF9 and TGFβ acted as mitogens for pre-supporting cells in *ex vivo* gonad cultures

Sertoli cells and granulosa cells are derived from common GATA4-expressing bivalent supporting cells in XY and XX E11.5 gonads. These supporting cell precursors are presumably responsive to growth factors present in the developing testis, which include FGF9, activin and TGFβ. To determine the relative abilities of FGF9, TGFβ and activin to drive proliferation of pre-supporting cells, we collected E12.5 XX gonads

and cultured them with combinations of FGF9 (50 ng/mL), activin (activin A and activin B each at 25 ng/mL) and TGF β (25 ng/mL) for 24 h. As XX supporting cells do not express high levels of SOX9, we used flow cytometry to analyse proliferation in GATA4+ cells. Moreover, as previous studies have demonstrated high levels of the cell cycle inhibitor p27^{KIP1} (p27) in non-proliferative XX supporting cells, we used flow cytometry to determine whether FGF9, activin or TGF β reduced p27 levels in the XX supporting cells of XX gonads.

Treatment of E12.5 XX gonads with FGF9 for 24 h induced a similar rate of proliferation in GATA4+ cells as that observed *in vivo* for SOX9+ Sertoli cells in E12.5 XY gonads ($23.8 \pm 2.3\%$ vs $19.5 \pm 10.2\%$ EdU positive respectively; Fig. 1B i compared with 1A),

demonstrating potent mitogenic activity for FGF9 in the foetal gonad. In addition, FGF9 significantly ($P < 0.001$) reduced the proportion of p27+ cells in the GATA4+ population and the average p27 fluorescence intensity in remaining p27+ cells (Fig. 1B ii, iii). TGF β also promoted a significant ($P < 0.05$) increase in proliferation and decreased the proportion of p27+ cells in the GATA4+ population and decreased p27 levels in the p27+ population (Fig. 1B). However, activin did not alter these parameters. Consistent with these data, combined treatment of E12.5 XX gonads with FGF9 (50 ng/mL), activin A/B (25 ng/mL each) and TGF β (25 ng/mL) (FAT) did not further increase proliferation of XX GATA4+ cells or further decrease p27 levels in the p27+ population compared with FGF9 treatment alone (Fig. 1B ii, iii).

Figure 2 Combined, FGF9, activin and TGF β repressed ovarian genes and promoted testicular characteristics in cultured E11.5 gonads. (A) qRT-PCR analysis of *Sox9*, *Cyp26b1*, *Foxl2* and *Rspo1* expression in E11.5 XX gonad/mesonephros tissues cultured for 72 h with control (C), FGF9, activin, TGF β and FAT media and XY samples treated with control media (C (XY)). (B) qRT-PCR analysis of *Sox9*, *Cyp26b1*, *Foxl2* and *Rspo1* expression in E11.5 XX gonad/mesonephros tissues cultured for 24 h with control (C) or FAT media and XY samples treated with control media (C (XY)). In (A and B), expression is relative to the XX control sample set at 1.0, normalised against *Mapk1*, *Sdha* and *Canx*. a, b, c and d are statistically different (ANOVA with multiple comparison test; $P < 0.05$). Values are expressed as average $\pm$ S.E.M., $n = 3-6$. (C) IF images of E11.5 XX (and XY control) gonad/mesonephros/whole gonad sections cultured for 72 h with control or FAT media and analysed with antibodies specific for laminin (cyan) and DPPA4 (red). Germ cells are marked by *Oct4*-GFP (green). Dashed white lines outline testis cord-like structures in XX gonads treated with FAT and testis cords in XY gonads. Scale bars: 50 μ m.

Combined, FGF9, activin and TGF β induced testicular characteristics in XX gonads

Although FGF9 is required for testis development, it is alone insufficient to initiate testis formation. As activin/TGF β signalling is also required for testis cord formation, we next tested whether combinations of FGF9, activin and/or TGF β could induce testis development in XX gonads. Constructing a multi-transgenic mouse model that overexpresses *Ggf9*, *Inhba*, *Inhbb* and *Tgfb1-3* in a spatiotemporal pattern that recapitulates normal gonad development was not considered feasible. We therefore examined the combined ability of FGF9, activin and TGF β to promote testis development in XX gonads using an established gonad organ culture system.

Gonad/mesonephros tissues were isolated from E11.5 fetuses and cultured with combinations of FGF9, activin and/or TGF β 1 or control medium for 72 h. To determine whether FGF9, activin and/or TGF β promoted expression of testis development genes, we performed qRT-PCR to measure the mRNA levels of *Sox9* and *Cyp26b1* and the ovarian genes *Foxl2* and *Rspo1* in XX gonads cultured for 72 h. Treatment of E11.5 XX gonads with FAT for 72 h resulted in upregulation of *Sox9* in XX gonads to levels comparable with those detected in XY gonads, but *Sox9* expression was unaffected by treatment with FGF9, activin or TGF β alone (Fig. 2A). Compared with XX controls, transcription of *Cyp26b1* increased two- to three-fold in the presence of FGF9 or FAT (paired *t*-tests; $P < 0.01$ and $P < 0.05$ respectively), but not with activin or TGF β alone (Fig. 2A). Transcription of *Rspo1* was significantly lower in XX gonads treated with FGF9, TGF β or FAT (Fig. 2A) compared with controls, but *Foxl2* levels were reduced only by FGF9 and FAT (Fig. 2A). Treatment of E11.5 XY gonads for 72 h with FGF9, activin, TGF β or FAT did not affect transcription of *Sox9*, *Cyp26b1* or *Rspo1*, but both FGF9 and FAT moderated levels of *Foxl2* (Supplementary Fig. 2A).

The response of these genes to growth factor treatment occurred early as treatment of E11.5 XX gonad/mesonephros complexes for 24 h with FAT was sufficient to drive increased transcription of *Sox9* and *Cyp26b1* and decrease levels of *Rspo1* and *Foxl2* (Fig. 2B). Despite FAT induction of *Sox9*, there was no effect on transcription of *Amh* or *Dhh*, which are also produced by Sertoli cells (Supplementary Fig. 2B), and involved in Müllerian duct regression and Leydig and peritubular myoid cell differentiation respectively. However, the foetal Leydig cell marker *Inhba*, which encodes activin A, was robustly induced by FAT after 24 or 72 h of culture, indicating that this gene may respond relatively directly to FAT (Supplementary Fig. 2C).

FAT promoted formation of testis cord-like structures in cultured E11.5 XX gonads

The induction of *Sox9*, *Cyp26b1* and *Inhba* expression and the repression of *Rspo1* and *Foxl2* (Fig. 2) indicated

that FAT might induce testicular development in E11.5 gonad/mesonephros cultures. To determine whether testis cords were forming in FAT-treated gonads, we performed IF for laminin, which delineates the basement membrane of testis cords. In XX gonads cultured in control medium for 72 h, laminin was distributed throughout the gonad in a discontinuous, unorganised pattern and did not enclose germ cells in cords (Fig. 2C, top panel). In contrast, groups of GFP-expressing germ cells were enclosed inside laminin-delineated spaces in E11.5 XX gonads treated for 72 h with FAT (Fig. 2C, middle panel), similar to germ cells within testis cords (Fig. 2C, bottom panel). Although these testis-like cords were not always distributed throughout the whole gonad, similar structures were not observed in control-treated XX gonads.

To determine whether key proteins involved in gonad development were altered by growth factor treatment, we cultured additional E11.5 XX gonads with or without FAT for 72 h and performed double IF using antibodies against laminin, SOX9, AMH, FOXL2 and the male germ line marker DPPA4. Consistent with our previous experiments, FAT induced laminin-defined cord-like structures containing germ cells that expressed relatively high levels of DPPA4 (Fig. 3A). Moreover, FAT-treated E11.5 gonads contained SOX9-expressing somatic cells that were associated with germ cells enclosed in cord-like structures (Fig. 3B), although substantially fewer SOX9+ cells were detected in FAT-treated XX gonads compared with control-treated XY gonads. By comparison, cells positive for SOX9 protein were very rarely detected in E11.5 XX gonads treated with FGF9 alone and were not detected in XX samples cultured in control medium (Fig. 3B; control sample shown). Although the number of FOXL2+ cells was reduced in FAT-treated gonads, some FOXL2+ cells remained and tended to group in cord-like structures associated with rare AMH+ cells (Fig. 3C).

Facilitating β -catenin signalling inhibited the testis-promoting activity of FAT

It is well established that β -catenin signalling promotes ovarian development and opposes testis development. As inhibition of GSK facilitates β -catenin signalling, we used a potent and highly specific GSK α/β inhibitor, Chir99021 (GSKi), to block GSK and test whether enhancing β -catenin signalling might reduce the ability of FAT to promote testicular features in XX gonads. Indeed, gonads cultured with FAT+GSKi were similar to XX control gonads with no laminin-defined cords apparent, low DPPA4 expression in germ cells, no detectable SOX9 or AMH-positive cells and numerous, scattered FOXL2-positive cells (Fig. 3A–C). Consistent with this, GSKi prevented FAT induction of *Sox9* and *Cyp26b1* transcription and led to a moderate recovery in *Foxl2* transcription to levels intermediate

Figure 3 FAT induced testicular characteristics in E11.5 XX cultured gonads, while potentiating β -catenin prevented these effects. (A–C) IF images of E11.5 XX (and XY control) gonad/mesonephros tissues cultured for 72 h with control, FAT or FAT-GSKi media and analysed with antibodies specific for (A) laminin (cyan) and DPPA4 (red); (B) germ cells (GFP), SOX9 (cyan); (C) AMH (cyan) and FOXL2 (red). Arrowheads indicate SOX9-positive cells (B) and arrows indicate AMH-positive cells (C). Germ cells are marked by *Oct4*-GFP. Dashed white lines outline testis cord-like structures in XX gonads treated with FAT and testis cords in XY gonads. Scale bars: 50 μ m. (D) qRT-PCR analysis of *Sox9*, *Cyp26b1*, *Foxl2* and *Rspo1* expression in E11.5 XX (and XY control; C (XY)) gonad/mesonephros tissues cultured for 72 h with control (C), FAT or FAT-GSKi media. Expression is relative to the XX control sample set at 1.0, normalised against *Mapk1*, *Sdha* and *Canx*. a, b and c are statistically different (ANOVA with multiple comparison test; $P < 0.05$). Values are expressed as average $\pm$ S.E.M., $n \geq 3$.

between the FAT and the XX control samples (Fig. 3D). However, GSKi did not rescue *Rspo1* transcription or prevent FAT induction of *Inhba* (Fig. 3D and Supplementary Fig. 2B respectively).

FAT promoted testicular characteristics more effectively in E11.5 XX gonads than in E12.5 XX gonads

Testis determination occurs within a critical developmental window through the onset of *Sry* and *Sox9* expression at E11.5. To determine whether testicular features could be promoted after E11.5, E12.5 XX gonad/mesonephros samples were cultured in control, FAT or FAT-GSKi media for 72 h. Culture of gonad/mesonephros samples for 72 h with FAT induced *Sox9* and *Cyp26b1* and repressed *Foxl2* and *Rspo1* transcription with similar efficiency to that observed in E11.5 XX gonads (Supplementary Fig. 2D). Similarly, GSKi blocked the ability of FAT to induce *Sox9* and *Cyp26b1*, but did not rescue expression of *Foxl2* or

Rspo1 (Supplementary Fig. 2D). However, IF analysis of E12.5 gonad/mesonephros samples cultured with FAT revealed very limited laminin organisation around germ cells, and neither SOX9+ nor AMH+ cells were detected in E12.5 gonad/mesonephros samples treated with FAT (not shown).

FGF9 and FAT induced expression of male germ line markers in XX gonads

Somatic cell signalling in the developing testis induces male germ line development, which is marked by the transcription of male germ line genes such as *Nanos2* and *Piwi2*, strong nuclear localisation of DPPA4 protein and germ cell entry into mitotic arrest. By contrast, germ cells in XX gonads are characterised by expression of *Stra8*, distinctive nuclear localisation of phosphorylated H2AX (γ H2AX) and entry into meiosis.

To determine the effects of growth factors on germ cell development, E11.5 and E12.5 XX gonads were

treated with FGF9, FAT, FAT-GSKi or control media for 72 h. Although *Nanos2* remained below detection levels, *Piwi2* and *Dppa4* transcription tended towards higher levels in E11.5 gonads treated with FGF9 or FAT for 72 h (Fig. 4A). *Piwi2* and *Nanos2* were significantly increased in E12.5 XX gonads treated for 72 h with FGF9 or FAT (Fig. 4A). Moreover, culture of E11.5 XY male gonad/mesonephros samples with FAT for 72 h also increased transcription of *Nanos2*, *Piwi2* and *Dppa4* (Supplementary Fig. 3). In addition, quantitative flow cytometric analyses demonstrated significantly increased levels of DPPA4 protein in germ cells of E11.5 XX gonad/mesonephros samples treated with FGF9 and FAT (Fig. 4B). Significantly, germ cells in E11.5 XX FAT-treated samples were also associated with rare AMH-positive cells indicating their localisation within testicular-like tissue (Figs 3C and 4C). Although the inclusion of GSKi only moderately affected *Piwi2* expression in XX gonads treated with FAT, GSKi blocked the ability of FAT to

increase *Nanos2* and *Dppa4* transcription (Figs 4A and 3B). Moreover, GSKi prevented FAT induction of DPPA4 protein expression as assessed using flow cytometry (Fig. 4B). Interestingly, DPPA4 protein levels were not significantly increased by 72 h of FGF9 or FAT treatment in E12.5 XX gonad/mesonephros, although a trend towards higher levels was observed, and these increases were blocked by GSKi (Fig. 4B).

FGF9 and FAT also modulated transcription and translation of STRA8. *Stra8* transcription was reduced four-fold in E11.5 and E12.5 XX gonad/mesonephros samples treated with FGF9 (unpaired *t*-tests; $P < 0.001$ and $P < 0.0001$ respectively), but *Stra8* was maintained at XX control levels in FAT- and FAT-GSKi-treated samples cultured from E11.5 and from E12.5 for 72 h (Fig. 4A). Despite this, quantitative flow cytometric analysis demonstrated that STRA8 protein was reduced to XY control levels in germ cells of E11.5 and E12.5 samples treated with either FGF9 or FAT (Fig. 4B). Moreover, the

Figure 4 FGF9 and FAT promoted male germ cell characteristics in XX germ cells. (A) qRT-PCR analysis of *Dppa4* (E11.5 only), *Stra8*, *Piwi2* (E11.5 and E12.5) and *Nanos2* (E12.5 only) expression in XX (and XY control) gonad/mesonephros tissues cultured for 72 h with control (C), FGF9, FAT or FAT-GSKi media. Expression is relative to the XX control sample set at 1.0, normalised against *Mvh* (*Ddx4*) to correct for germ cell number in each sample. a, b and c are statistically different (ANOVA with multiple comparison test; $P < 0.05$). Values are expressed as average $\pm$ s.e.m., $n = 3-6$. (B) Quantitative flow cytometric analysis of DPPA4 and STRA8 levels in Oct4-GFP-positive germ cells of E11.5 and E12.5 XX (and XY control) gonad/mesonephros sample tissues cultured for 72 h with control, FGF9, FAT or FAT-GSKi media. a, b and c are statistically different (ANOVA with multiple comparison test; $P < 0.05$, values are expressed as average $\pm$ s.e.m.; $n = 3-8$). (C) IF images of E11.5 XX (and XY control) gonad/mesonephros tissues cultured for 72 h with control, FAT or FAT-GSKi media and analysed with antibodies specific for AMH (cyan) and STRA8 (red). Arrows indicate AMH-positive cells. Germ cells are marked by Oct4-GFP. Dashed white lines outline testis cord-like structures in XX gonads treated with FAT and testis cords in XY gonads. Scale bar: 50 μm.

ability of FGF9 and FAT to repress STRA8 protein levels was partially alleviated by GSKi in E11.5 samples and completely abolished in E12.5 samples cultured for 72 h (Fig. 4B). These data were supported by IF analyses of STRA8 in gonad/mesonephros samples cultured under the same conditions (Fig. 4C).

FGF9 induced mitotic arrest in XX germ cells

Combined, these data indicated that FGF9 and FAT blocked germ cell entry into meiosis and promoted expression of male germ line markers in both XX and XY gonads, whereas enhanced β -catenin signalling opposed these outcomes. However, definitive cell biological markers of male and female germ line development are the entry of germ cells into mitotic arrest at G_0/G_1 , and meiosis at G_2/M of the cell cycle respectively. To determine the relative abilities of FGF9 and FAT to regulate meiosis and mitotic arrest in germ cells, we extended treatment of E11.5 gonad/mesonephros

samples to 96 and 120 h. We used flow cytometry to separate germ cells based on their expression of MVH and measured cell cycle state based on DNA content using propidium iodide staining and proliferation based on EdU incorporation. In addition, we used flow cytometry to measure the percentage of germ cells that were positive for γ H2AX, which is highly enriched in meiotic germ cells.

In E11.5 XY gonad/mesonephros samples cultured in control media for 96 h, 78% of germ cells were in G_0/G_1 , whereas 9% were in G_2/M (Fig. 5A i and ii), and this did not change after 120 h of culture (Fig. 5B i and ii). By comparison, in XX control gonads, 66% of germ cells were in G_2/M by 120 h and 66% were positive for γ H2AX (Fig. 5B ii and iii). Combined, these data demonstrate that germ cells in XY and XX control gonads entered mitotic arrest or meiosis respectively, although with a slight delay compared with germ cells *in vivo*.

In XX gonads cultured with FGF9, 28% of germ cells were in G_0/G_1 after 96 h (Fig. 5A i), whereas 49% were

Figure 5 FGF9 induced mitotic arrest, whereas FAT maintained pluripotent gene expression in foetal germ cells. (A–C). Flow analysis of germ cells in G_0/G_1 and G_2/M of the cell cycle and germ cells enriched for γ H2AX in E11.5 and E12.5 XX and XY (control only; C (XY)) gonad/mesonephros tissues cultured for 96 or 120 h with control (C), FGF9 or FAT media. A. E11.5 + 96 h culture (γ H2AX not analysed); B. E11.5 + 120 h C. E12.5 + 96 h. a, b, c and d are statistically different (ANOVA with multiple comparison test; $P < 0.05$; values are expressed as average $\pm$ S.E.M.; $n = 6$ for 96 h, $n = 4$ for 120 h). (D) qRT-PCR analysis of *Nodal*, *Oct4*, *Nanog* and *Sox2* expression in E12.5 XX (and XY control) gonad/mesonephros tissues cultured for 72 h with control (C), FGF9 or FAT media. Expression is relative to the XX control sample set at 1.0, normalised against *Mvh* (*Ddx4*) to correct for germ cell number in each sample. a, b and c are statistically different (ANOVA with multiple comparison test; $P < 0.05$; values are expressed as average $\pm$ S.E.M., $n \geq 3$).

in G_0/G_1 after 120 h (Fig. 5B i), indicating that FGF9 promoted entry of XX germ cells into mitotic arrest with around two-thirds of the efficiency, and a slight delay compared with XY controls. By comparison, in FAT-treated samples 41% of germ cells were in G_0/G_1 at 96 h (Fig. 5A i), but this dropped to 31% at 120 h (Fig. 5B i). Consistent with these findings, treatment of XX gonad/mesonephros tissues with FGF9 for 120 h reduced the percentage of germ cells in G_2/M and the percentage expressing γ H2AX (Fig. 5B ii, iii). However, neither the percentage of XX germ cells in G_2/M nor the proportion of γ H2AX+ cells decreased in the presence of FAT after 120 h of culture (Fig. 5B ii, iii). Combined, these data indicate that continued culture with FGF9 is more effective than FAT at inducing germ cells into G_0/G_1 arrest.

Although FAT treatment induced very limited morphological change in E12.5 XX gonads, transcription of the male somatic and germ line genes increased and ovarian markers decreased. Therefore, we next tested whether FGF9 or FAT altered the balance of mitotic arrest and meiosis in gonad/mesonephros samples cultured for 96 h from E12.5. As expected, germ cells entered mitotic arrest and meiosis with high efficiency in XY and XX control-treated samples, with 89 and 92% in G_0/G_1 and G_2/M respectively (Fig. 5C i, ii). Compared with E11.5, the ability of FGF9 to block germ cell entry into meiosis was lost in E12.5 XX gonad/mesonephros samples, with 93% of germ cells in G_2/M and 3.6% in G_0/G_1 after 96 h (Fig. 5C). However, the proportion of germ cells in G_2/M was moderately reduced in FAT-treated samples compared with XX controls (Fig. 5C ii), although the percentage of germ cells expressing γ H2AX in FAT, FGF9 and XX control samples was similar (Fig. 5C iii).

FAT maintained expression of the core pluripotency genes in germ cells

The core network regulating pluripotency is repressed between E13.5 and E15.5 in both XX and XY germ cells, although *Nanog* is maintained for a day longer in XY compared with XX germ cells. *Nodal* is expressed in XY germ cells during this period and has been implicated in maintaining pluripotency. Therefore, we used qRT-PCR to determine whether FGF9, FAT and FAT-GSKi modulated expression of *Nodal*, *Oct4*, *Sox2* and *Nanog* in E12.5 XX gonads treated for 72 h. Transcription of *Nodal*, *Sox2* and *Nanog* was higher in E12.5 XX gonads treated with FAT for 72 h than in XX and XY control samples (Fig. 5D). In FGF9-treated samples, transcription of *Nodal* was similar to that in XX and XY controls, whereas *Oct4* and *Nanog* were transcribed at intermediate levels, and *Sox2* was transcribed at high levels (Fig. 5D). Similarly, increased expression of *Nodal*, *Nanog* and *Oct4* was detected in E11.5 XY gonad/mesonephros samples treated with FAT, but not FGF9, TGF β or activin alone for 72 h (Supplementary Fig. 4).

Discussion

Growth factors FGF9, activin and TGF β promote testicular characteristics in XX gonads

In this study, we have demonstrated that both FGF9 and, to a more limited extent, TGF β , promote proliferation of supporting cells in XX gonads but activin alone did not. In addition, we provided evidence that culture of XX gonads with FGF9, activin and TGF β repressed ovarian development and induced transcription of *Sox9* and limited expression of SOX9 protein. This was associated with FAT-induced formation of laminin-delineated cords that were morphologically similar to early testis cords. Moreover, FGF9 and FAT directly or indirectly blocked female germ line development while promoting male germ line development, and FAT maintained high levels of germ cell pluripotency. The testis-promoting activity of FAT was significantly reduced by blocking GSK, consistent with a role for β -catenin signalling in opposing the activity of FAT in this context. Although FGF9 was less effective at inducing testis development than FAT, it was moderately more effective at blocking meiosis and promoting mitotic arrest in XX germ cells and did not maintain germ line pluripotency to the same extent as FAT. Together, these data identified a cooperative role for FGF9, activin and TGF β in inducing testicular characteristics in cultured XX gonads.

Gene knockout studies have demonstrated that FGF9 signalling is essential for testis development, but deletion of activin or TGF β genes have relatively mild effects (Shull *et al.* 1992, Colvin *et al.* 2001, Schmahl *et al.* 2004, Kim *et al.* 2006, 2007, Bagheri-Fam *et al.* 2008, Memon *et al.* 2008, Archambeault & Yao 2010, Moreno *et al.* 2010, Mendis *et al.* 2011). Consistent with this, this study demonstrated that *ex vivo* exposure of XX gonads to FGF9 promoted changes in XX gonad characteristic of the developing testis, including supporting cell proliferation, repression of ovarian genes and promotion of male germ line development. However, FGF9 alone was insufficient to drive *Sox9* transcription or the formation of laminin-delineated testis cords to the extent achieved with FAT, demonstrating a significant contribution from activin and TGF β . This is consistent with a previous study in which intact foetal gonads were exposed to FGF9 in *ex vivo* cultures (Schmahl & Capel 2003), although FGF9 alone was capable of inducing SOX9 in dissociated gonadal cells or at the periphery of XX gonads when exposed to FGF9-coated beads (Kim *et al.* 2006).

Several factors may explain the ability of FGF9 to induce SOX9 in dissociated gonadal cells or in cells close to FGF9 coated beads, but not in whole gonads. One possibility is that β -catenin signalling is dispersed in dissociated cells or is less effective at the gonadal periphery, but remains intact in whole gonads. Alternatively, FGF9 levels may be insufficiently increased in whole gonad cultures exposed to FGF9 in

the medium compared with local FGF9 levels achieved using beads. Nonetheless, activin and TGF β contributed to the induction of *Sox9* when applied in combination with FGF9, although they were also individually insufficient to induce *Sox9* expression or testis development in cultured XX gonads. Combined, these data indicate that FGF9, activin and TGF β act together to promote *Sox9* transcription and some testicular characteristics in intact cultured XX gonads and limited expression of SOX9 and AMH protein. This conclusion is consistent with previous observations that activin/TGF β signalling is required for testis cord formation and Sertoli cell proliferation in E11.5-E12.5 XY gonads (Miles *et al.* 2013) and the established role for FGF9 in testis development (Lin & Capel 2015).

Although we observed increased *Sox9*, *Cyp26b1* and *Inhba* transcription and some SOX9+ and AMH+ cells in FAT-treated XX gonads, *Dhh* was not upregulated, indicating that Sertoli cell development was restricted in this system. Similarly, while SOX9+ and AMH+ cells were detected, their numbers were low, also indicating that Sertoli cell differentiation was limited. This presumably explains why *Amh* transcription was not increased when the whole gonad/mesonephros cell population was analysed using qRT-PCR. Together, these observations indicate that although FAT induced some testicular characteristics in XX gonads, the extent of testicular development was limited.

A role for TGF β and activin in testis organisation?

One of the more prominent features induced by FAT was the substantial morphological reorganisation of gonadal cells characterised by aggregation of germ cells with supporting cells in laminin-delineated structures similar to testis cords. These morphological changes could occur via the supporting cells, as they proliferate and differentiate into Sertoli-like cells and drive cellular reorganisation, or could be driven by growth factor-stimulated cell reorganisation and basement membrane deposition and/or organisation. However, cellular and basement membrane organisations were strongly affected by FAT, whereas Sertoli cell differentiation was incomplete. It therefore seems likely that FGF9, activin and TGF β actively support cord formation through enhanced deposition and/or organisation of basement membrane, rather than indirectly through extensive Sertoli cell differentiation. Consistent with this, TGF β and activin have been associated with basement membrane deposition in other systems (Whiting *et al.* 2003, Krag *et al.* 2007, Botta *et al.* 2012). Cord formation is also likely to involve supporting cell proliferation, which was driven in XX gonads treated with either FGF9 or FAT at levels equivalent to Sertoli cell proliferation in XY gonads. Moreover, TGF β also promoted supporting cell proliferation in XX gonads, albeit with less potency than FGF9. However, as FGF9 was sufficient to drive

maximal supporting cell proliferation but not extensive deposition of laminin, supporting cell proliferation *per se* appears insufficient to account for the presence of testis cords in FAT-treated XX gonads.

Enhancing β -catenin moderates the testis-promoting activity of FGF9, activin and TGF β

It is well established that WNT4/ β -catenin signalling opposes activity of the testis-determining pathway, favouring ovarian development (Chassot *et al.* 2014). In this study, blocking GSK abolished the ability of FGF9 and FAT to induce expression of *Sox9* and *Cyp26b1*; however, expression of the ovary-promoting gene *Rspo1* was not restored, and *Foxl2* transcription was only partially restored. The failure to restore *Rspo1* is perhaps not surprising as GSK phosphorylation of β -catenin occurs downstream of RSP01. However, the factors limiting the ability of GSKi to fully restore *Foxl2* transcription remain unclear.

Interestingly, the foetal Leydig cell-expressed gene *Inhba* was strongly induced by FAT but was not suppressed by blocking GSKi, indicating that this pathway is not subject to repression by β -catenin. Consistent with the ability of β -catenin to inhibit testis development, GSKi also blocked the formation of laminin-delineated testis cords and the occurrence of SOX9 and AMH-positive cells in E11.5 gonads treated with FAT. However, in a related study, blocking GSK in XX foetal gonads increased supporting cell proliferation in the presence of FGF9 (Gustin *et al.* 2016). Taken together, these observations indicate that although β -catenin blocks FAT induction of testis development, it does so by blocking growth factor induction of testis differentiation genes rather than restricting supporting cell proliferation.

FGF9 promotes male germ line differentiation and germ cell mitotic arrest within a critical window between E11.5 and E12.5

Sex-specific development of germ cells is dependent on the surrounding somatic cell environment. This is achieved in part by FGF9, which represses germ cell expression of *Stra8* and entry into meiosis, while promoting expression of male germ line markers, including *Nanos2* (Barrios *et al.* 2010, Bowles *et al.* 2010, Wu *et al.* 2013). However, TGF β /activin signalling is also directly or indirectly required for male germ line development (Miles *et al.* 2013, Wu *et al.* 2013). Consistent with this, treatment of E11.5 XX gonad/mesonephros samples with FGF9 or FAT increased expression of the male germ cell-expressed genes *Nanos2*, *Piwil2* and *Dppa4* and high levels of DPPA4 protein.

Although FGF9 can reduce *Stra8* transcription and entry of germ cells into meiosis (Bowles *et al.* 2010),

the ability of FGF9 to promote germ cell mitotic arrest has not been studied. In this study, flow cytometric analyses demonstrated a greater proportion of germ cells in G₀/G₁ in XX gonads treated with FGF9 from E11.5 than in either XX controls or FAT-treated samples, consistent with a role for FGF9 in promoting germ cell mitotic arrest. Moreover, the percentage of germ cells expressing γ H2AX was significantly lower in FGF9-treated gonads than in XX control gonads. Despite this, the ability of FGF9 to induce mitotic arrest in foetal germ cells was lost when gonad/mesonephros samples were cultured from E12.5, demonstrating that FGF9 primarily induces mitotic arrest in XX germ cells in the critical window between E11.5 and E12.5, corresponding to the male sex-determining period for XY germ cells (Adams & McLaren 2002).

Interestingly, although FGF9 reduced *Stra8* transcription in XX germ cells, FAT failed to do so, indicating that activin/TGF β signalling negates the ability of FGF9 to transcriptionally repress *Stra8*. Despite this, both FGF9 and FAT reduced STRA8 protein expression as assessed by quantitative flow cytometry and IF, indicating that control of STRA8 activity is achieved at both the transcriptional and translational levels. However, although FAT reduced STRA8 protein with efficiency similar to FGF9, the percentage of germ cells in G₂/M and expressing γ H2AX was only partly reduced in FAT-treated samples. This is perplexing as it indicates that germ cells enter meiosis in the absence of STRA8 in FAT-treated samples. Nevertheless, as *Stra8* transcription remained high in FAT-treated samples, it is possible that sufficient protein persisted during the earlier stages of FAT-treated cultures to enable germ cell entry into meiosis.

FAT enhances germ line pluripotency

Despite the greater ability of FAT to promote testicular development at the somatic level, with the exception of inducing mitotic arrest and blocking entry into meiosis, FGF9 and FAT appeared to promote male development in XX germ cells with similar efficiency. Although, FAT induced mitotic arrest in germ cells more effectively than FGF9 in XX gonads treated from E11.5 for 96 h, this pattern was reversed in XX gonads treated for 120 h. It remains unclear why fewer germ cells were induced into G₀/G₁ in E11.5 samples treated with FAT, but one possibility is that continued exposure to FAT maintains germ cells in a relatively undifferentiated state and causes arresting germ cells to re-initiate proliferation. Consistent with this, transcription of *Nodal*, *Nanog* and *Oct4* was maintained at higher levels in XX samples treated for 72 h with FAT than FGF9 or control media. It therefore appears that activin and/or TGF β contribute significantly to maintaining germ cell pluripotency, a conclusion consistent with observed roles for

activin/TGF β /Nodal signalling in maintaining *Nanog* expression and pluripotency in XY germ cells (Spiller *et al.* 2012, Miles *et al.* 2013), the epiblast and in human embryonic stem cells (Mesnard *et al.* 2006, Vallier *et al.* 2009). However, it also seems likely that activin/TGF β interact with FGF signalling to maintain pluripotency, as FGF9 can promote derivation of pluripotent EG cells (Durcova-Hills *et al.* 2006), and in this study, FGF9 alone was sufficient to partially maintain germ cell pluripotency in XX germ cells. Moreover, FAT increased transcription of pluripotency genes in XX samples treated for 72 h, whereas individual treatments using FGF9, activin and TGF β had no effect.

Pluripotency is normally repressed in the developing germ line and maintained during formation of germ cell tumours, consistent with a requirement to downregulate pluripotency genes to allow germ line differentiation (Cook *et al.* 2009, Krentz *et al.* 2009, Western 2009, Western *et al.* 2010). *Fgf9* is repressed in the developing testis between E12.5 and E14.5 (Bowles *et al.* 2010, Miles *et al.* 2013), indicating that continued exposure may be detrimental for germ line development. However, *in vivo* regulation of *Fgf9* differs from that of *Inhba* (Activin A) and TGF β in that, *Inhba* increases in somatic cells, while TGF β 1 is upregulated in the germ cells of the developing testis between E12.5 and E15.5 (Miles *et al.* 2013). Moreover, FAT and FGF9 also induced male development in XX germ cells, consistent with observed roles for FGF9 and activin in promoting male germ line differentiation (Bowles *et al.* 2010, Wu *et al.* 2013). Taken together, these observations indicate that FGF9, TGF β and activin act in a complex network that regulates both differentiation and pluripotency in foetal germ cells, and the balance of these factors is critical for ensuring appropriate male germ line development.

Conclusions

Male sex determination is centrally dependent on the transcription factors SRY and SOX9, which activate signalling pathways involved in testis differentiation. By demonstrating that FGF9, activin and TGF β can partly initiate testis development, this study provides insight into the complex inductive processes underpinning sex determination and early gonad differentiation in mice. Male sex determination is considered to involve transition across a 'threshold' after which testis development is ensured. Growth factors provide an attractive mechanism through which the male sex-determining threshold may be achieved. Moreover, activity of FGF9, activin and TGF β is modulated by ovarian factors such as β -catenin, potentially enhancing dosage-responsive outcomes. This may be of particular relevance for understanding reproductive disorders in patients in whom gonadal environment may be altered

during development, leading to partially disrupted gonad differentiation, reduced fertility and/or germ cell tumours (Skakkebaek *et al.* 2001). In addition, signalling mechanisms involving the FGF, activin/TGF β and β -catenin pathways are highly conserved between species and are broadly used to modulate developmental processes. It is interesting to speculate that similar signalling mechanisms may regulate gonad development in a broad range of situations, particularly non-mammalian species in which sex is determined through genetic or environmental mechanisms not involving Sry.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-16-0293>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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