Evidence that activin A directly modulates early human male germline differentiation status

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Abstract

Disrupted fetal germline development underpins testicular germ cell neoplasia, which is increasing worldwide. The complex signaling milieu during normal testis development includes TGFβ superfamily ligands; this study tests the hypothesis that, activin A, a TGFβ superfamily member, can influence gonocyte development. The human seminoma-derived cell line, TCam-2, a model of fetal gonocytes, was cultured with activin A (1.25–25 ng/mL) for 48 h, or with 5 ng/mL activin A for short- (6, 24, and 48 h) and long-term (13 days) exposures, and downstream targets measured by qRT-PCR. Transcripts that exhibited significant dose-dependent responses to activin A included the early germ cell markers KIT, NODAL, and CRIPTO (NODAL co-receptor and activin inhibitor) which all increased and the differentiation marker DNMT3L which decreased. After 48 h, KIT, NODAL, and CRIPTO levels were significantly higher, while the differentiation marker NANOS2 was significantly lower. Interestingly, activin A exposure also significantly reduced both transcript and protein levels of the PIWI/piRNA pathway component DNMT3L. Because TCam-2 cells produce the activin inhibitor CRIPTO, CRIPTO was reduced using siRNA prior to activin A exposure. This selectively increased KIT in response to activin A. Other ligands present in the fetal testis (BMP4, FGF9, TGFβ1, and TGFβ2) induced distinct effects on germline marker expression. This study showed that activin A can directly modulate germline markers in this human gonocyte-like cell, promoting a less-differentiated phenotype. Additional findings indicate evidence of signaling crosstalk between activin A and NODAL, leading to target-specific effects on gonocyte differentiation.

Introduction

The rising incidence of testicular dysgenesis syndrome (TDS) has been well-documented worldwide. TDS is broadly manifested as male infertility, impaired spermatogenesis, cryptorchidism, hypospadias, and testicular germ cell tumours (TGCT), with the latter predominantly forming as a gonocyte-like seminoma or a differentiated non-seminoma subtype (Skakkebaek et al. 2016). TDS is understood to be a consequence of dysregulated fetal testis development, resulting from in utero exposure to agents such as endocrine disruptor chemicals (BPA, phthalates, pharmaceuticals, etc.) and/or genetic mutations. TGCTs are consider to arise under such conditions that impair normal fetal germ cell (gonocyte) development (Rajpert-De Meyts et al. 2016, Skakkebaek et al. 2016). To further our understanding of how testicular pathologies affecting germline development arise, this study examines how changes in the local environment may affect the trajectory of human gonocyte maturation.

Sperm production in adulthood depends on the correct progression of germ cell development beginning in fetal life; this can be tracked by monitoring sequentially expressed genes and the genomic methylation profiles in germ cells. In humans, primordial germ cells (PGCs) are specified at gestational week (GW) 2–3, and by GW 4, they express pluripotency genes (POU5F1 (OCT3/4), NANOG), germ cell specification genes (SOX17, BLIMP1), and other germ cell markers (KIT, DAZL, and DDX4). Germ cells colonise the gonads by GW 5 and male sex determination occurs at around GW 6 in response to SRY expression in Sertoli cells (Tang et al. 2016, Del Valle et al. 2017). Gonocytes begin to enter mitotic arrest from GW 9, coupled with an upregulation in differentiation genes including NANOS2; however, a heterogeneous population of both mitotic and quiescent germ cells are present until at least GW 25 (Li et al. 2017). The germ cell population is developmentally heterogeneous in fetal life (Gaskell et al. 2004, Li et al. 2017, Sohni et al. 2019). The transition of gonocytes into cells with features of pre-spermatogonia continues through the second and third trimesters of gestation and after birth, with most germ cells transforming into type-A dark spermatogonium by about 9 months after...
birth (Hutson et al. 2013); OCT4-positive gonocytes can persist in the testis up till at least 6 months after birth (Mitchell et al. 2008). The signaling cues that drive gonocyte transitions between these states over this long duration are unknown.

A unique feature of early germ cell development is the global epigenetic reprogramming which involves removal of DNA methylation in the migrating PGCs from GW 4 to GW 11, and establishment of new, sex-specific DNA methylation marks from GW 16 (Guo et al. 2015). During this time, unmethylated germline genomic regions are protected from transposable element activity by the PIWI/piRNA pathway. This involves both the degradation of transposable element transcripts into piRNAs within the cytoplasm and genomic DNA methylation of these elements via the actions of DNA methyltransferases DNMT3L and DNMT3A (Aravin et al. 2008). Transcription of PIWI/piRNA genes encoding TDRD and PIWIL proteins is upregulated from GW 7 to 9 (Guo et al. 2015, Tang et al. 2015) and their actions are needed for full fertility in adulthood. These, and the many other processes essential for gametogenesis, depend on coordinated cellular development controlled by multiple signaling factors and pathways which remain to be deciphered.

Activin A and NODAL are members of the Transforming Growth Factor-beta (TGFβ) superfamily, which includes many ligands important for normal growth of the fetal testis (reviewed in Young et al. 2015). TGFβ superfamily members have a common ligand structure and signal through shared, as well as distinct moieties, initiated by activation of serine/threonine kinase transmembrane receptors (reviewed in Miller & Hill 2016). NODAL utilizes the same Type 1 and Type 2 receptors and intracellular Smads (SMAD2/3) as activin A. However, NODAL additionally requires the co-receptor CRINTO (encoded by TDGF1) (Miller & Hill 2016). At the cell surface, the presence of CRINTO antagonizes activin A activity, while also facilitating NODAL signalling, as demonstrated initially in HEK293T cells (Gray et al. 2003, Kelber et al. 2008). Downstream target gene activation can be governed by their relative levels, as demonstrated in mouse embryonic cells and in P19 embryonal carcinoma cells in which this occurs via SMAD2/4-mediated chromatin remodeling (Lee et al. 2011, Coda et al. 2017). Importantly, there is no clear knowledge of how these two ligands interact functionally in fetal gonads, despite the demonstration of fundamental roles for each in fetal germline maturation. The outcome of NODAL signaling in human fetal testis germ cells is the promotion of their proliferation and pluripotency factor expression (Jorgensen et al. 2018), with similar observations in mouse (Souquet et al. 2012, Spiller et al. 2012). In the human, there is currently no specific role assigned to activin A, but in the mouse, the absence of activin A results in increased gonocyte numbers at birth (Mendis et al. 2011). Important, new findings regarding this signaling pathway challenge long-standing dogma about receptor subunit utilization and highlight the need for context-dependent observations to understand how they act (Christian & Heldin 2017, Ramachandran et al. 2018).

In the fetal human germline and testis, several activin/NODAL signaling components have been detected (Anderson et al. 2002, Jorgensen et al. 2018). The INHBA subunit is detectable in fetal Leydig cells, as are receptor proteins in human gonocytes, indicating that human germline cells can respond directly to locally produced TGFβ superfamily ligands (Anderson et al. 2002). Furthermore, human gonocyte and spermatogonial stem cell behaviors are affected by specific ligand inhibitors in testis cultures, demonstrating germline responsiveness within the testicular milieu (Jorgensen et al. 2018, Moraveji et al. 2019).

TGCTs are understood to originate from fetal germ cells that have impaired differentiation and ultimately form either seminoma or non-seminoma tumours. Seminomas contain relatively uniform undifferentiated gonocyte-like cells, while non-seminomas contain pluripotent embryonal carcinoma cells and a range of differentiated derivatives (Rajpert-De Meyts et al. 2016). The TCam-2 cell line was derived from a primary seminoma sample (Mizuno et al. 1993) and shares many characteristics with fetal germ cells, including the presence of TGFβ superfamily signaling components (Dias et al. 2009, Young et al. 2011) and responsiveness to BMP4, activin, and retinoic acid (Young et al. 2011). Their differentiation in response to BMP4 has been carefully documented in xenotransplantation studies in mice (Nettersheim & Schorle 2017, Nettersheim et al. 2019). These cells also retain features of seminomas, including aneuploidy and hypomethylation, and expression of key genes including KIT, KITL, POUSF1 and NANO2. The lack of methylation on both alleles of two imprinted genes, H19 and IGF2, indicates that the progenitor to this line arose from a fetal germ cell prior to the establishment of paternal imprints (de Jong et al. 2008); however, TCam-2 cells are more highly methylated than typical seminomas (Wermann et al. 2010, Nettersheim et al. 2013). Such features demonstrate the suitability of TCam-2 cells for both in vitro and in vivo studies of seminoma biology, while providing an important, accessible model of the early human male germline.

With the goal of further understanding how fetal male germline fate is determined, this study assessed the impact of activin A and other TGFβ molecules on the expression of genes relevant to human gonocyte differentiation using the seminoma-derived TCam-2 cell line. The study addresses the hypothesis that activin A can directly modulate the gonocyte transcriptome and tests the potential for its crosstalk with NODAL to affect germline differentiation.
Materials and methods

TCam-2 cell culture

TCam-2 cells were maintained as previously (Young et al. 2011), in RPMI1640 medium (Thermo Fisher Scientific) containing 10% fetal bovine serum (FBS; Bovogen, Keilor East, Australia), 25 units/mL of penicillin, and 25 µg/mL of streptomycin (Thermo Fisher Scientific) at 37°C in 5% CO₂ in air. For all experiments, 100,000 cells were seeded into individual wells of 6-well plates, unless otherwise stated.

Growth factor dose response testing

Cells were grown to approximately 75% confluency, then media was replaced with serum-free media for overnight culture. The next day, 1.25 to 20 ng/mL of activin A, TGFβ1, TGFβ2, BMP4, FGF9 (Table 1), or volume-matched vehicle control were added in fresh serum-free media for 24 or 48 h, then the cells were harvested as described subsequently for transcript measurement. Two independent experiments were performed as a screen, with experimental duplicates for all growth factors. Following this, three independent experiments with experimental duplicates were performed for 1.25–5 ng/mL activin A.

Activin A treatments

TCam-2 cells at approximately 75% confluency were treated with 5 ng/mL of recombinant activin A (R&D Systems) or an equal volume of vehicle (Table 1). In cultures containing serum, activin A was added to the media on the day following plating. For long-term cultures (13 to 21 days), medium containing activin A was replaced daily and 1.0×10⁵ cells were passaged into new wells every 84 h. At every passage, cell numbers were determined using a hemocytometer. For shorter time-course experiments, cells were exposed for 6, 24, and 48 h to 5 ng/mL recombinant activin A or vehicle in serum-free media, then collected for transcript analysis. Five independent experiments were performed with experimental duplicates.

Table 1 Reagents for cell culture and immunofluorescence.

<table>
<thead>
<tr>
<th>Growth factors</th>
<th>Company</th>
<th>Cat #</th>
<th>Vehicle</th>
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<tr>
<td>Activin A</td>
<td>R&amp;D Systems</td>
<td>338-AC</td>
<td>4 mM HCl</td>
</tr>
<tr>
<td>Nodal</td>
<td>R&amp;D Systems</td>
<td>3218-ND</td>
<td>0.4% BSA in 4 mM HCl</td>
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<tr>
<td>BMP4</td>
<td>R&amp;D Systems</td>
<td>314-110-P-010</td>
<td>0.4% BSA in 4 mM HCl</td>
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<td>FGF9</td>
<td>R&amp;D Systems</td>
<td>7399-F9</td>
<td>Water</td>
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<tr>
<td>TGFβ1</td>
<td>Promokine (Heidelberg, Germany)</td>
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<td>Water</td>
</tr>
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<td>TGFβ2</td>
<td>Promokine (Heidelberg, Germany)</td>
<td>C63498</td>
<td>Water</td>
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</table>

Primary antibodies

Anti-DNMT3L (rabbit monoclonal) Abcam ab194094 1:100
Anti-OCT4 (goat polyclonal) Santa Cruz Biotechnology Sc-8628 1:100

Secondary antibodies

Donkey anti-goat Alexa Fluor-488 Thermo Fisher A11055 0.25
Donkey anti-rabbit Alexa Fluor-647 Santa Cruz Biotechnology A31573 0.25
Goat anti-rabbit Alexa Fluor-546 Santa Cruz Biotechnology A11010 1:500 (IF)

CRIPTO knockdown using siRNA

TCam-2 cells were grown to 60% confluency, then media replaced with RPMI medium with 5% FBS lacking penicillin-streptomycin. Cells were immediately transfected with Silencer® Select Pre-designed siRNA construct targeting CRIPTO (TDGF1; s#13592, Thermo Fisher Scientific) or scrambled control using Lipofectamine 3000 (Thermo Fisher Scientific), according to manufacturer protocols. Transcript reduction of >70% was achieved at all siRNA concentrations tested (6.25, 12.5, and 25 pmol) relative to control samples; 6.25 pmol was used for subsequent transfection experiments. Twenty-four hours post-transfection, cells were serum-starved overnight then exposed to 5 ng/mL activin A, 300 ng/mL human recombinant Nodal, or respective vehicles for 24 h in serum-free conditions. This dose was chosen based on a previous study (Nettersheim et al. 2015) and on an initial dose response test that identified an effect on key target transcripts, LEFTY1, LEFTY2, and NODAL. Cells were collected for transcript analysis from four independent experiments.

RNA extraction, cDNA synthesis, and quantitative RT-PCR (qRT-PCR)

Following culture, cells were washed with 2 mL PBS, then collected into 1 mL Trizol reagent (Invitrogen) for RNA extraction as per the manufacturer’s protocol. Genomic DNA was removed using DNA-Free (Invitrogen). RNA concentrations and purity were measured on a NanoDrop (Thermo Fisher Scientific). RNA concentrations were measured with 200 ng RNA with 200 Units SuperScript III Reverse Transcriptase (Thermo Fisher Scientific), 50 ng random primers, and 500 ng oligo dT primers (Promega) per sample. A control lacking reverse transcriptase was generated for every sample to assess potential gDNA contamination.

Power SYBR Green Master Mix (Thermo Fisher) and specific primer pairs (Table 2; Integrated DNA Technologies, Coralville, IA, USA) were used for transcript measurements in 384 well plates; cDNA was diluted 1:20, and every sample was measured in triplicate. Real time PCR was conducted on the Applied Biosystems 7900HT Fast Real-time PCR System (Gandé Genomics Centre, Monash Health Translation Precinct, Clayton, AU) and data generated and analysed using
Table 2  Primer pairs for qPCR (SYBR Green).

<table>
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<tr>
<th>Gene</th>
<th>Accession</th>
<th>Forward primer (5′–3′)</th>
<th>Reverse primer (5′–3′)</th>
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<tr>
<td>PIWIL1</td>
<td>NM_004764.4</td>
<td>TCCTAGGCAGAGAGGGAGGCAG</td>
<td>GACCGAGAGATGTAGTGAGG</td>
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<tr>
<td>PIWIL2</td>
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<td>ACGATGCGAGGTGGTGGCTCTC</td>
<td>GGCGCTAGTGGTGGTGGCTC</td>
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<td>PIWIL4</td>
<td>NM_152431.2</td>
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<td>GGGCTGCGTGGTGGGCTGTG</td>
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<td>TDRD1</td>
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<tr>
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<tr>
<td>MOV101L</td>
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<td>CAGACATGACGAGAGGAGATGC</td>
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<td>CAGACATGACGAGAGGAGATGC</td>
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<td>DNMT3B</td>
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<td>DNMT3L</td>
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<td>NODAL</td>
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<td>CAGACATGACGAGAGGAGATGC</td>
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<td>TDC1F (CRIPTO)</td>
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<td>ACGAGCTCGTGGAGGACTGAC</td>
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<tr>
<td>LEFT1</td>
<td>NM_020997.4</td>
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<td>LEFT2</td>
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<td>SOX17</td>
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<td>NONO2</td>
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<td>RPLP0</td>
<td>NM_001002.3</td>
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</table>

*From Nettersheim et al. (2015); ^from Coufal et al. (2009).*

SDS software (Applied Biosystems). Relative quantification was determined from a standard curve generated by serial dilutions. Target transcript levels were normalised to the RPLP0 housekeeper transcript, which remained unchanged in response to experimental conditions compared with controls. Across experiments, the s.d. of RPLP0 cycle threshold values was less than 0.5. Five independent experiments were performed with experimental duplicates; each averaged to yield a single data point.

Flow cytometry

TCam-2 cells were cultured as described previously and treated for 24 and 48 h with 5 ng/mL activin A or vehicle in serum-free media, following overnight serum starvation. Media was removed, wells washed with 2 mL phosphate buffered saline (PBS), then cells detached using 0.1% trypsin-EDTA (Invitrogen). Following centrifugation and supernatant removal, cells were resuspended in 4% paraformaldehyde (PFA)/PBS and fixed for 15 min at room temperature (RT). Cells were washed three times in permwash (1× saponin-based permeabilisation reagent (ThermoFisher) in 1% BSA/PBS) and stored in permwash for no more than 1 week before antibody staining and flow cytometry. Cells were centrifuged and resuspended in permwash containing 5% donkey serum (Sigma) for 15 min, then collected by centrifugation and resuspended in permwash with anti-DNMT3L, anti-OCT4, or concentration-matched IgG isotype controls (rabbit and goat, respectively) (Table 1; 45 mins at RT). Following two PBS washes, cells were incubated with donkey anti-rabbit AlexaFluor-647 and donkey anti-goat AlexaFluor-488 (45 min at RT; Table 1). After two washes, cells were resuspended in permwash containing 1 µg/mL propidium iodide (Sigma-Aldrich) and analysed on a BD Biosciences FACSCANTO-II at Monash FlowCore, MHTP Node. Single, intact cells were gated according to forward scatter vs width characteristics, and DNMT3L- and OCT3/4-positive cells were identified by comparison to staining with IgG isotype controls. Mean fluorescence intensity was measured for the DNMT3L-positive population and for the entire population for OCT3/4 in single intact cells using FlowJo X.0.7 software (Ashland, OR, USA). Four independent experiments were performed with experimental duplicates.

Immunofluorescence

One hundred thousand cells were seeded onto 12 mm glass coverslips in 12-well plates in RPMI/10% FCS/Pen-Strep. Once 75% confluent, cells were serum-starved overnight, then fixed immediately in 4% PFA for 15 min at RT, washed twice, and stored in PBS at 4°C until staining, or treated with 5 ng/mL activin A or vehicle control for 48 h, and then fixed as described. All following steps were performed at RT. Nonspecific antibody binding was blocked by incubation in 0.5% BSA/PBS for 30 min, then cells were permeabilised in 0.1% Triton X-100 (Merck) in PBS for 10 min. Cells were incubated overnight with an anti-DNMT3L antibody (Table 1) in 0.5% BSA/PBS. Cells were washed four times in PBS for 5 min each, then incubated with goat anti-rabbit AlexaFluor-546 (Table 1) for 3 h. After four washes, cells were incubated with 1.7 µg/mL DAPI (ThermoFisher) for 10 min, washed three times in PBS, mounted under GVA (Genemed, San Francisco, CA, USA), and set overnight at 4°C. Imaging was performed at Monash Micro Imaging (MHTP, Clayton, VIC, Australia) using an Olympus FV1200 confocal microscope.

Statistical analysis

Statistical analysis was performed using GraphPad Prism Software. All data are presented as mean ± s.d., unless
otherwise stated. Statistical differences were determined by a student’s t-test or one-way ANOVA if data were normal following the Shapiro–Wilk normality test. If not normally distributed, a Mann–Whitney test or Kruskal–Wallis test was performed. Significance was determined if $P < 0.05$.

**Results**

**TCam-2 cells respond to activin A in a dose-dependent manner**

Exposure of TCam-2 cells to 5 ng/mL activin A for 24 h was previously shown to increase KIT levels (Young et al. 2011). To identify the dose at which a maximal response was obtained, TCam-2 cells were initially treated with 1.25 to 20 ng/mL activin A. Transcripts associated with gonocyte differentiation were measured after 48 h of culture. Doses of 10 to 20 ng/mL activin A resulted in no difference in transcript levels compared to that measured with 5 ng/mL (data not shown), identifying 5 ng/mL as the lowest concentration of activin A needed to achieve maximal effect. In subsequent experiments, TCam-2 cells were treated with 1.25 to 5 ng/mL of activin A (Fig. 1A). KIT and CRIPTO were significantly higher (1.5- and 1.2-fold, respectively) and DNMT3L was significantly lower (0.7-fold) when cultured with 1.25 ng/mL or higher. NODAL was significantly elevated (2-fold) with doses of >2.5 ng/mL of activin A. The significant difference between KIT, DNMT3L, and NODAL at 2.5 and 5 ng/mL demonstrates there is a dose-dependent transcriptional response to activin A for these genes (Fig. 1A). Activin A levels in the fetal testis are currently unknown; however, 5 ng/mL is the highest concentration measured in homogenates of postnatal mouse testes. This peak value was measured in samples collected at birth (Barakat et al. 2008), indicating that levels at and below this concentration are of physiological relevance.

**Activin A upregulates early germline markers and downregulates differentiation-associated transcripts**

The response of the early germ cell markers KIT, NODAL, and CRIPTO and the germ cell differentiation markers NANOS2 and DNMT3L were examined in a time-course experiment at 6, 24, and 48 h of exposure to 5 ng/mL activin A (Fig. 1B). KIT was significantly elevated following activin A treatment compared to in control (vehicle) samples at 24 and 48 h; however, the KIT transcript levels declined over time in both sample groups indicating the potential for KIT expression to be modulated by other factors such as media, cell density, or growth. The transcript encoding NODAL, which can maintain germline pluripotency marker expression (Jorgensen et al. 2018), was significantly higher (3- to 4-fold) than in controls at 6, 24, and 48 h. CRIPTO, encoding the NODAL co-receptor and also a downstream target of NODAL (Souquet et al. 2012), was...
significantly elevated at 48 h. In contrast, transcription of the male germ cell differentiation marker, *NANOS2*, was reduced to 0.35-, 0.34-, and 0.26-fold of control levels in activin A-treated samples 6, 24, and 48 h after treatment (Fig. 1B).

**Activin A reduces DNMT3L transcript and protein levels in TCam-2 cells**

Following 24 and 48 h of activin A treatment, *DNMT3L* was 0.62- and 0.48-fold of control levels, respectively (Fig. 2A). Consistent with this, flow cytometry revealed a significant decrease in the proportion of cells positive for DNMT3L after 48 h of culture (from 41.6% in vehicle to 23.5% with activin A). The absence of a change in the mean fluorescent intensity (MFI) within the DNMT3L-positive population at 24 or 48 h (Fig. 2B) indicates that there is no decrease in levels within cells producing this protein. In accord with these flow cytometry data, immunofluorescence demonstrated that DNMT3L was heterogeneously expressed in the TCam-2 population prior to treatment and after 48 h in culture. There appear to be fewer DNMT3L-positive cells following activin A treatment (Fig. 2C). As these data clearly demonstrate that the TCam-2 cell population is heterogeneous, at least in respect to DNMT3L, we investigated whether the pluripotency marker OCT4 exhibited a similarly heterogeneity. However, more than 90% of the cell population was positive for OCT4, and no change in this proportion or the MFI was noted at 24 or 48 h in control or activin-treated cultures (Fig. 2D), indicating the effect of activin A on DNMT3L is selective.

**Most downstream effects of activin A are not dependent on NODAL upregulation**

Because activin A induces *NODAL* transcription in TCam-2 cells (Fig. 1), we considered that activin effects

![Figure 2](https://rep.bioscientifica.com)

*Figure 2* DNMT3L and OCT4 expression in TCam-2 cells. (A) qRT-PCR analysis of *DNMT3L* in serum-starved TCam-2 cells exposed to 5 ng/mL Activin A (black) or vehicle control (white) for 6, 24, and 48 h. Five independent experiments with duplicate samples were performed. Each sample value was normalised to *RPLP0* and averaged to yield a single data point. (B) DNMT3L in TCam-2 cells exposed to 5 ng/mL Activin A or vehicle control for 24 and 48 h was measured via flow cytometry and the percentage of DNMT3L-positive cells and mean fluorescent intensity (MFI) of the positive population were measured. Data are expressed as mean ± s.d. Significant differences between vehicle and treated for each time-point are indicated by asterisk (**P < 0.01**). (C) Representative images of serum-starved TCam-2 cells on coverslips at time zero or exposed to activin A and vehicle for 48 h stained with anti-DNMT3L (red) and DAPI (Blue) at 20x and 40x magnification. Scale bars represent 100 µM (20x) and 50 µM (40x), and insets are negative controls where primary antibody was omitted. (D) The percentage of OCT4-positive cells and the MFI was measured by flow cytometry in TCam-2 cells treated with 5 ng/mL activin A (black bars) or control (white bars) for 24 and 48 h (n = 3). Data are presented as mean ± s.d.
on target genes such as KIT and DNMT3L could either be directly induced by activin A or indirectly affected by NODAL signalling. TCam-2 cells also produce CRIPTO (Spiller et al. 2016), which blocks activin signaling by binding the shared Type 1 receptor subunit, ALK4 (Gray et al. 2003). To test whether activin A activation of target genes in TCam-2 cells is affected by NODAL upregulation or by the presence of CRIPTO, we employed an siRNA strategy to reduce CRIPTO levels and thereby inhibit NODAL signaling (Fig. 3A).

To establish that NODAL signaling is possible in TCam-2 cells, 300 ng/mL NODAL was added to serum-starved TCam-2 cells for 24 hrs. The elevation of downstream target genes LEFTY2 (56.5-fold, \( P = 0.0562 \)) and NODAL (1.8-fold; \( P < 0.01 \); Fig. 3B) confirmed NODAL activity. To determine if CRIPTO knockdown disrupted NODAL signaling, 300 ng/mL NODAL was added following siRNA transfection. CRIPTO was successfully reduced by siRNA targeting in both vehicle and NODAL-treated TCam-2 cells (86.5% and 88% knockdown, respectively, compared with controls; Fig. 3C). While NODAL-induced transcription of LEFTY2 (28.1-fold; \( P < 0.05 \)) and NODAL (1.6-fold; \( P = 0.0512 \)) occurred in the scrambled-control samples, NODAL did not induce either LEFTY2 or NODAL in CRIPTO-siRNA samples (Fig. 3C), indicating disruption of NODAL signaling.

We next used this approach to test whether activin A induction of target genes was affected by NODAL/CRIPOT signaling by adding 5 ng/mL activin A to TCam-2 cells 40 h after CRIPTO siRNA transfection (Fig. 3A). CRIPTO knockdown was maintained in siRNA-transfected cells regardless of the presence or absence of exogenous Activin A (Fig. 3D). In addition to NODAL,
which is induced by activin A treatment (Fig. 1), we measured LEFTY1 and LEFTY2, which can be induced by either NODAL or activin A in mouse embryonic stem cells (Lee et al. 2011). Activin A treatment of scrambled siRNA controls resulted in a 910-fold increase in LEFTY2, a 12-fold increase in LEFTY1, and a 3.9-fold increase in NODAL. Moreover, when CRIPTO was reduced by siRNA, activin A treatment still resulted in a 542-fold induction of LEFTY2 (P<0.05), 10-fold induction of LEFTY1 (P<0.05), and a 4-fold increase in NODAL (P<0.0001), indicating that endogenous CRIPTO synthesis was insufficient to block activin A signaling in TCam-2 cells and that activin A could induce a clear and highly robust expression of LEFTY2, LEFTY1, and NODAL independent of CRIPTO.

To determine whether activin A modulation of other transcripts relevant to human gonocyte development was affected by CRIPTO reduction, the early germline markers KIT and SOX17 and the differentiation marker DNMT3L were also measured. SOX17 was significantly increased by activin A in both scramble- and CRIPTO siRNA-transfected cells, and DNMT3L was significantly lower in both (Fig. 3D). The CRIPTO siRNA did not alter activin A effects on SOX17 or DNMT3L, indicating that CRIPTO does not modulate the inductive potency of activin A in this context. Only KIT was significantly higher in activin A-treated CRIPTO knockdown samples compared with activin A-treated scramble controls, demonstrating a negative impact of CRIPTO on KIT transcription (Fig. 3D) and revealing a transcript-dependent effect of CRIPTO inhibition on activin A signaling. Collectively, these data show that activin A can directly influence transcription of key development genes in TCam-2 cells, and probably in early human male germ line cells, and that its actions are selectively affected by CRIPTO.

**Activin A levels affect components required for de novo DNA methylation and piRNA regulation**

Transcripts strongly upregulated in differentiating gonocytes encode proteins required for de novo DNA methylation, such as DNMT3A and DNMT3L, and for piRNA synthesis, including PIWIL and TDRD family members. Because activin A exposure decreased DNMT3L transcript and the proportion of cells expressing the protein (Fig. 2), qRT-PCR was used to measure transcriptional responses by a range of additional piRNA pathway and de novo DNA methylation genes in TCam-2 cells. We compared transcript levels obtained in our qRT-PCR analysis of untreated cells with those reported previously as RNASeq data in human GW 7 PGCs and in TCam-2 cells (Irie et al. 2015; datasets GSM1466229 and GSM1466230). These expression
profiles were generally quite consistent between our study and previously reported RNASeq data. **Dnmt3a**, **Dnmt3b**, and several **Tdrd** mRNAs were similar in PGCs and TCam-2 cells, while **Mov10l1** expression is not detectable in TCam-2 cells. **Piwil1**, **Piwil2**, **Mael**, and **Mov10l1** levels were more than 100-fold higher in PGCs compared to TCam-2 cells, while **Dnmt3l** was 70-fold lower (Fig. 4A).

To assess how transcripts encoding proteins in the DNA methylation and piRNA pathways were affected by activin a, TCam-2 cells were treated with activin a for 6, 24, and 48 h. Exposure to activin a moderately reduced **Tdrd1** and **Dnmt3l** and increased **Dnmt3a** and **Tdrd5**, but did not affect **Tdrd9**, **Tdrd7**, or **Piwil4** (Fig. 4B).

**LINE1 levels are unaffected by activin A exposure**

The piRNA pathway serves a crucial role in repressing retrotransposon activity during de novo DNA methylation. This includes the methylation of repetitive sequences, such as LINE elements, through processes that require both **Dnmt3l** and **Tdrd1**. As activin A exposure decreased both **Dnmt3l** and **Tdrd1** transcripts and decreased the percentage of **Dnmt3l**-positive cells, we proposed that **LINE1** expression may increase in TCam-2 cells as a result. However, while **LINE1 (L1orf2)** levels were high in TCam-2 cells (61% of **Rplp0** level, Fig. 4A), activin A exposure had no effect at 6, 24, or 48 h (Fig. 4B).

As global DNA demethylation requires up to 6 days in mouse ES cells (Walter et al. 2016), a longer-term exposure was undertaken to assess whether the decrease in **Dnmt3l** correlated with increased retrotransposon activity. Serum was absent from short-term cultures (up to 48 h) to avoid masking activin A responses; however, TCam-2 cells require it to sustain normal growth for periods longer than 3 days (not shown). **Dnmt3l** was also reduced by activin A treatment for 24 h in the presence of 10% serum (Fig. 5A), confirming the negative impact of activin A on **Dnmt3l** levels shown in Fig. 2. After 13 days of culture in medium containing 10% serum, both **Dnmt3l** and **Tdrd1** transcripts were significantly lower in activin A-treated samples (0.39 and 0.46-fold respectively) compared with controls; however, **L1orf2** expression was unaffected (Fig. 5B). Although TCam-2 cell numbers were comparable between activin A-treated and control groups at the first and second passages, the number of activin A-treated TCam-2 cells was lower starting at the third passage, on the second week of culture (Fig. 5C). The activin A-treated cells appeared slightly flatter than controls, but no evidence of increased cell death was observed (Fig. 5D).

**Other ligands in the developing testis modulate germ cell development transcripts in TCam-2 cells**

Other signaling factors present in the fetal testes were tested for their impact on this suite of germ cell differentiation markers. TGFβ1, TGFβ2, BMP4, and...
FGF9 were each added to TCam-2 cells for 24 h at doses from 1.25–20 ng/mL. Only low doses of either TGFβ1 or TGFβ2 significantly increased NODAL, while only TGFβ1 significantly increased DNMT3A and TDRD9. BMP4 significantly decreased both DNMT3L and CRIPTO at all doses and decreased TDRD1 at 5–20 ng/mL. FGF9 significantly reduced KIT at higher doses (5–20 ng/mL), TDRD9 at 1.25 and 5–20 ng/mL, and increased DNMT3L at 1.25–2.5 ng/mL (Fig. 6).

Discussion
To understand why male infertility rates are increasing, we need to learn what governs survival and differentiation of the human germline. A recent analysis of first trimester human fetal gonads demonstrated that NODAL and activin A signaling is important for gonocyte support, with the authors concluding that its dysregulation may influence the risk of TGCT formation (Jorgensen et al. 2018). The present study was designed to further examine the impact of local signaling cues on human fetal germ cell developmental transitions.

The seminoma-derived TCam-2 cell line retains markers of seminomas and fetal germ cells. It is an established human gonocyte model and is useful for investigating the roles of TGFβ superfamily ligands, which are produced by fetal somatic cells. TCam-2 cells produce few TGFβ superfamily ligand transcripts
(BMP7, INHBC, GDF3, and GDF15), but contain most signaling machinery and respond to activin A and BMP4 (Young et al. 2011, Irie et al. 2015). TCam-2 cells have been extensively used as a model of seminomas and their sensitivity to the microenvironment has been well documented. When transplanted into germ-cell-ablated mouse testes, they retain seminoma characteristics; however, when transplanted into the flank or brain of immunodeficient mice, TCam-2 cells undergo reprogramming to an embryonal carcinoma-like cell (Nettersheim et al. 2012, 2015), a process dependent on the presence of SOX2 and FOXA2 (Nettersheim et al. 2019). This outcome highlights their responsiveness to environmental cues. Outcomes from the present study provide evidence that activin A alters the human germ cell transcriptome, promoting a less-differentiated marker profile identified by the upregulation of KIT, NODAL, and CRIPTO and the decrease in differentiation markers DNMT3L and NANOS2. Heterogeneity in DNMT3L expression between TCam-2 cells within a culture reinforces the concept that this cell line is phenotypically flexible and heterogeneous, similar to early germ cells. Individual research groups should continue to consider this when using this important cell line, particularly since it has the key feature of a transformed cell line which permits multiplication outside of the normal niche.

KIT is of critical importance to fetal germ cell differentiation (reviewed in Mithraprabhu & Loveland 2009). It is involved in the migration, survival, and proliferation of murine primordial germ cells and is present on humans gonocytes up to GW 21 (Robinson et al. 2001, Kerr et al. 2008). KIT is re-expressed in the human postnatal testis by differentiated type B spermatogonia (Di Persio et al. 2017). Activating KIT mutations are detected in a quarter of seminomas, and protein levels are high in both germ cell neoplasia in situ (GCNIS) and seminomas (reviewed in Rajpert-De Meyts & Skakkebaek 1994, Rajpert-De Meyts et al. 2016). The observation that activin A induces higher levels of KIT transcripts in TCam-2 cells at both 24 and 48 h extends our earlier findings (Young et al. 2011), but contrasts with the outcome for seminoma fragments cultured for 48 h in which activin A reduced both KIT transcript and protein levels (Jorgensen et al. 2014). This unexpected finding highlights the potential for aberrant intrinsic responses specific to activin and TGFβ superfamily signaling to sustain testicular germ cell tumours.

Additional outcomes in this study indicate that activin A levels may selectively affect synthesis of the epigenetic machinery that is normally highly upregulated in the early differentiating male germine. DNMT3L transcript and protein and the TDRD1 transcript were reduced by activin A exposure. These are required for normal male fertility, with their absence in mice causing dysregulated or delayed de novo methylation and reduced germ cell numbers (Bourchí’s & Bestor 2004, Webster et al. 2005, Chuma et al. 2006, La Salle et al. 2007, Shoji et al. 2009, Niles et al. 2013). We do not know how synthesis of the piRNA machinery is controlled in the fetal testis; our finding that activin A can reduce DNMT3L and TDRD1 suggests that activin A may contribute to this important process.

The importance of altered epigenetic profiles and involvement of piRNA machinery in testicular germine tumour aetiology and progression has been examined in several studies. Both seminomas and non-seminomas exhibit a global piRNA loss, decreased levels of PIWILs and TDRD1, and LINE1 hypomethylation (Ferreira et al. 2014, Rouge et al. 2015), conditions that indicate TGCTs have a diminished capacity for retrotransposon suppression. DNMT3L was identified as upregulated in advanced-stage seminomas (Matsuoka et al. 2016). Because DNMT3L and TDRD1 downregulation can result in reduced genomic methylation, we predicted that activin A exposure would lead to methylation loss and therefore increased transposable element expression in TCam-2 cells. However, no change in L1ORF2 levels was measured following long-term activin exposure; we hypothesize this is due to the reduction by 50%, rather than complete DNMT3L loss. These results suggest that TCam-2 cells can be a practical model in which to conduct analysis of the mechanisms regulating PIWI/piRNA machinery synthesis and the outcomes of its dysregulation.

This study provides new information about the complex relationship between activin and NODAL and its physiological relevance for the male germine. Activin A stimulated significant increases in NODAL and the NODAL inhibitors, LEFTY1 and LEFTY2 in TCam-2 cells, which could significantly affect NODAL activity. This result was previously demonstrated in the mouse P19 embryonic carcinoma cell line (Coda et al. 2017) and in murine embryonic stem cells (Lee et al. 2011). In longer-term studies lasting several weeks, BMP inhibition was required for NODAL signaling and for TCam-2 cell reprogramming from a seminoma to embryonal carcinoma-like cell (Nettersheim et al. 2015, 2016). In the present short-term studies, we observed that NODAL alone was sufficient to evoke significant increases in NODAL and LEFTY2 transcript levels. Interestingly, although TCam-2 cells responded to NODAL, activin A induced a much greater elevation of the downstream transcripts encoding NODAL negative regulators. While CRIPTO reduction had minimal effect on activin A-induced transcript changes, the significant elevation in KIT provides evidence of a transcript-dependent effect of CRIPTO on activin A signaling. It is intriguing that the levels of NODAL, INHBA, and LEFTY transcripts vary by approximately 2.5-, 20-, and 30-fold, respectively, during the first trimester (Jorgensen et al. 2018). This may define a window for exposure to environmental disruptions that could affect key germine maturation events, which have been shown to include

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As a key driver of germ cell pluripotency in both mouse and human germ cells, NODAL functions downstream of FGF9 in mice (Spiller et al. 2012, Wu et al. 2013). Male E11.5 urogenital ridges cultured with FGF9 exhibit Cripto and Nodal upregulation; however, isolated E11.5 germ cells exposed to FGF9 upregulated Cripto, but not Nodal (Spiller et al. 2012). In TCam-2 cells exposed to FGF9, neither NODAL nor Cripto were upregulated. This result correlates with a role for the somatic cell microenvironment in mediating germ cell responsiveness to FGF9, highlighting how crucial the correct timing of somatic cues may be for normal germline differentiation.

In conclusion, this study demonstrates the capacity for activin A to selectively modulate germline marker expression in the human seminoma-derived line TCam-2. This approach has enabled investigations of signaling crosstalk within the TGFB superfamily and provided evidence of its impact on the machinery that controls epigenetic modification of the germline. By investigating the cellular transitions which occur in response to signaling cues, experiments with TCam-2 cells will enable hypothesis generation and testing that will ultimately allow us to decipher what interactions within the testis microenvironment govern normal and pathological events affecting the male germline.

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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Author contribution statement
K L L, J C Y, S W, and S C M designed the experiments. S C M, S W, and J C Y performed the experiments. S C M, S W, K L L, and P W wrote the manuscript.

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