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ORIGINAL ARTICLE ***Andrology***

WNT signalling in the normal human adult testis and in male germ cell neoplasms

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STUDY QUESTION: Is WNT signalling functional in normal and/or neoplastic human male germ cells?

SUMMARY ANSWER: Regulated WNT signalling component synthesis in human testes indicates that WNT pathway function changes during normal spermatogenesis and is active in testicular germ cell tumours (TGCTs), and that WNT pathway blockade may restrict seminoma growth and migration.

WHAT IS KNOWN ALREADY: Regulated WNT signalling governs many developmental processes, including those affecting male fertility during early germ cell development at embryonic and adult (spermatogonial) ages in mice. In addition, although many cancers arise from WNT signalling alterations, the functional relevance and WNT pathway components in TGCT, including germ cell neoplasia *in situ* (GCNIS), are unknown.

STUDY DESIGN, SIZE, DURATION: The cellular distribution of transcripts and proteins in WNT signalling pathways was assessed in fixed human testis sections with normal spermatogenesis, GCNIS and seminoma (2–16 individuals per condition). Short-term (1–7 h) ligand activation and long-term (1–5 days) functional outcomes were examined using the well-characterised seminoma cell line, TCam-2. Pathway inhibition used siRNA or chemical exposures over 5 days to assess survival and migration.

PARTICIPANTS/MATERIALS, SETTING, METHODS: The cellular localisation of WNT signalling components was determined using *in situ* hybridisation and immunohistochemistry on Bouin's- and formalin-fixed human testis sections with complete spermatogenesis or germ cell neoplasia, and was also assessed in TCam-2 cells. Pathway function tests included exposure of TCam-2 cells to ligands, small molecules and siRNAs. Outcomes were measured by monitoring beta-catenin (CTNNB1) intracellular localisation, cell counting and gap closure measurements.

MAIN RESULTS AND THE ROLE OF CHANCE: Detection of nuclear-localised beta-catenin (CTNNB1), and key WNT signalling components (including WNT3A, *AXIN2*, TCF7L1 and TCF7L2) indicate dynamic and cell-specific pathway activity in the adult human testis. Their presence in germ cell neoplasia and functional analyses in TCam-2 cells indicate roles for active canonical WNT signalling in TGCT relating to viability and migration. All data were analysed to determine statistical significance.

LARGE SCALE DATA: No large-scale datasets were generated in this study.

LIMITATIONS, REASONS FOR CAUTION: As TGCTs are rare and morphologically heterogeneous, functional studies in primary cancer cells were not performed. Functional analysis was performed with the only well-characterised, widely accepted seminoma-derived cell line.

WIDER IMPLICATIONS OF THE FINDINGS: This study demonstrated the potential sites and involvement of the WNT pathway in human spermatogenesis, revealing similarities with murine testis that suggest the potential for functional conservation during normal

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spermatogenesis. Evidence that inhibition of canonical WNT signalling leads to loss of viability and migratory activity in seminoma cells suggests that potential treatments using small molecule or siRNA inhibitors may be suitable for patients with metastatic TGCTs.

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Key words: human spermatogenesis / testicular germ cell tumour / WNT signalling / TCFL1 / TCFL2 / Axin2

Introduction

The WNT signalling pathway exerts pleiotropic influences on fundamental cellular processes in many organisms throughout life (Logan and Nusse, 2004; Chien *et al.*, 2009a; MacDonald *et al.*, 2009; van Amerongen and Nusse, 2009; Ramakrishnan and Cadigan, 2017; Grainger and Willert, 2018). Canonical pathway activation occurs following binding of ligands, such as WNT3A, to receptor complexes. By inducing dissociation of a multi-subunit 'destruction complex' that includes adenomatous polyposis coli (APC), Axin (itself an active pathway target), casein kinase 1 (CK1) and glycogen synthase kinase 3 (GSK3), this binding stabilises the central mediator of canonical WNT signalling, beta-catenin, CTNNB1. Stable CTNNB1 then accumulates and translocates into the nucleus to activate target gene promoters via interaction with key transcription factors including LEF1, TCF1, TCF7L1 and TCF7L2 (Barker, 2008; MacDonald *et al.*, 2009; Clevers and Nusse, 2012; Valenta *et al.*, 2012). Hence CTNNB1 nuclear localisation is a hallmark of active canonical WNT signalling (Barker and van den Born, 2008; Fodde and Tomlinson, 2010), although canonical signalling can be active even if nuclear CTNNB1 is not readily detected (Fodde and Tomlinson, 2010).

Multiple lines of evidence demonstrate the importance of regulated WNT actions in determining spermatogenic cell fate. In the adult mouse testis, active canonical WNT signalling in post-mitotic mouse germ cells is evident from CTNNB1 nuclear localisation, and acute pathway disruption reduces survival and progression through post-mitotic spermatogenic stages (Li *et al.*, 2005; Chang *et al.*, 2011; Kerr *et al.*, 2014; Dong *et al.*, 2015). Regulation of WNT signalling is also important for maintenance of spermatogonia in the postnatal mouse testis (Chassot *et al.*, 2017).

The most common type of testicular germ cell tumours (TGCTs) arise from disturbances of embryonic/foetal development of the testis that arrest maturation of gonocytes into pre-spermatogonia. The arrested gonocytes transform into a relatively quiescent germ cell neoplasia *in situ* (GCNIS); then events associated with puberty drive their proliferation, and ultimately progression, into seminoma and/or non-seminomatous tumours (Skakkebaek *et al.*, 1987, 2001). GCNIS and seminoma cells maintain foetal germ cell transcriptional and functional characteristics, while non-seminomas are heterogeneous tumours composed of embryonic stem cell-like cells (embryonal carcinoma), trophoblastic elements (choriocarcinoma) or yolk sac tumour cells and somatically differentiated tissues in teratoma (Gashaw *et al.*, 2007; Young et al., 2011; Ulbright *et al.*, 2016). There is evidence of altered WNT signalling associated with testicular tumour progression and treatment resistance (Peng *et al.*, 1995; Honecker *et al.*, 2004; Vladusic *et al.*, 2010; Fabijanovic *et al.*, 2016; Lafin *et al.*, 2019). Mutations or deletions of *AXIN1*, *APC* and *FAT1*, which negatively regulate WNT pathway, were identified in a subset of cisplatin-resistant TGCT (Bagrodia *et al.*, 2016). CTNNB1 (beta-catenin) was identified at variable levels in TGCTs (Chovanec *et al.*, 2018), and TCF7L1 was identified nearly exclusively in non-seminomas (Bu *et al.*, 2019); however, little is known of the function of the pathway in the testicular tumour environment.

The present study addresses the current gap in knowledge about how and when WNT signalling contributes to normal and neoplastic germ cell fate in the adult human testis. Several histological approaches are employed to establish the cellular localisation profiles of transcripts and proteins involved in WNT pathway activities, as a means to determine where the pathway is active and to consider whether the roles identified in genetically modified mice can be logically expected to be relevant to human testes. A survey of GCNIS and seminoma specimens addresses whether particular stages and cell types may be directly affected by WNT ligands. Non-seminomas were not studied because of loss of germ cell phenotype. The functional implications of pathway inhibition are examined using the TCam-2 seminoma cell line, to assess if this is an appropriate target for therapeutic interventions.

Materials and methods

Human tissue sections and cell lines

Sections of normal adult human testis with complete spermatogenesis were obtained with informed consent from adult male volunteers, with sample collection approved by the Monash University Human Research Ethics Committee. Testicular cancer samples consisting of GCNIS and seminomas, including some regions with complete spermatogenesis, were obtained from the Department of Growth & Reproduction, Rigshospitalet in Copenhagen. The number of samples of each type assessed in this study is outlined in Table I. Non-seminomas, which do not retain germ cell phenotype, were not included in this study. Permission for the use of the archived paraffin-embedded specimens was obtained from the Regional Committee for Medical Research Ethics Denmark to E.R.-D.

TCam-2 cells were originally derived from a primary human seminoma (described in Mizuno *et al.*, 1993; de Jong *et al.*, 2008; Young *et al.*, 2011). The SW480 human colorectal carcinoma cells (originally described in Leibovitz *et al.*, 1976) contain a truncating APC mutation and are a well-characterised model of active canonical WNT signalling (Faux *et al.*, 2004).

L-cells (American Type Tissue Collection (ATCC) CRL-2648), L-Wnt3a (ATCC CRL-2647) and L-Wnt5a (ATCC CRL-2814) cell lines were obtained from the ATCC. Conditioned medium was collected as described (Chen *et al.*, 2003; Willert *et al.*, 2003).

Table I Summary of immunohistochemistry survey on human testes.

Marker	Testis tissue type	Number of independent samples assessed	Summary—cells	Summary—signal
TCF1	Normal human testis	16	Spermatogonia	++
			Sertoli cells	+/−
	GCNIS	13	GCNIS	+
			Sertoli cells	+
			Immune infiltrate	+++
	Seminoma	11	Seminoma	-
			Immune infiltrate	+++
LEF1	Normal human testis/region	16	Spermatogonia	++
			Spermatocytes	+/−
			Sertoli cells	−
			Leydig cells	++
	GCNIS	10	GCNIS	−
			Sertoli cells	−/+
			Immune infiltrate	+++
	Seminoma	12	Seminoma	−
			Immune infiltrate	+++
WNT3a	Normal human testis/region	19	Spermatogonia	+++
			Spermatocytes	+++ (stage-specific)
			Sertoli cells	+/−
	GCNIS	10	GCNIS	−
	Seminoma	12	Seminoma	−
			Immune infiltrate	++
TCF7L1	Normal human testis/region	3	Post-mitotic germ cells	++
	GCNIS	3	GCNIS	++
			Sertoli cells	−
	Seminoma	3	Seminoma	++
TCF7L2	Normal human testis/region	3	Post-mitotic germ cells	+++
	GCNIS	3	GCNIS	+++
			Sertoli cells	−
	Seminoma	3	Seminoma	+++
CTNNB1	Normal human testis/region	2	Post-mitotic germ cells	+++
	GCNIS	6	GCNIS	−
	Seminoma	12	Seminoma	−

The table presents a summary of IHC results from a range of normal and neoplastic human testes. Only cell types of note are included; all other cell types not mentioned in these sections were negative. Signal strength (i.e. −, negative: +/−, faint; +++, strong) indicates the signal strength in >50% of that particular cell type).

TCam-2 and SW480 culture and treatment with WNT agonists and antagonists

TCam-2 cells were maintained in RPMI1640, and SW480 and L-cells (including L-Wnt3a and L-Wnt5a lines) were cultured in DMEM (both Gibco, Gaithersburg, USA). All cell lines were cultured at 37°C in 5% CO_2 in air, with penicillin/streptomycin (Gibco) and 10% foetal calf serum (FCS; Quantum Scientific, Australia). For expression, survival and proliferation assays, TCam-2 cells were plated at 1–5 × 10^5 cells per well (depending on the prospective culture period) in six-well dishes (Nunc, Rochester, USA) in media with serum. For immunofluorescence migration assays, cells were plated in 12- or 24-well plates either with or without coverslips. Following 24 h of growth, cultures were grown under different conditions: either L-cell conditioned media or media containing growth factors and serum supplements, as specified in the Results section. Recombinant human WNT3A or WNT5A (R&D systems, MN, USA, resuspended in PBS/0.1% bovine serum albumin (BSA)) was added at a final concentration of 100 ng/ml; IWR-1 (Calbiochem, Darmstadt, Germany, suspended in DMSO) was added to 50 or 100 μM and CCT036477 (Sigma-Aldrich, MO, USA, suspended in DMSO) tested at 20, 2 and 0.2 μM. All cultures included appropriate vehicle-only controls.

In situ hybridisation

The cDNAs corresponding to target genes *CTNNB1* and *AXIN2* were generated by reverse transcriptase-PCR (primers listed in Supplementary Table SI) and ligated into the pGemT Easy Vector (Promega, Madison, WI, USA). Constructs were transformed into DH5α bacterial cells, verified using colony PCR screening, and subsequently sequenced. Sense and antisense DIG-labelled cRNA probes were synthesised by *in vitro* transcription from the plasmids with digoxigenin-UTP (Roche Diagnostics, Basel, Switzerland) using T7 and SP6 RNA polymerases (Promega, Madison, WI, USA). Prior to hybridisation, sections were treated with Proteinase K (0–2 ng/ml) for 30 min at 37°C. Hybridisation was performed with 100–300 ng cRNA probe at 60°C overnight. Anti-digoxigenin antibody conjugated to alkaline phosphatase (1:1000, Roche Diagnostics) was applied and probe

binding was detected as a deep purple colour using alkaline phosphatase substrate (BCIP/NBT, Thermo Scientific, Waltham, MA, USA). Sections were counterstained with Harris haematoxylin and mounted under a glass coverslip with Glycerol Vinyl Alcohol (GVA) aqueous mounting solution (Zymed, San Francisco, CA, USA). Each experiment was performed three times on multiple independent samples.

Northern blot analysis

RNA from TCam-2 cells, SW480 cells and whole day 12.5 mouse embryos was isolated using Trizol® (Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. RNA (20 µg/lane) was separated on a 1.2% formaldehyde gel, followed by overnight transfer to Hybond XL membranes (Amersham Biosciences Pty., Ltd, Sydney, Australia). Transcript sizes were determined by comparison to an RNA ladder (New England Biolabs, Ipswich, MA, USA) (Supplementary Fig. S1A). Membranes were blocked using Ultrahybe buffer (Life Technologies) at 68°C for 1 h and hybridised in Ultrahyb buffer (Life Technologies) with 25 ng/ml antisense DIG-labelled cRNA probes overnight at 68°C. Membranes were washed at 68°C with 2× SSC/0.1% SDS and 0.1× SSC/0.1% SDS. Probe binding was detected using anti-digoxigenin antibody conjugated to alkaline phosphatase (1:1000). Signals were detected using CDP-Star substrate (Roche Diagnostics) followed by exposure to X-ray film.

Immunohistochemistry

Immunohistochemistry (IHC) was performed on sections of normal human testis and samples containing GCNIS and seminoma. CTNNB1 IHC was performed exactly as described previously (Kerr et al., 2014). For all other antibodies, optimisation of signal-to-noise ratios for each antibody was evaluated through a series of antigen retrieval protocols including 50 mM glycine (pH 3.5, >90°C maintained in an 800W microwave for 7 min), and 10 mM citrate buffer (pH 6.0, >90°C maintained in pressure cooker for 10 min). Antibody and technical details are outlined in Supplementary Table SII. Tissue sections were treated with 3% hydrogen peroxide (Merck Millipore, Germany) for 5 min followed by blocking with normal goat serum (5% in Tris buffered-saline (TBS)) for 20 min, then incubated overnight with primary antibody, as listed in Supplementary Table SII. Subsequent steps were performed at room temperature, with TBS washes (3 × 5 min) between incubations. Primary antibody binding was detected for CTNNB1, TCF7L1 and TCF7L2 using a biotinylated goat anti-rabbit IgG secondary antibody (Life Technologies; 1:500 dilution, 1 h) and the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA), according to the manufacturer's instructions, followed by detection of antibody binding as a brown precipitate following development with 3,3′-diaminobenzidine (DAB; DAKO, Campbellfield, Australia). TCF1, LEF1 and WNT3a staining was detected with a secondary rabbit MP-7401 (Impress reagent kit, Vector Labs) followed by development with DAB substrate (Vector Labs). All slides were counterstained with Harris haematoxylin (Sigma-Aldrich, St Louis, MO, USA). Tissue sections were mounted under glass coverslips using DPX (BDH Laboratories, Poole, UK) and dried overnight. Optimisation of signal-to-noise ratios for each antibody assessed was evaluated individually, as outlined in Supplementary Table SII, and scoring was performed by a qualified pathologist, with identification of likely immune cell infiltrates based on observations in Klein et al. (2016). All antibodies were tested by Western hybridisation, as previously described (Young et al., 2011) (Supplementary Fig. S1B).

Immunofluorescence analysis of CTNNB1, TCF7L1 and TCF7L2 localisation in TCam-2 cells

TCam-2 cells were plated at 2.5 × 10^5 cells per well in 24-well plates and cultured for 24–48 h. The media were replaced with either L-cell, L-Wnt3a or L-Wnt5a-conditioned media for 1, 4 or 7 h. Following treatment, cells were rinsed with PBS and fixed in 4% paraformaldehyde in PBS for 10 min. Two subsequent rinses with PBS were followed by blocking for 1 h at room temperature in PBS/0.1% BSA. Cells were permeabilised in PBS/0.1% Triton-X 100 and then incubated overnight at room temperature with CTNNB1 antibody (1:300 PBS/0.1% BSA; BD Biosciences, San Jose, CA, USA). Cells were then washed 3× in PBS, and antibody binding detected with an Alexa Fluor 488-coupled goat anti-mouse secondary antibody (1:500 in PBS/0.1% BSA; Invitrogen, Carlsbad, CA, USA). Negative staining (omission of primary antibody) controls were included in every experiment, with no signal observed in any of these. Subcellular localisation (Fn/c) of CTNNB1 protein detected by immunofluorescence was determined using Image J analysis of confocal images (BioRad 1024ES confocal with a Nikon TE300 microscope and 60× oil objective). For each treatment, five fields with 3–7 cells/field were analysed at each time point. Data are derived from three independent experiments. Individual Fn/c values were plotted to show variance across cell populations.

To detect TCF7L1 and TCF7L2 proteins, TCam-2 cells were plated in 12-well plates on to coverslips. Cells were allowed to attach overnight, then the cells were subjected to immunofluorescence analysis as above. TCF7L1 and 2 antibody (1:100 PBS/0.1% BSA; Abcam, USA) binding was detected as above with the Alexa Fluor 488-coupled goat anti-rabbit secondary antibody (1:500 in PBS/0.1% BSA; Invitrogen, Carlsbad, CA, USA), followed by confocal imaging.

siRNA-mediated knockdown of TCF7L1 and 2

For siRNA experiments, TCam-2 cells were seeded in three 6-well plates at a density of 2.0 × 10^5 cells/well, then incubated overnight at 37% (5% CO_2) in RPMI containing 10% FCS + 0.5% penicillin/streptomycin. Once TCam-2 cells had adhered and reached 60–70% confluency, media containing 10% FCS was replaced with RPMI + 5% FCS (without antibiotics). Transfections were performed using the Lipofectamine™ RNAiMAX system (ThermoFisher, Carlsbad, USA) according to the manufacturer's instructions. Pre-designed small interfering RNAs (Silencer select siRNA, ThermoFisher) were used to target TCF7L1 (ThermoFisher, 5 nmol, Cat #4392420, ID s37905) and TCF7L2 (ThermoFisher, 5 nmol, Cat #4392420, ID s13881). These constructs were transfected in TCam-2 cells independently or in combination and used at a final concentration of 12.5 pmol. Untreated TCam-2 cells and Silencer Select Negative control siRNA (control) (ThermoFisher, 40 nmol, Cat #4390844) were used as controls. Transfected TCam-2 cells were collected 1, 3 and 5 days post-transfection in TRIzol for RNA analysis. Gene knockdown was validated by quantitative reverse transcription–polymerase chain reaction

(qRT–PCR). These experiments were repeated three independent times.

Quantitative reverse transcription–polymerase chain reaction

TCam-2 cell RNA was extracted using the Qiagen RNeasy extraction kit (Qiagen, Hilden, Germany), then treated with DNaseI (Ambion, Austin, TX) according to the manufacturer's specifications. Superscript III reverse transcriptase (100 U) and random hexamer primers (Applied Biosystems, Foster City, USA) were combined in a 20 µl reaction with 500 ng DNA-free total RNA to generate cDNA. Negative control reactions without Superscript III or water controls were included for each sample. SYBR-Green PCR master mix (8 µl, Roche Diagnostics) and 8 µl diluted cDNA were combined with 500 nM each of the forward and reverse (Supplementary Table SI) primers. PCR was performed on a 7900HT real-time system (Applied Biosystems, Gandel Charitable Trust Sequencing Centre, Monash Institute for Medical Research) at 95°C for 10 min, with 45 cycles of amplification at 95°C for 15 s, and 62°C for 30 s. Reactions were performed in triplicate, and relative quantification values averaged. The amplified products of all primer pairs were verified by sequencing and dissociation curve analysis. Relative standard curve analysis (SDS 2.0 software; Applied Biosystems) was used to generate a correlation of threshold values (crossing points) with target mRNA levels. Three independent experiments were performed for each primer set, with error bars indicating standard error of the mean (SEM).

Cell number and viability assays

To determine the effect of WNT agonist/antagonist treatments on viability, TCam-2 cells were harvested using 0.1% trypsin/versine, rinsed in PBS and resuspended in PBS/10% FCS containing 0.05 mg/ml propidium iodide (PI: 5 mg/ml, Sigma). The viable:non-viable cell ratio based on PI incorporation was measured on a FACSCanto II Analyser, gated at 670 nm (B670A filter) at the Monash University Bioplatform Flowcore Facility. At least three independent experiments were performed for each treatment and time point, and the results are graphed as the percentage of non-viable cells, relative to control sample values. To determine specific treatment effects on cell number, TCam-2 cells were dissociated into a single-cell suspension with 0.1% trypsin/versine, then enzymic activity was halted by adding FCS (to final concentration of 10%)/PBS and the entire well contents collected for counting. Relative cell counts were determined through cytometric flow rate analysis (cell count/minute) using a FACSCanto II Analyser (Flowcore Facility, Monash University). Each sample was counted twice and values were averaged. At least three independent experiments were performed for each treatment and time point, and the results were graphed as relative cell numbers normalised to control values. For all culture experiments, error bars indicate SEM, with unpaired, two-tailed *T*-tests, Mann–Whitney post-test, performed with Prism™ software to determine statistical significance, as indicated in the figure legends.

Migration assay

TCam-2 cells were grown to confluence in 12-well plates. A single horizontal scratch wound was generated across the well using a pipette tip. Cells were washed once in serum-free medium to remove debris, and media with or without inhibitors was then added to the cells, or an siRNA transfection was performed (as above). Indicator marks were inscribed on the plate bottom to designate a specific region of the wound for subsequent imaging. For this assay, cells were incubated in media containing 5% serum containing either no inhibitor, DMSO (vehicle control), 100 µM IWR-1 or 2 µM CCT036477, or siRNAs as indicated. Plates were photographed at 0 h, then at 1, 3 and 5 days post-wound to assess migration. Percentage of gap size normalised to 0 h was determined by measuring wound area using Image J. TCam-2 cells plated concurrently were assessed at these time points to confirm that the different treatments (specifically in 5% serum, see above) did not elicit a difference in cell number. All assays were performed on at least three separate occasions, with $n = 3$ for each condition in each experiment.

Results

Localisation of key WNT pathway signalling components in the adult human testis indicates canonical WNT signalling is active in spermatogenic cells

Detection of *CTNNB1* transcripts using *in situ* hybridisation identified a signal in all germ cells (Fig. 1A), with a particularly intense *AXIN2* signal in spermatogonia and spermatocytes (Fig. 1B). Many WNT signalling pathway transcripts are detected in the normal human testis, as well as in seminoma and the TCam-2 seminoma cell line (Supplementary Fig. 2A and B).

To localise CTNNB1, a pan-CTNNB1 antibody was used to detect both the phosphorylated and unphosphorylated ('activated') protein isoforms, permitting the tracking of alterations in subcellular location that are indicative of functional changes. The CTNNB1 protein was detected in all germ cell types; however, only post-mitotic germ cells, including all round spermatids, exhibited nuclear-localised CTNNB1 (Fig. 1C). Distinct WNT3A, TCF7L1 and TCF7L2 signals were detected in spermatogonia, spermatocytes and round spermatids (Fig. 1D, G and H), with fainter signals detected for TCF1 and LEF1 in a more restricted pattern (Fig. 1E and F). Table I summarises the findings from all immunohistochemical analyses.

GCNIS and seminomas express WNT signalling components but lack detectable nuclear CTNNB1

The CTNNB1 protein signal was readily detected in cells within GCNIS tubules, including GCNIS and Sertoli cells (Fig. 2A), and in seminoma cells (Fig. 3A), but was detected in the cytoplasm and the cell membrane. There was no evidence of detectable nuclear protein, either in GCNIS or in seminoma cells (Figs 2A and 3A). Nuclear TCF7L1 and TCF7L2 were readily detected within both GCNIS (Fig. 2E and F) and seminoma cells (Fig. 3E and F) cells. Neither TCF1 nor LEF1 was readily detectable in either GCNIS or seminoma cells (Fig. 2C and D), however, nuclear TCF1 and LEF1, along with WNT3A, were consistently detected in regions devoid of seminoma cells within small, strongly stained cells that appear to be infiltrating

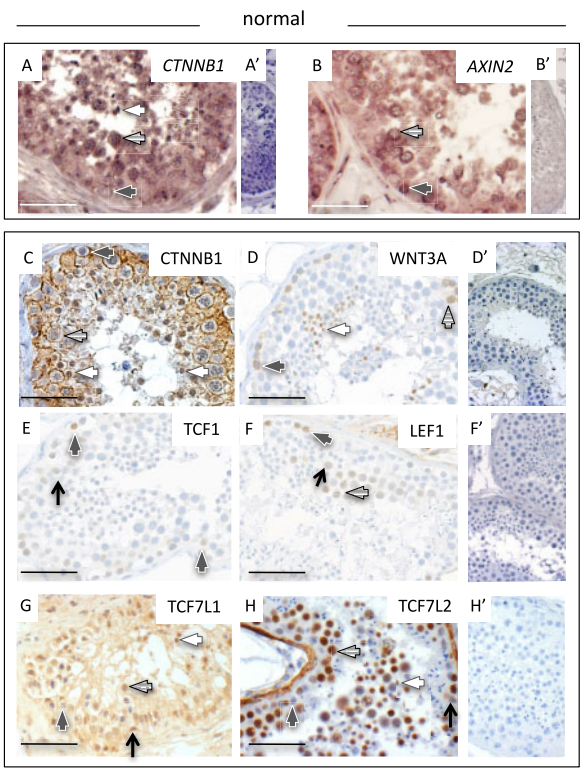

Figure 1. Detection of WNT signalling components in specific cell types within the normal adult human testis. (**A**, **B**) CTNNB1 and AXIN2 transcripts exhibit dynamic expression throughout human spermatogenesis. (A) *CTNNB1* mRNA was detected by *in situ* hybridisation (ISH) on normal adult human testis sections. Little signal was detected in spermatogonia (grey arrow), with the strongest signal in spermatocytes (hatched arrow) and spermatids (white arrow). (B) *AXIN2* transcripts were detected most strongly in spermatogonia (grey arrow) and spermatocytes (hatched with *CTNNB1* or *AXIN2* sense cRNA probes. (**C**) CTNNB1 protein is present in all germ cells and predominantly nuclear in post-meiotic germ cells. (C) CTNNB1 protein was detected in the cytoplasm of spermatogonia (grey arrow) and spermatocytes (hatched arrow), but was nuclear localised in spermatids (white arrow). (**D–H**) WNT signalling components are detected in specific cell types within the normal adult human testis. WNT3A (D) was detected in the cytoplasm of spermatogonia, in specific spermatocyte subsets (hatched arrow), as well as in round spermatids (white arrow). WNT signalling mediators TCF-1 (E) and LEF-1 (F) were each detected in some spermatogonia (grey arrows) and some Sertoli cell (black arrows) nuclei, while LEF-1 was detected additionally in spermatocyte (hatched arrows) nuclei. (G, H) The most striking signals were detected with the intracellular mediators TCF7L1 and 2, with strong signal detected in all post-mitotic germ cells (spermatogonia: grey arrows; spermatocytes: hatched arrows; and spermatids: white arrows); overlapping with the CTNNB1 pattern. (**D′–H′**) Panels represent controls lacking primary antibody. Images are representative of RNA detection by ISH or protein detection by IHC in two independent human testes. Scale bars indicate 50 μm.

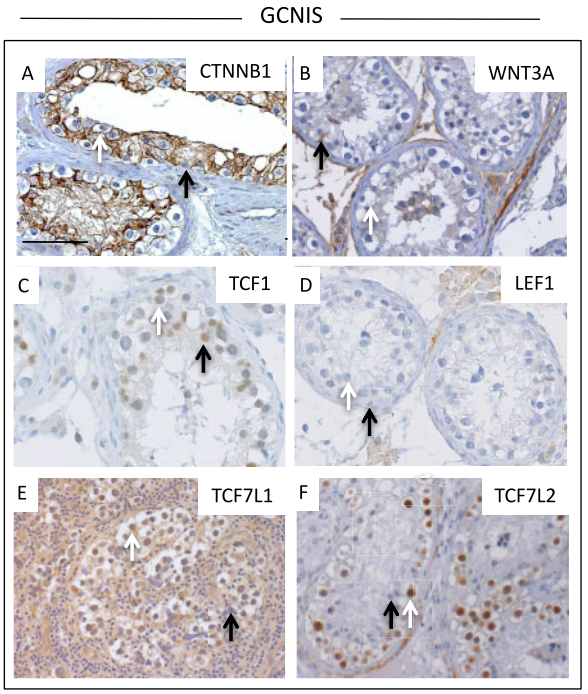

Figure 2. IHC on sections of GCNIS highlights the differential cellular localisation of several components of the WNT signalling pathway. (**A, B**) CTNNB1 protein is present in the cytoplasm of GCNIS cells. In GCNIS tubules, CTNNB1 protein was detected in the cytoplasm of GCNIS (white line arrow) and Sertoli (black line arrow) cells. Little WNT3A is detected in GCNIS tubules. In GCNIS tubules, WNT3A was detected at low levels in the Sertoli (black line arrow) cell cytoplasm, with a strong signal in interstitial regions proximal to tubules. (**C–F**) WNT signalling components are detected in CIS and Sertoli cells of CIS tubules. TCF-1 was detected in the nucleus of both GCNIS (white line arrows) and Sertoli (black line arrow) cells within GCNIS tubules, with no LEF-1 detected in CIS cells and only faintly in rare Sertoli cell nuclei. In contrast, strong TCF7L1 and TCF7L2 signals were present in GCNIS cell nuclei, with no signal detected in Sertoli cell nuclei. TCF7L1 signal was detected in the cytoplasm of most regions of the GCNIS section; however, TCF7L2 signal was specifically detected only in the GCNIS cells. Images are representative of protein detection by IHC in multiple independent samples (see Table I). Scale bars indicate 50 μm and are representative for all the others. Arrows indicate representative cells.

immune cells (Fig 3B–D) (Klein *et al.*, 2016). Nuclear TCF1 was also present at a low level in Sertoli cells within GCNIS tubules (Fig. 2C).

TCam-2 cells are responsive to canonical WNT stimulation

TCam-2 cells normally exhibit membrane-associated CTNNB1 (Fig. 4A), mirroring observations of seminoma cells in sections (Fig. 3A). However, TCam-2 cells responded to canonical WNT ligand exposure with a rapid nuclear accumulation of CTNNB1 (Fig. 4B–D).

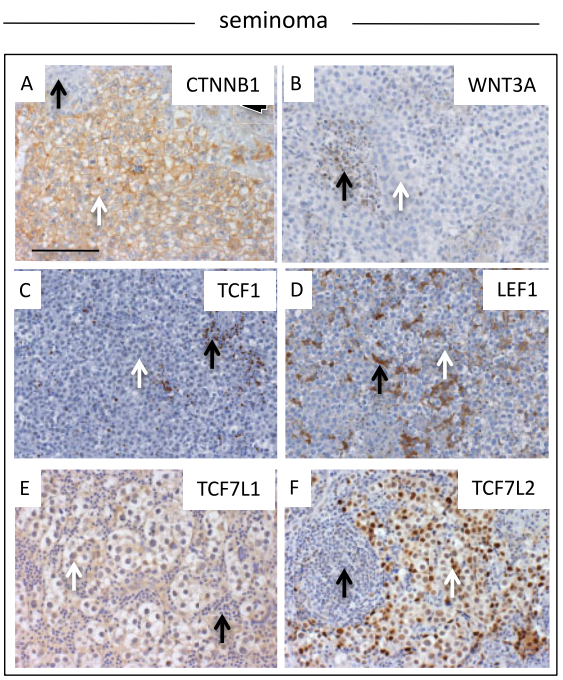

Figure 3. IHC on sections from multiple independent seminomas highlights the differential cellular localisation of several components of the WNT signalling pathway. (**A**) CTNNB1 protein was readily detected in the cytoplasm of seminoma cells (white arrow), with little nuclear localisation evident. Regions surrounding the seminoma (black arrow) appeared negative for CTNNB1, and likely represent immune infiltrate and/or areas of fibrosis. (**B**) WNT3A protein was not detected in seminoma cells (white arrow), but was strongly detected in surrounding cells that likely represent immune infiltrate (black arrows). (**C**) TCF-1 was not detected in seminoma cells (white arrows). (**D**) LEF1 was not detected in seminoma cells, and in contrast to CIS cells, However, for both TCF1 and LEF1, a strong signal was evident in surrounding regions (black arrows), again potentially from immune infiltrate. Images are representative of protein detection by IHC in multiple independent samples (Table I). (**E, F**) The seminoma cells exhibited very strong TCF7L1 and L2 signal. Scale bar indicates 50 μm and is representative for all the others. Arrows indicate representative cells.

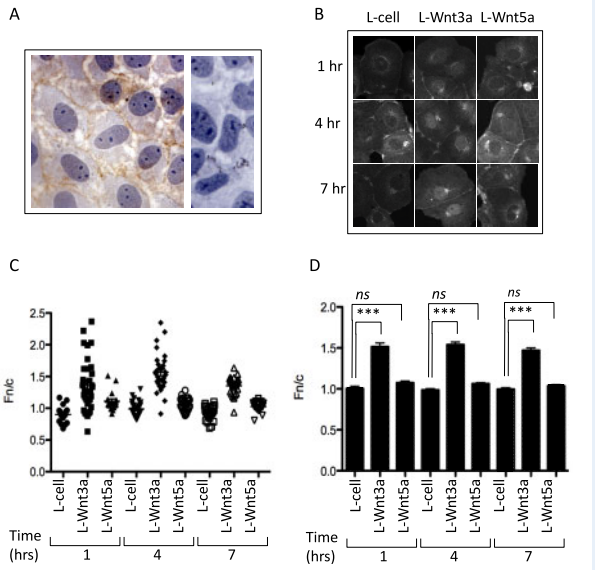

Figure 4. TCam-2 cells exhibit a rapid, ligand-specific response to canonical WNT pathway activation. (**A**) Nuclear CTNNB1 is not detected in TCam-2 cells. Left panel: CTNNB1 protein detected using immunocytochemistry in TCam-2 cells. Right panel: negative control, lacking primary antibody. (**B**) TCam-2 cells were treated with conditioned medium from either control L-cells or L-cells overexpressing Wnt3a (L-Wnt3a) or Wnt5a (L-Wnt5a) for the indicated times prior to image analysis of CTNNB1 subcellular localisation detected using indirect immunofluorescence. (**C**) Quantitation of Fn/c data from one representative experiment showing CTNNB1 subcellular localisation at three timepoints in response to treatment conditions. Fn/c ratios >1 indicate nuclear accumulation, plotted to show response variation across each population. (**D**) Combined Fn/c values from three replicate experiments. Statistical significance determined through unpaired, two-tailed *T*-test comparison of L-Wnt3a and L-Wnt5a treatments to controls at each timepoint. Wnt3a exposure elicited a significant increase in CTNNB1 nuclear accumulation at all three time points relative to all other groups. Error bars indicate SEM, and significance was determined through unpaired, two-tailed *T*-tests, with Mann–Whitney post-test. *P*-values are presented as $^{***}P < 0.0001$, *ns* is non-significant.

Active WNT signalling is associated with either high levels of cellular proliferation or, alternatively, suppression of proliferation in different cancer cell types (Zhan *et al.*, 2017). In this seminoma model cell line, exposure to neither the canonical WNT3A nor the non-canonical WNT5A ligand affected cell numbers over 5 days, independent of the presence of FCS (Fig. 5A and B).

Intracellular canonical WNT signalling antagonists significantly affect TCam-2 growth, viability and migration

The response of TCam-2 cells to WNT antagonists acting at two distinct sites in this pathway was determined: increased destruction complex stability (IWR-1) and inhibition of CTNNB1–TCF/LEF interaction (CCT036477) (Huang *et al.*, 2009; Bao *et al.*, 2012; Jarde *et al.*, 2013). In contrast to the response to ligand addition, a significant negative impact on cell number was observed following exposure to both IWR-1 and CCT036477 (Fig. 5C–F).

Finally, wound healing assays were used to assess the impact of canonical WNT pathway antagonism on migration in TCam-2 cells. To control for the potential that proliferation rather than migration led to a reduced gap size, cells were cultured under conditions that do not support proliferation (5% serum), and cell numbers were counted on parallel samples to confirm this. Significant reductions in gap closure rates (to only around 60% of original gap size) were measured in the presence of either IWR-1 or CCT036477 (Fig. 6A and B). No

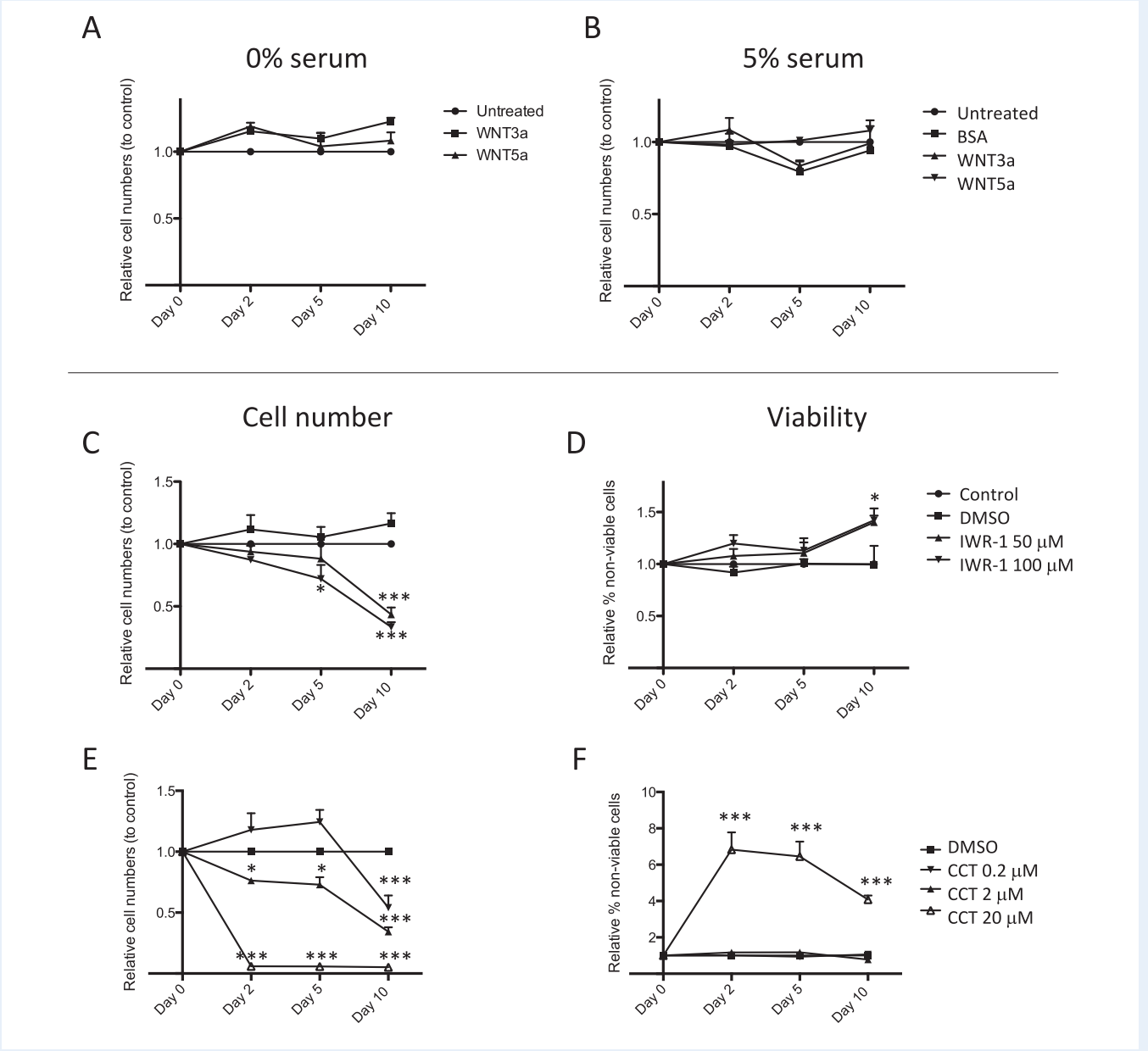

Figure 5. TCam-2 cells exhibit functional responses to inhibition of canonical WNT signalling. TCam-2 cells were exposed to a range of WNT pathway inhibitors, and their functional responses (cell numbers and viability) were assessed. (**A**) Cell numbers, 100 ng/ml recombinant ligands in serum-free media (to avoid activation of signalling pathways by factors in serum). (**B**) Cell numbers, 100 ng/ml recombinant ligands in 5% serum to allow for growth tracking over 10 days. (**C**, **D**) Cell numbers and viability in response to 50 µM and 100 µM IWR-1. (**E**, **F**) Cell numbers and viability in 20, 2 and 0.2 µM CCT036477. For all experiments, cell numbers were assessed by flow cytometry, and normalised at each timepoint to untreated or control cells. Graphs are presented as mean values, with error bars indicating SEM, and significance was determined through unpaired, two-tailed *T*-tests, with Mann–Whitney post-test. $^{*}P < 0.05$ and $^{***}P < 0.001$.

significant impact on cell number was recorded under these growth conditions (Fig. 6C)

A TCam-2 RNA-seq dataset (Kim *et al.*, 2014) contained evidence that transcript levels of *TCF7L1* and *TCF7L2* are relatively high in this cell line, compared to *LEF1* and *TCF1* (Fig. 7A), correlating with the ready detection of the TCF7L1 and TCF7L2 using IHC. Their presence in the nucleus of TCam-2 cells was demonstrated by immunofluorescence (Fig. 7B). The siRNA-mediated knockdown of either *TCF7L1* or *TCF7L2* (Fig. 7C) elicited a significant decrease in the migration activity of TCam-2 cells (Fig. 7D).

Discussion

This study shows for the first time the potential for the WNT pathway to play a significant role in normal human spermatogenesis. Based on

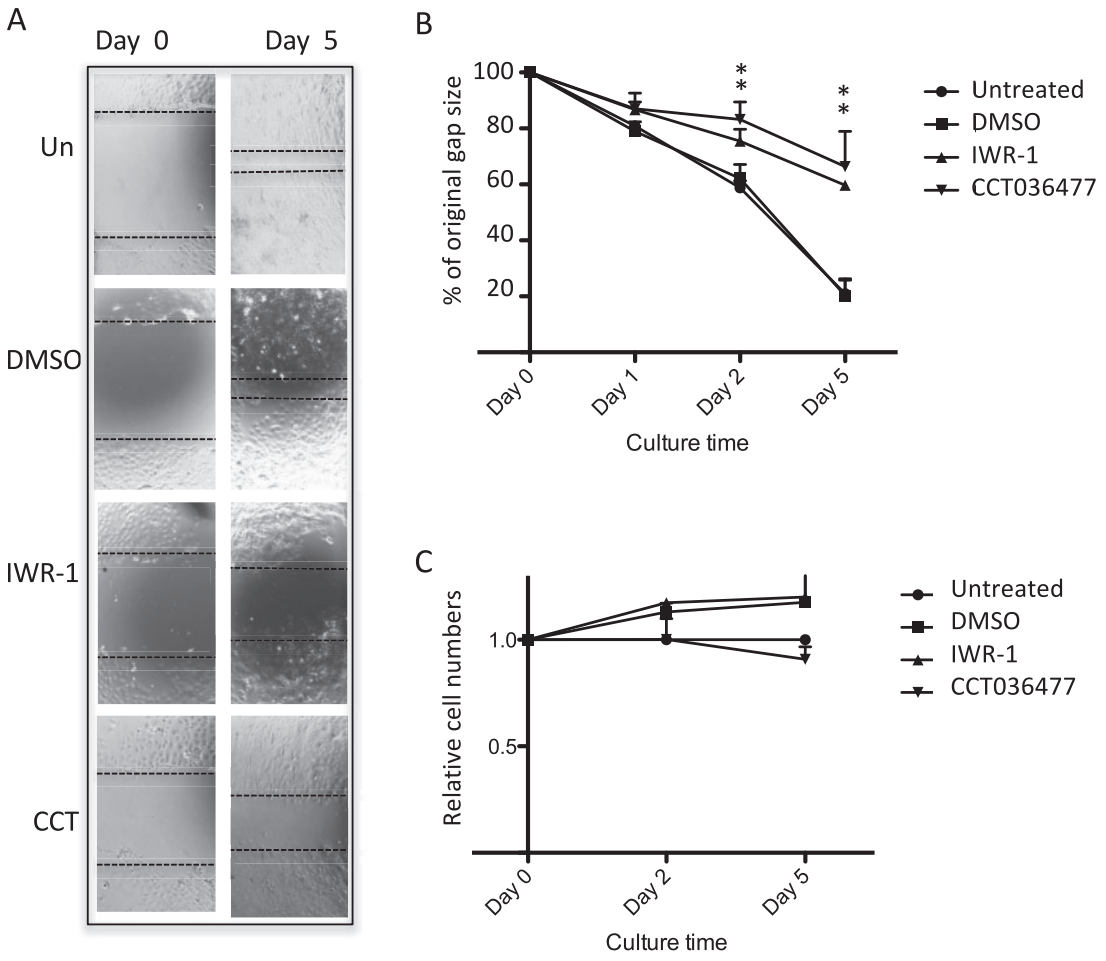

Figure 6. Suppression of WNT signalling inhibits migration in TCam-2 cells but does not affect cell numbers when cultured in 5% serum. (**A**, **B**) TCam-2 cells were cultured in 5% serum to support migration. A scratch wound was generated, and standard growth media was replaced with media with or without inhibitors as follows: no treatment, DMSO (vehicle control), 100 μM IWR-1 or 2 μM CCT035477. Images were taken immediately (Day 0) and 1, 2 and 5 days later. Measured gap width is presented relative to the gap present at time 0. Relative wound healing rates were calculated at each timepoint, with significant differences between controls and inhibitor-treated samples present by Day 2. Images taken with 4× objective. (**C**) Cells plated simultaneously in identical conditions were collected at Days 2 and 5 to assess cell numbers. No significant difference in cell numbers arose from the different treatments, indicating that, in the presence of serum, WNT inhibitors do not affect cell number but do impact cell migration. Graphs are presented as mean values, with error bars indicating SEM, and significance was determined through unpaired, two-tailed *T*-tests, with Mann–Whitney post-test. $^{*}P < 0.05$.

the common expression profile of the key WNT signalling mediator, CTNNB1, we propose that its roles are comparable to that observed experimentally in the mouse (Kerr *et al.*, 2014; Chassot *et al.*, 2017). In the normal adult human testis, while *CTNNB1* transcripts are detected in all germ cells, the strongest signal is evident in spermatocytes and round spermatids, correlating with CTNNB1 nuclear localisation and likely activation of canonical WNT signalling in these cells. The expression analysis further indicates that early germ cell signalling is most likely mediated through an AXIN2-responsive pathway as demonstrated in the mouse (Takase and Nusse, 2016) via TCF7L1 and TCF7L2.

This study adds important data on the cellular localisation of AXIN2, which was most pronounced in a subset of spermatogonia and in primary spermatocytes. This contrasts with the predominantly post-meiotic nuclear localisation of CTNNB1. AXIN2 was reported as a negative regulator of the WNT pathway that acts by reducing CTNNB1 stability; the results presented here indicate there is a functional switch to CTNNB1-mediated signalling during adult spermatogenesis in both mouse and human testes. Despite the distinct differences between some aspects of mouse and human spermatogonial stem cells (Fayomi and Orwig, 2018), it is interesting to consider recent single-cell RNA-sequencing data obtained from analysis of mouse and human testes (Hermann *et al.*, 2018), which demonstrate consistency of transcript expression in spermatogonia and spermatocytes in both species and are in accord with the WNT pathway data presented in this study. In mice, forced activation of WNT/CTNNB1

A

Sample	LEF1	TCF7 (TCF1)	TCF7L1 (TCF3)	TCF7L2 (TCF4)
TCam-2 (1)	0	372	2136	1185
TCam-2 (2)	3	433	2531	1313

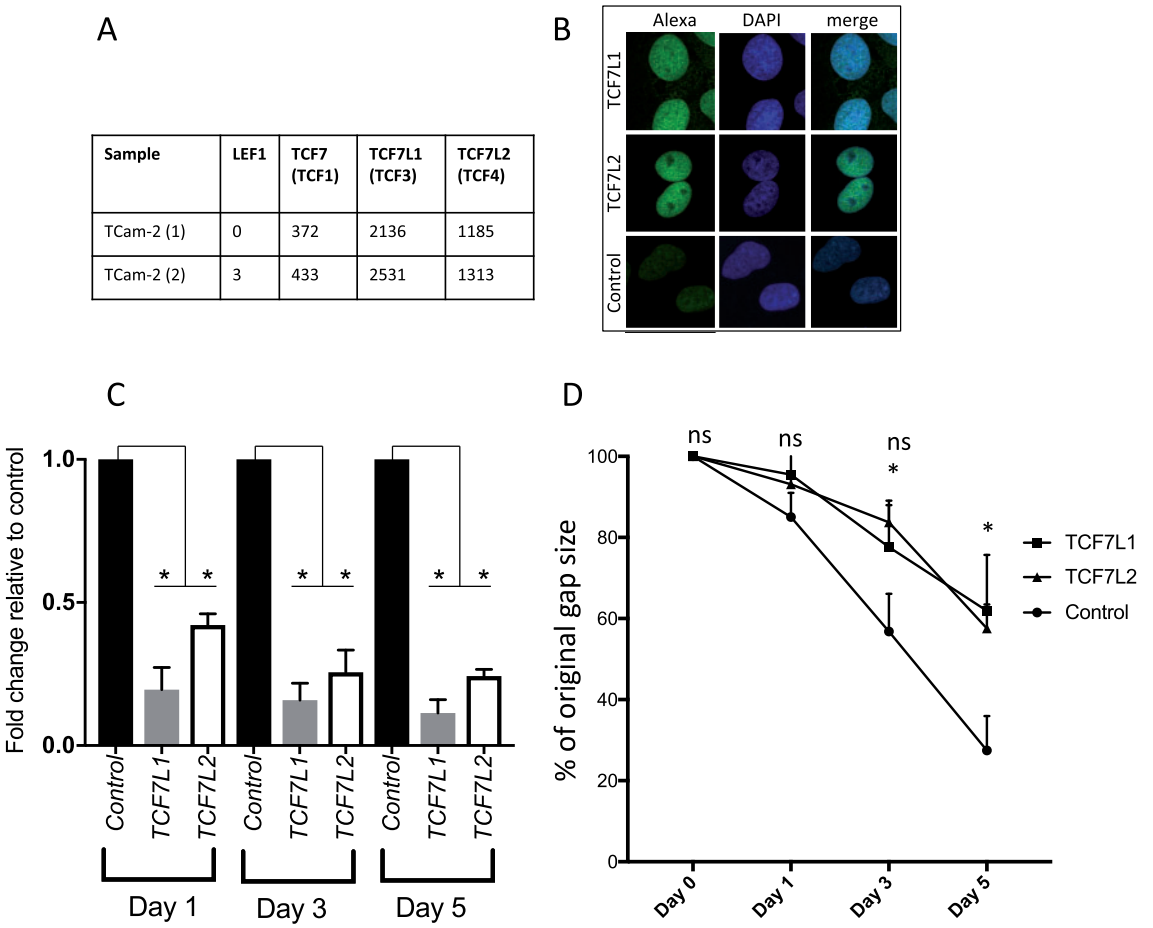

Figure 7. Suppression of TCF7L1 and TCF7L2 in TCam-2 cells leads to a significant decrease in migratory ability. (**A**) The Surani (Kim *et al.*, 2014) TCam-2 RNA-seq dataset was interrogated for the presence of *LEF1*, *TCF1*, *TCF7L1* and *TCF7L2* transcripts. Of the two samples tested, *LEF1* and *TCF1* were detected at low levels, whereas *TCF7L1* and *TCF7L2* were detected at 10-fold higher levels than *TCF1*. These transcriptional data closely mirror the seminoma IHC data (Fig. 3). (**B**) Immunofluorescence was performed on TCam-2 cells to detect TCF7L1 and TCF7L2 proteins. Both proteins were readily detected in the nucleus of TCam-2 cells. Control indicates immunofluorescence performed without primary antibody. (**C**) Two independent siRNA constructs targeting each of *TCF7L1* and *TCF7L2* transduced into TCam-2 cells resulted in significant decreases in target transcript levels (one example shown) compared to the (scrambled) control. Graphs are presented as mean values, with error bars indicating SEM, and significance was determined through unpaired, two-tailed *T*-tests, with Mann–Whitney post-test. $*P < 0.05$. (**D**) TCam-2 cells transduced with either TCF7L1 or TCF7L2 siRNA constructs were subjected to a wound healing assay. Healing was measured daily over 5 days, with significant inhibition of healing (decreased gap size reduction) exhibited by the *TCF7L1* and *TCF7L2* knockdown samples. Graphs are presented as mean values, with error bars indicating SEM, and significance was determined through two-way ANOVA. $*P < 0.05$.

pathway results in increased spermatogonial proliferation and spermatocyte loss by apoptosis (Chassot *et al.*, 2017); therefore, the need for balanced signalling to support ongoing spermatogenesis documented in the mouse (Kerr *et al.*, 2014) requires further investigation in the human.

There is considerable functional evidence that mouse spermatogenesis relies on carefully balanced WNT signalling. Individual spermatogonial subtypes exhibit different outcomes in the postnatal testis following activation of either canonical (self-renewal) or non-canonical (morphological changes/cell motility) WNT signalling (Golestaneh *et al.*, 2009; Yeh *et al.*, 2011). In addition, a cell-intrinsic WNT inhibitor, SHISA6, expressed in spermatogonial stem cells, sustains GDNF-positive cells and prevents their differentiation. This is proposed to act via cytoplasmic sequestration of CTNNB1 by E-cadherin (Tokue *et al.*, 2017). Of potential relevance to the time of human TGCT emergence, mouse embryonic germ cells also have a clear requirement for correctly balanced WNT signalling; the restriction of GSK3-mediated CTNNB1 phosphorylation *in vivo* and resulting CTNNB1 nuclear localisation delays cell cycle progression and significantly reduces germ cell numbers between E11.5 and E13.5 (Kimura *et al.*, 2006). In addition, the forced transgenic activation of WNT/CTNNB1 in foetal gonocytes causes apoptosis-mediated male germ cell depletion (Li *et al.*, 2014; Chassot *et al.*, 2017). The involvement of WNT signalling in development of embryonic and early postnatal

mouse germ cells provides a foundation for studies into the pathogenesis of neoplastic transformation of germ cell in the human testis (reviewed in Atlasi *et al.* (2014)).

The pattern of WNT signalling component expression is apparently not preserved in neoplastic germ cells, which generally exhibit the expression profile of primordial germ cells/foetal gonocytes rather than mature male germ cells (Sonne *et al.*, 2009). The absence of nuclear CTNNB1 protein in GCNIS and TGCT samples reported here is partly in agreement with an earlier the report of Honecker *et al.* (2004) in which only faint cytoplasmic protein was observed in GCNIS cells. However, unlike that report, we did not observe CTNNB1 within seminoma cell nuclei. Our result is in agreement with another study (Chovanec *et al.*, 2018), which reported no detection of nuclear protein in any germ cell tumour subtype. In that study, a signal was detected in 52% of seminoma samples, appearing as membrane-associated, a typical location for cytoplasmic CTNNB1 bound to cadherins (Nguyen *et al.*, 2019). The heterogeneous distribution of CTNNB1 would fit with the proposition that seminoma tumours are themselves heterogeneous, for example, with regard to DNA methylation (Pedersen *et al.*, 2016) or changing in response to the local and diverse immune milieu (Klein *et al.*, 2016), which may influence tumour progression (Loveland *et al.*, 2017). The absence of nuclear CTNNB1 cannot be interpreted to indicate an absence of pathway activation (Fodde and Tomlinson, 2010), and so the future interrogation of other WNT pathway components will be informative.

While there are a number of mouse studies addressing the functional role of WNT signalling in the normal mouse testis, similar functional studies of the testicular neoplasms cannot be performed in the mouse as rodents do not develop tumours analogous to human GCNIS and seminoma (Batool *et al.*, 2019). Our functional analyses using the human TCam-2 cell line to model seminoma indicate canonical WNT signalling is fundamental to supporting processes of proliferation, viability and migration that may be of relevance to TGCT progression. TCam-2 cells mount a rapid response to acute canonical WNT signalling activation. In conditions that reduce CTNNB1 nuclear localisation or activity, these cells lose both viability and migratory ability in longer-term cultures (days). These outcomes can be mediated through increased destruction complex function, by inhibition of the CTNNB1–TCF interaction, or through direct pathway signalling inhibition, as demonstrated via siRNA-mediated knockdown of TCF7L1 or TCF7L2.

In general, the outcomes of WNT pathway dysregulation in cancers are highly cancer-type-dependent, either increasing proliferation/progression or inhibiting tumour growth (Derksen *et al.*, 2004; Chien *et al.*, 2009b; Pearson *et al.*, 2009). In light of the TCam-2 responses reported here, the development of targeted therapeutics to down-regulate WNT-mediated signalling, through shRNA/siRNA and small molecule-mediated modulation of WNT pathway activity (Polakis, 2012), is of particular relevance. The small molecule pathway inhibitors from the IWR and CCT families, as used in this study, are already being considered for their therapeutic potential in the treatment of osteosarcoma (Martins-Neves *et al.*, 2018), lymphoma (Mathur *et al.*, 2015) and colorectal cancers (Jarde *et al.*, 2013; Lee *et al.*, 2015). However, future investigations into WNT down-regulation in TGCT have to carefully consider a possibility of inducing cisplatin resistance.

This study has provided new evidence that WNT signalling is active and dynamic during normal human spermatogenesis and also relevant to germ cell cancers. The findings demonstrate the potential for using strategies that regulate CTNNB1-mediated canonical WNT signalling to influence fundamental behaviours of relevance to both normal and neoplastic male germline cells, such as proliferation, viability and migration. Further work is needed to understand how somatic cells, including the immune cells abundant in some seminomas, may influence tumour progression by affecting WNT signalling activity. It is anticipated that the observations here, which identify the presence of key WNT mediators of gene activation in TGCTs, will encourage the consideration of WNT signalling in the quest to understand how the tumours develop and spread.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Authors' roles

J.C.Y.: study conception and design, experimental data acquisition and analysis/interpretation, drafting of the manuscript, manuscript revision, final manuscript approval. G.K.: experimental data acquisition and analysis/interpretation, manuscript revision, final manuscript approval. D.M.: experimental data acquisition and analysis/interpretation, drafting the manuscript, manuscript revision, final manuscript approval. J.E.N.: experimental data acquisition and analysis/interpretation, manuscript revision, final manuscript approval. E.R.-D.: study conception and design, experimental data analysis/interpretation, provision of key materials, manuscript revision, final manuscript approval. H.E.A.: study conception and design, experimental data analysis/interpretation, manuscript revision, final manuscript approval. K.L.L.: study conception and design, experimental data analysis/interpretation, drafting of the manuscript, manuscript revision, final manuscript approval.

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Conflicts of interest

All authors declare no conflicts of interest with respect to the current study.

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