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Constitutive WNT/CTNNB1 activation triggers spermatogonial stem cell proliferation and germ cell depletion

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ABSTRACT

The differentiation of germ cells into oogonia or spermatogonia is the first step that eventually gives rise to fully mature gametes. In the female fetal gonad, the RSPO1/WNT/CTNNB1 signalling pathway is involved in primordial germ cell proliferation and differentiation into female germ cells, which are able to enter meiosis. In the postnatal testis, the WNT/CTNNB1 pathway also mediates proliferation of spermatogonial stem cells and progenitor cells. Here we show that forced activation of the WNT/CTNNB1 pathway in fetal gonocytes using transgenic mice leads to deregulated spermatogonial proliferation, and exhaustion of the spermatocytes by apoptosis, resulting in a hypoplastic testis. These findings demonstrate that a finely tuned timing in WNT/CTNNB1 signalling activity is required for spermatogenesis.

1. Introduction

Canonical WNT/CTNNB1 (β-catenin) signalling is activated by the binding of a WNT ligand, like WNT3A, to a multiproteic complex composed by Frizzled receptor and LRP5/6 co-receptors. This binding induces the sequestration of CTNNB1 inhibitors, preventing the proteolysis of and therefore stabilizing CTNNB1 in the cytoplasm. This promotes its translocation into the nucleus where it forms a transcriptional complex with TCF/LEF transcription factors. This complex in turn up-regulates the expression of various target genes, the identity of which depends on the cellular context (de Lau et al., 2014; Niehrs, 2012).

The WNT/CTNNB1 pathway is involved in many developmental processes such as primary embryonic axis formation (McMahon and Moon, 1989), segmentation and patterning in *Drosophila melanogaster* (Baker, 1988; Sampedro et al., 1993; van den Heuvel et al., 1989), bone and muscle development (Borello et al., 2006; Chen et al., 2005; Tajbakhsh et al., 1998), sex determination (Chassot et al., 2008; Maatouk et al., 2008), and specification of the germline lineage. In mice, the canonical WNT/CTNNB1 signalling promotes the epiblast to respond to BMP4 signalling, leading to specification of primordial germ cells (PGCs) (Ohinata et al., 2009). One of the WNT/CTNNB1 targets is the mesoderm and notochord transcription factor T (Brachyury) (Yamaguchi et al., 1999). In the mouse epiblast, T induces the expression of *Blimp1* and *Prdm14*, the earliest markers of PGC commitment (Aramaki et al., 2013). The PGCs then migrate through the hindgut and dorsal mesentery to colonize the genital ridges at around 10.5 days *post coitum* (d*pc*) in mice (Ginsburg et al., 1990; McLaren and Durcova-Hills, 2001). PGCs proliferate during their migration prior to sex determination (Campolo et al., 2013). Once PGCs have reached the gonads, they become regulated by both intrinsic and environmental factors. The RNA helicase MVH is required for PGC proliferation in XY gonads, (Tanaka et al., 2000), whereas the secreted protein RSPO1 activates the WNT/CTNNB1 signalling to enhance PGC proliferation in XX gonads (Chassot et al., 2011). Following their proliferation, PGCs differentiate according to their somatic environment (Adams and McLaren, 2002; Hu et al., 2015; McLaren and Southee, 1997; Palmer and Burgoyne, 1991). In XX gonads, PGCs become oogonia and enter meiosis around 13.5 d*pc* (Adams and McLaren, 2002; McLaren and Southee, 1997). In XY gonads, PGCs become gonocytes by 12.5 d*pc* (Best et al., 2008; McLaren and Southee, 1997), then are arrested in G0/G1 at 14.5 d*pc* and remain quiescent until approximately postnatal days 1–2 (1–2 days *post-partum* or d*pp*) (Dolci and De Felici, 1990; Western et al., 2008).

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Between 0 and 2 d*pp*, gonocytes migrate from their original central position in the seminiferous cords toward the periphery, resume proliferation and transform into heterogeneous populations of spermatogonia (Vergouwen et al., 1991; Western et al., 2008). One of these populations promptly differentiates into KIT-positive spermatogonia and gives rise to the first round of spermatogenesis. The other population gives rise to the spermatogonia stem cells (SSC) (Nakagawa et al., 2010). In the adult mice, the prevailing model considers that spermatogonia in the single cell state, known also as A single (As) spermatogonia, are the SSC (Huckins, 1971; Oakberg, 1971). Upon division, As spermatogonia give rise to two paired (Ap) spermatogonia, then to chains of 4–32 aligned (Aal) spermatogonia interconnected by intercellular bridges. Together As, Ap, and Aal are referred to as "undifferentiated spermatogonia", and As retain a potential for self-renewal (de Rooij, 2001; Yoshida, 2009, 2012). Then Aal spermatogonia undergo transition without division into A1 spermatogonia, which in turn generate A2, A3, A4, intermediate, B spermatogonia by mitotic divisions. Collectively, A_1 to B spermatogonia are referred to as "differentiating spermatogonia" and express KIT membrane receptor (Schrans-Stassen et al., 1999). They are irreversibly committed to differentiation (Chiarini-Garcia and Russell, 2001; Yoshida et al., 2006). Then B spermatogonia divide to generate preleptotene (i.e., premeiotic) spermatocytes. The latter undergo differentiation to spermatozoa through a series of steps giving rise firstly to primary (leptotene, zygotene, pachytene, and diplotene) and secondary spermatocytes, then to round, elongating spermatids and eventually spermatozoa. The differentiating germ cells are arranged into concentric layers from the basement membrane to the lumen of the seminiferous tubules. Premeiotic cells are present in the most peripheral germ cell layer, while meiotic spermatocytes form the central layers and post-meiotic cells (round and elongating spermatids) the layers closest to the lumen (reviewed in (de Rooij and Russell, 2000)).

WNT/CTNNB1 signalling regulates both spermatogonial stem cell proliferation in the adult testis (Takase and Nusse, 2016), and oogonia proliferation and differentiation in the fetal ovary (Chassot et al., 2011), suggesting a very conserved role of this pathway in germ cell physiology. However, whereas activation of the canonical WNT signalling was occasionally detected in somatic cells such as Sertoli cells, peritubular myoid cells and blood vessel, WNT/CTNNB1 was mainly activated in the undifferentiated spermatogonia (Chassot et al., 2011; Takase and Nusse, 2016), suggesting that the activation of this signalling pathway must be tightly controlled to allow spermatogenesis to proceed. To address the consequences of forcing the activation of the WNT/CTNNB1 pathway in spermatogenesis, we investigated spermatogonia development and spermatocyte formation by using a gain-of-function mutation. Our study showed that forced expression of *Ctnnb1* gene in XY gonocytes stimulates spermatogonia proliferation, blocks their differentiation and induces apoptosis in spermatocytes, leading to spermatogenesis arrest, and to a seminiferous epithelium containing only Sertoli cells and undifferentiated spermatogonia after one month of age.

2. Material and methods

2.1. Mouse strains and genotyping

The experiments described herein were carried out in compliance with the relevant institutional and French animal welfare laws, guidelines and policies. All the experiments were approved by the French ethics committee (Comité Institutionnel d'Ethique Pour l'Animal de Laboratoire; number NCE/2011-12). All mice were kept on a mix background 129/C57BL/6J. Mouse lines were obtained from the Jackson Laboratory. The *Ddx4:Cre* *Ctnnb1*$^{ex3/+}$ and *mT/mG* mice were described previously and genotyped as reported (Gallardo et al., 2007; Harada et al., 1999; Muzumdar et al., 2007). Genotyping was performed using DNA extracted from tail tips or ear biopsies of mice.

2.2. Histological analyses

Anesthetized animals were perfused with a 4% (w/v) paraformaldehyde solution, and testes were dissected, fixed in Bouin's solution overnight, and then embedded in paraffin on the Experimental Histopathology Platform of iBV, CNRS UMR7277-INSERM U1091-UNS. Microtome sections of 5 µm thickness were stained with hematoxylin and eosin (H & E) according to standard procedures. Pictures were taken with an MZ9.5 microscope (Leica) coupled with a DHC490 camera (Leica) and Leica application suite V3.3.0 software, and processed with Adobe Photoshop.

2.3. Quantitative PCR analyzes (QPCR)

Individual gonads were dissected in Phosphate Buffered Saline (PBS) from 2 and 3 d*pp* animals. RNA was extracted using the RNeasy Qiagen kit, and reverse transcribed using the RNA RT-PCR kit (Stratagene). Primers and probes were designed by the Roche Assay Design Center (https://www.rocheappliedscience.com/sis/rtpcr/upl/adc.jsp). All real-time PCR assays were carried out using the LC-Faststart DNA Master kit Roche according to the manufacturer's instructions. QPCR was performed on cDNA from one gonad and compared to a standard curve. QPCR were repeated at least twice. Relative expression levels of each sample were quantified in the same run and normalized by measuring the amount of *Sdha* cDNA (which represents the total gonadal cells).

2.4. Statistical analysis

For each genotype (n=3), the mean of these 3 absolute expression levels (i.e. normalized) was calculated and graphs of QPCR results show fold of change +SEM. All the data were analysed by unpaired one-sided Student's *t*-test using Microsoft Excel. Asterisks highlight the pertinent comparisons and indicate levels of significance: *P < 0.05, **P < 0.01 and ***P < 0.001. Data are shown as mean + SEM.

2.5. In-situ hybridization

Samples were fixed with 4% (w/v) paraformaldehyde overnight and then processed for paraffin embedding. Microtome sections of 7 µm thickness were processed for in *situ* hybridization. *Stra8* digoxigenin–labelled riboprobe was synthetized and in situ hybridization analyses were performed as described in (Chassot et al., 2011). Imaging was performed on MZ9.5 microscope (Leica) coupled with a DHC490 camera (Leica) and Leica application suite V3.3.0 software, and processed with Adobe Photoshop.

2.6. Immunological analyses

Samples were fixed with 4% (w/v) paraformaldehyde overnight and then processed for paraffin embedding. Microtome sections of 7 µm thickness were processed for immunostaining. Immunofluorescence analyses were performed as described in (Chassot et al., 2011). The following dilutions of primary antibodies were used: DDX4 (cat 13840, Abcam) 1:200, WT1 (clone 6F-H12, Dako) 1:300, AMH (C-20, cat sc6886, Santa Cruz) 1:200, SOX9 (cat HPA001758, Sigma) 1:500, SALL4 (cat 29112, Abcam) 1:200, αSMA (cat A5228, Sigma) 1:500, GFRα1 (cat GT15004, Immune System) 1:200, Phospho Histone H2AX (cat 16193, Millipore) 1:300, MKi67 (clone SP6, cat 9106, Thermo-Scientific) 1:200, CDH1 (cat 610182, BD Transduction Laboratories) 1:100, CyclinD1 (Booster Biological Technology) 1:300, CASP3 (cat AF835, R & D Systems) 1:200, KIT (cat CBL1360, Millipore) 1:300, GFP (cat TP401, Torrey Pines Biolabs), 1:750, 3-bETA HSD (P-18 cat 30820 Santa Cruz), 1:200. Slides were counterstained with 4',6-