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Epithelial Wnt/βcatenin signalling is essential for epididymal coiling

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ABSTRACT

Organ shape and size are important determinants of their physiological functions. Epithelial tubes are anlagen of many complex organs. How these tubes acquire their complex shape and size is a fundamental question in biology. In male mice, the Wolffian duct (WD; postnatally known as epididymis) undergoes an astonishing transformation, where a straight tube only a few millimetres long elongates to over 1000 times its original length and fits into a very small space, due to extensive coiling of epithelium, to perform the highly specialized function of sperm maturation. Defective coiling disrupts sperm maturation and leads to male infertility. Recent work has shown that epithelial cell proliferation is a major driver of WD coiling. Still, very little is known about the molecular signals involved in this process. Testicular androgens are known regulators of WD development. However, epithelial androgen receptor signalling is dispensable for WD coiling. In this study, we have shown that Wnt signalling is highly active in the entire WD epithelium during its coiling, and is limited to only a few segments of the epididymis in later life. Pharmacological and genetic suppression of Wnt signalling inhibited WD coiling by decreasing cell proliferation and promoting apoptosis. Comparative gene expression analysis identified Fibroblast growth factor 7 (Fgf7) as a prime Wnt target gene involved in WD coiling and *in vitro* treatment with Fgf7 protein increased coiling of WDs. In summary, our work has established that epithelial canonical Wnt signalling is a critical regulator of WD coiling and its precise regulation is essential for WD/epididymal differentiation.

1. Introduction

Tubular organs perform many vital functions including transport of liquids and gases (Hogan and Kolodziej, 2002). In males, the reproductive tract ductal system is mainly required for the maturation, storage and transport of sperm (Joseph et al., 2009). Wolffian duct (WD), a precursor of the male reproductive tract ductal system, is present as a straight tube during early stages of embryonic development (13.5 day post coitum, dpc, in mice) (Joseph et al., 2009). During development in mice, WD undergoes extensive growth to reach over a meter in length from its initial size of a few millimetres, and fits into a small confined space adjacent to the testis by extensive coiling (Joseph et al., 2009). This growth is primarily driven by proliferation of WD epithelial cells (Dyche, 1979). The shape and size of differentiated WD, epididymis, is essential for male fertility (Murashima et al., 2015). Sperm released by the testis are relatively immotile and lack the ability to recognise female eggs and acquire these essential features within the epididymis (Joseph et al., 2009).

Previous work from our lab and others has shown that Wnt signalling is one of the major pathways involved in the development of the mammalian urogenital system (Carroll et al., 2005; Tanwar et al., 2010a). Wnt ligands are highly expressed in precursors of both male (WD) and female (Müllerian duct: MD) reproductive tracts, and their deletion leads to the defective development of reproductive organs (Carroll et al., 2005; Tanwar et al., 2010a). Mutations in human *WNT4* gene result in agenesis of MD and loss of female reproductive tract tubal organs (oviduct, uterus and upper vagina) (Biason-Lauber et al., 2004), which is phenocopied by the *Wnt4* deleted mice (Vainio et al., 1999). *Wnt9b* null mice fail to develop male (epididymis) and female reproductive tract ductal organs (Carroll et al., 2005). Loss of *Wnt7a* leads to persistence of the female duct with WD in male mice (Parr and McMahon, 1998). These studies highlight the significance of Wnt signalling in genesis of male and female reproductive tract tubal organs. The gene knockout models in these studies show defects in the early stages of WD or MD development combined with abnormalities in other organ systems, but the role of Wnt signalling in WD coiling and differentiation was not studied.

Before sex determination, the same urogenital system is present in both males and females. After sexual differentiation,

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bipotential gonads acquire sex specific features and male gonad start secreting testosterone, which is responsible for the stabilization of WD (Joseph et al., 2009). Androgen receptor (AR) is present in both male and female WDs but lack of its ligand leads to regression of female WDs (Welsh et al., 2009). External testosterone supplementation is sufficient for restoring partial development of female WDs but unable to stimulate coiling (Welsh et al., 2009). Examination of WDs from animals with chemical or genetic ablation of AR signalling showed that androgen signalling is not essential for WD cell proliferation (Welsh et al., 2009). This view is further supported by findings that WD epithelial cell proliferation and normal epididymal coiling occurs in mice with WD epithelium specific deletion of *AR* (Murashima et al., 2011). Non-androgenic factors are clearly involved in WD proliferation and coiling, but their identity is still unclear.

In this study, we have identified that Wnt signalling is highly active during the key stages of WD development and differentiation. Using chemical and genetic approaches to alter the levels of Wnt signalling, we showed that this signalling pathway is a major regulator of WD proliferation and is essential for it's coiling.

2. Materials and methods

2.1. Mouse genetics and husbandry

Mice used in the present study were maintained on C57BL/6;129SvEv mixed genetic background and were housed under standard animal housing conditions. All procedures for mice experimentation were approved by the Animal Care and Ethics Committee at the University of Newcastle. TCF/Lef:H2B/GFP (Ferrer-Vaquer et al., 2010) mice were obtained from the Jackson lab (ME, USA) and maintained by crossing with wild type mice and hereafter referred as TCFGFP. Pax8-rtTA (Traykova-Brauch et al., 2008) mice were bred with tetO-Cre mice to generate Pax8-rtTA; tetO-cre mice and were named as LC1cre. LC1cre,Ctnnb1$^{fl/fl}$ (Huelsken et al., 2001), Ctnnb1^{tm1Mmt} (Harada et al., 1999) and Gt(ROSA)26Sor$^{tm1Sor/J}$ (Soriano, 1999) mice were used to generate LC1cre;Ctnnb1$^{fl/fl}$, LC1cre;Ctnnb1$^{fl(ex3)/fl(ex3)}$ and LC1cre;Lacz$^{fl/fl}$ mice, and were referred to as Ctnnb1$^{\Delta/\Delta}$, Ctnnb1$^{\Delta(ex3)/\Delta(ex3)}$, Lacz$^{\Delta/\Delta}$ after doxycycline treatment, respectively. To induce recombination, these mice were treated with doxycycline (1 mg/ml in 1% sucrose drinking water) from 13.5 dpc to 1-day post-natal. Genotyping was performed on DNA extracted from ear clips of adult mice and tail snips from embryos using REDExtract-N-Amp™ Tissue PCR Kit (Sigma, MO, USA) using standard PCR protocols. The sequence of genotyping primers is listed in Table S1. Whole mount β-galactosidase/Lacz staining was performed as described in (Tanwar et al., 2012). Foetuses of known age were produced by housing female mice with males of proven fertility overnight and vaginal plugs were checked following morning. The day when vaginal plug was seen was considered as 0.5 dpc. Genital ridges and genitourinary tract organs were collected at 13.5, 15.5, 18.5 dpc and 1-day post-natal. Gross pictures were obtained using Nikon SMZ25 stereoscope (Coherent Scientific, SA, Australia).

2.2. Organ culture

Foetal genital ridges were cultured as previously described with minor modifications (Tanwar et al., 2010b). Briefly, 15.5 dpc male genital ridges were isolated from TCFGFP 15.5 dpc time pregnant mice and cultured in DMEM/F12 medium (Sigma) supplemented with 10% fetal bovine serum (Interpath, Vic, Australia), 1% L-Glutamine (Sigma) and 1% Penicillin-Streptomycin (Lonza, Vic, Australia) on top of Whatman Nuclepore Polycarbonate Track-Etch Membrane (0.8 μm; Australian Scientific, NSW, Australia) at an air-liquid interface. The following Wnt inhibitors were added to culture media: 10 μM Niclosamide (Osada et al., 2011) or 100 μM IWR1 (Karner et al., 2010) or 5 μM PKF118–310 (Leow et al., 2010) or 10 μM bicalutamide (De Gendt et al., 2009) or 20 μM iCRT3 (Lee et al., 2013) or vehicle (DMSO). Cultures were maintained in incubator at 37 °C, with 5% CO_2, for 3 days and the medium was changed every 24 h. At the end of the experiments, genital ridges were gently collected and fixed overnight in 4% paraformaldehyde (PFA, Electron Microscopy Sciences, ProSciTech, QLD, Australia) at 4 °C. These tissues were then used to perform whole mount immunofluorescence (WIF) or processed for paraffin embedding and sectioning. WD only cultures were performed by modifying a previously published protocol for urinary bladder culture (Ola et al., 2011). WDs were cultured on matrigel (Cultrex reduced growth factor basement membrane extract, BioScientific, NSW, Australia) in a Falcon eight well chamber slide (In Vitro Technologies, Vic, Australia). Culture conditions and tissue processing were similar to as described above.

2.3. Whole mount immunofluorescence (WIF)

WIF was performed by modifying a previous protocol (Laird et al., 2011). Briefly, fixed genital ridges were permeabilized in 0.1% Triton X-100 in PBS. Ridges were then dehydrated and rehydrated through a gradient of ethanol (25, 50, 75 and 100%) followed by four washes with PBST of 20 min each. Tissues were then blocked with blocking solution (1% bovine serum albumin +0.2% skim milk+0.3% Triton X-100 in PBS) for one hour at room temperature. Following this, primary antibodies against CK8/Troma I [1:250; Developmental Studies Hybridoma Bank, IA, USA (DHSB)]; active βcatenin (1:200; Cell Signalling Technology, MA, USA); PH3 (1:200, Millipore, MA, USA) were added and incubated overnight at 4 °C. Tissues were then washed four times, 30 minutes each, with PBSMT (2% skim milk+0.5% Tween-20 in PBS) and incubated in AlexaFluor labelled secondary antibodies (1:250; Jackson ImmunoResearch Labs, PA, USA) for one hour at room temperature. Tissues were then washed three times with PBST, 30 min each, mounted in buffered glycerol and images were acquired using Nikon SMZ25 stereoscope.

2.4. Histology, immunohistochemistry (IHC) and TUNEL

Protocols for histological and IHC analysis of gonadal ridges are described in our previous study (Tanwar et al., 2010a). The following primary and secondary antibodies were used: βcatenin (BD Transduction Labs); Cyclin D1, LEF1, TCF1, Ecadherin (Cell Signalling Technology, MA, USA); AR, FGF7 (1:250; Santa Cruz Biotechnology, CA, USA); CK8 (DSHB); αSMA (Sigma, MO, USA) and AlexaFluor secondary antibodies (1:250; Jackson ImmunoResearch Labs). TUNEL assay was performed as per the manufacturer's instructions (Millipore). Pictures were taken with Olympus DP72 microscope at the same gain and exposure time for both control and mutant tissue samples. For the assessment of total number of PH3- and TUNEL-positive cells, images were collected at 20× from three independent areas of the WDs/epididymides of at least three different mice and counted using ImageJ (National Institutes of Health, USA). The percentages of proliferating and apoptotic cells were calculated as the fraction of CK8 and PH3- and CK8 and TUNEL-positive cells divided by the total number of CK8-positive cells.

2.5. Mouse WNT signalling array

Total RNA was isolated from 13.5 ($N=60$), 15.5 ($N=38$) and 18.5 dpc ($N=24$) WDs of wild type embryos using RNeasy® Micro kit (Qiagen, Vic, Australia) following manufacturer's instructions. First