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NRG1 signalling regulates the establishment of Sertoli cell stock in the mouse testis

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ABSTRACT

Testis differentiation requires high levels of proliferation of progenitor cells that give rise to two cell lineages forming the testis, the Sertoli and the Leydig cells. Hence defective cell cycling leads to testicular dysgenesis that has profound effects on androgen production and fertility. The growth factor NRG1 has been implicated in adult Leydig cell proliferation, but a potential function in the fetal testis has not been analysed to date. Here we show that *Nrg1* and its receptors *ErbB2/3* are already expressed in early gonadal development. Using tissue-specific deletion, we further demonstrate that *Nrg1* is required in a dose-dependent manner to induce proliferation of Sertoli progenitor cells and then differentiated Sertoli cells. As a result of reduced numbers of Sertoli cells, *Nrg1* knockout mice display a delay in testis differentiation and defects in sex cord partitioning. Taken together *Nrg1* signalling is essential for the establishment of the stock of Sertoli cells and thus required to prevent testicular hypoplasia.

1. Introduction

During embryogenesis, androgens produced by the testis are critical regulators of Wolffian ducts that give rise to male reproductive genitalia and male traits (Fluck et al., 2011; Shima and Morohashi, 2017). The synthesis of androgens requires two cell types: in fetal Leydig cells, cholesterol is converted into androstenedione that in turn is transformed into androgens in Sertoli cells (Shima et al., 2013). Both cell types originate from proliferating progenitor cells located in the coelomic epithelium (Schmahl et al., 2000). Proliferation of these progenitors occurs at low levels prior to sex determination and is under the control of *Six1/4*, as well as the insulin and WNT signalling pathways (Fujimoto et al., 2013; Pitetti et al., 2013; Chassot et al., 2012). After ingression into the male gonad, progenitor cells differentiate into fetal Leydig cells or Sertoli cells, a process that requires the expression of the sex-determining gene *Sry* and its target *Sox9* (Karl and Capel, 1998; Sekido and Lovell-Badge, 2008; Chaboissier et al., 2004). Additional proliferation of progenitor cells is then stimulated by FGF9, a growth factor secreted by the Sertoli cells (Schmahl et al., 2004). At 12.5 d*pc* in the mouse, proliferation in the coelomic epithelium returns to a basal level and progenitor cell invasion into the testis stops (Schmahl et al., 2000). Thus, the basal stock of Sertoli cells is yet established and its number determines the testicular size. Consequently, defects in progenitor cell proliferation leads to testicular dysgenesis (Schmahl and Capel, 2003). The identification of the signalling pathways underlying progenitor cell expansion is therefore crucial to better understand the occurrence of this pathology.

As testis differentiation proceeds, vascular endothelial cells migrating from the mesonephros and across the gonad promotes testis cord partitioning and assembly of the coelomic vessel at the surface of the gonad (Coveney et al., 2008; Combes et al., 2009). The development of sex cords is completed by deposition of a basement membrane by the smooth muscle like peritubular myoid cells (Tung et al., 1984).

After birth, the fetal Leydig cells are replaced by adult Leydig cells that produce all the enzymes required for androgen synthesis (Mendis-Handagama et al., 1987). Their number increases concomitantly with the level of LH (Luteinizing hormone) and this in turn leads to the expression of Neuregulin1 (NRG1), a transmembrane protein belonging to the epidermal growth factor family (Umehara et al., 2016). In addition to its role in adult Leydig cell proliferation, NRG1 also promotes spermatogonial proliferation in postnatal testes (Umehara et al., 2016; Zhang et al., 2011). Interestingly, *Nrg1* is expressed in the interstitial

Abbreviations: dpc, days post-coitum; dpp, days post-partum; LH, Luteinizing Hormone

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cells of the embryonic testis from 12.5 d*pc* onwards (Jameson et al., 2012), but its role during testicular morphogenesis has remained elusive. Here we assess the contribution of *Nrg1* in testis development by analysing the consequences of *Nrg1* conditional ablation in XY embryonic gonads.

2. Materials and methods

2.1. Mouse strains and genotyping

All experiments were carried out in compliance with the relevant institutional and French animal welfare laws, guidelines and policies and have been approved by the French ethics committee and Ministry of Education and Research (APAFIS#3772-2016012215521604v3). All mouse lines were kept on a mixed 129/C57Bl6/J background. *Wt1-Cre*$^{Tg/+}$; *Nrg1*$^{fl/fl}$ or *Wt1-CreER*$^{T2/+}$; *Nrg1*$^{fl/fl}$ and controls (*Nrg1*$^{+/+}$, *Nrg1*$^{fl/+}$ *or Nrg1*$^{fl/fl}$) were obtained by crossing *Nrg1*$^{fl/fl}$ females with *Wt1-Cre*$^{Tg/+}$ or *Wt1-CreER*$^{T2/+}$ males and then *Nrg1*$^{fl/fl}$ or *Nrg1*$^{fl/+}$ females with *Wt1-Cre*$^{Tg/+}$; *Nrg1*$^{fl/+}$ or *Wt1-CreER*$^{T2/+}$; *Nrg1*$^{fl/+}$ (Zhou et al., 2008; Li et al., 2002). *ErbB2*$^{fl/fl}$ were mated with *ErbB3*$^{fl/fl}$ mice to obtain *ErbB2*$^{fl/fl}$; *ErbB3*$^{fl/fl}$ and then the *Wt1-CreER*$^{T2/+}$ was added by a cross-breeding scheme similar to that described above in order to obtain *Wt1-CreER*$^{T2/+}$; *ErbB2*$^{fl/fl}$; *ErbB3*$^{fl/fl}$ mice (Zhou et al., 2008; Garratt et al., 2000; Sheean et al., 2014). *Wt1-Cre*$^{Tg/+}$; *Nrg1*$^{fl/fl}$; *Rspo1*$^{+/-}$ mice were bred by crossing *Wt1-Cre*$^{Tg/+}$; *Nrg1*$^{fl/fl}$ females with *Rspo1*$^{-/-}$ males and next *Nrg1*$^{fl/+}$; *Rspo1*$^{+/-}$ females with *Wt1-Cre*$^{Tg/+}$; *Nrg1*$^{fl/+}$; *Rspo1*$^{+/-}$ males (Chassot et al., 2008). *Wt1-Cre*$^{Tg/+}$; *Nrg1*$^{fl/fl}$; *Rspo1*$^{-/-}$ mice were generated by crossing *Nrg1*$^{fl/fl}$; *Rspo1*$^{+/-}$ females with *Wt1-Cre*$^{Tg/+}$; *Nrg1*$^{fl/fl}$; *Rspo1*$^{+/-}$ males. Activation of the Cre recombinase in *Wt1-CreER*$^{T2/+}$; *Nrg1*$^{fl/fl}$ and *Wt1-CreER*$^{T2/+}$; *ErbB2*$^{fl/fl}$; *ErbB3*$^{fl/fl}$ females was carried out by oral gavage to pregnant females with 4 mg of tamoxifen (Sigma-Aldrich) dissolved in 90% corn oil (Sigma-Aldrich) and 10% ethanol, per 20 g of body weight. The stage of tamoxifen administration is noted in the corresponding figure legend. Embryos were collected from timed matings. The presence of a vaginal plug in the morning was used to indicate mating and was designated as 0.5 d*pc*. Embryos were staged by counting the number of tail somites (ts) with 8 ts corresponding to 10.5 d*pc* and 18 ts to 11.5 d*pc* (Hacker et al., 1995). Genotyping was performed using DNA extracted from tail tips or ear biopsies of mice and performed as described in (Zhou et al., 2008; Li et al., 2002; Garratt et al., 2000; Sheean et al., 2014; Chassot et al., 2008). Fertility tests were performed using 4-6 months-old *Wt1-Cre*$^{Tg/+}$; *Nrg1*$^{fl/fl}$ males mated with *Nrg1*$^{fl/fl}$ females.

2.2. Histological analysis

Testes were fixed in Bouin solution overnight and embedded in paraffin. Five µm thick sections were stained with Periodic-Acid-Schiff and analysed using a MZ9.5 (Leica) microscope coupled with a DHC490 (Leica) camera and application suite V3.3.0 (Leica) software and processed with Adobe Photoshop. Macroscopic views were performed with a MZ16 (Leica) microscope coupled with a DHC490 (Leica) camera and application suite V3.3.0 (Leica) software and processed with Adobe Photoshop.

2.3. In-situ hybridization

Tissue samples were fixed in 4% paraformaldehyde in PBS overnight at 4 °C, embedded in paraffin and hybridizations were carried out essentially as described in (Chassot et al., 2008). *Nrg1* digoxigenin–labelled riboprobe was synthetized using 576 bp matching sequence (Fig. 1E). *ErbB2* riboprobe was generated by PCR amplification of gonadal cDNA using the FErbB2: 5′-tggtccagcctgagccatgg-3′ and RErbB2: 5′-acttgtccaaagggtctcg-3′ primers and cloning in the pCR®II-TOPO® vectors (Invitrogen life technologies). *ErbB3* riboprobe was kindly provided by Michael Wegner. Post-hybridization washes were performed in 100 mM maleic acid pH7.5, 150 mM NaCl, 0.1% (v/v) tween-20 (MABT). Imaging was performed using a MZ9.5 (Leica) microscope coupled with a DHC490 (Leica) camera and application suite V3.3.0 (Leica) software and processed with Adobe Photoshop.

2.4. Immunofluorescence analyses on sections

Tissue samples were fixed in 4% paraformaldehyde overnight at 4 °C and embedded in paraffin. Microtome sections of five µm thickness were used for immunofluorescence experiments as described in (Chassot et al., 2008). The dilutions of primary antibodies are reported in Table S5. Slides were counterstained with DAPI diluted in the mounting medium at 10 µg/ml (Vectashield, Vector Laboratories) to visualize the nuclei. They were analysed with a motorized Axio ImagerZ1 microscope (Zeiss) coupled with an Axiocam Mrm camera (Zeiss) and processed with Axiovision LE (Zeiss) and Adobe Photoshop.

2.5. Wholemount immunohistochemistry

Gonads were fixed in 4% paraformaldehyde for 1 h and incubated in 30% Methanol - 3.75% H_2O_2. Nonspecific epitopes were blocked using 5% milk powder for 5 h. The samples were then incubated with anti-PECAM1 (1:50) and visualized with 0.05% DAB - 0.015% H_2O_2 according to standard procedures. The analysis was performed using a MZ16 (Leica) microscope coupled with a DHC490 (Leica) camera and application suite V3.3.0 (Leica) software and processed with Adobe Photoshop.

2.6. Wholemount immunofluorescence

Gonads were fixed in 4% paraformaldehyde overnight at 4 °C and were incubated in washing/blocking solution (1% Donkey serum, 1% Triton X-100, 3% BSA in PBS). After incubation with AMH (1:200, sc6886, Santa Cruz Biotechnology) and CD144 antibodies (1:200, 550548, BD Pharmigen) for 48 h and overnight in washing/blocking solution, secondary antibodies were incubated overnight (1:500, Life Technologies). Following overnight in washing/blocking solution, samples were dehydrated in methanol (25%–100% in PBS) and were clarified in 1:1 Methanol: BABB solution (1:2 Benzyl Alcohol, 1.09626.1000, Millipore; Benzyl Benzoate, B9550, Sigma) and in BABB solution. Imaging was performed on a LSM 880 inverted Axio Observer confocal microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) using a Plan Apo 10X dry NA 0.45. Images were acquired using an Argon LASER at 488 nm and a DPSS 561 nm. Fluorescence emission was detected on a descanned GaAsp PMT, and z acquisitions were performed using a piezo z-drive. Images were processed with Imaris 9.1 Bitplane.

2.7. Reverse transcription PCR analysis

Individual gonads were dissected from the mesonephros in PBS at 13.5 d*pc* and immediately frozen at −80 °C. RNA was extracted using the PureLink™ RNA Mini kit (Ambion), and reverse transcribed using the MMLV reverse transcriptase (28025-013, Invitrogen). The primers used were *Nrg1* 5′-cgagtgcttcatggtgaagg-3′ and 5′-cgaccaccaacagggcgata-3’. Thirty cycles of PCR were sufficient to visualize the PCR products. The band of 120 bp was extracted using the QIAquick PCR Purification Kit (28104, Qiagen) and sequenced by Eurofins Genomics.

2.8. Quantification of cells

Paraffin sections of five µm thickness were processed for immunostaining experiments using the MKI67 antibody or and DAPI to identify proliferative cells and nuclei respectively. Additional proliferation analyses were performed on the same sections by way of 5-Bromo-2′-deoxy-Uridine labelling and detection using an appropriate