Critical role of *Emx2* in the pluripotency – differentiation transition in male gonocytes via regulation of FGF9/NODAL pathway

Ma Tian-Zhong¹, Chen Bi¹, Zhang Ying², Jing Xia¹, Peng Cai-Ling¹, Zhang Yun-Shan¹, Huang Mei-Wen¹ and Niu Yan-Ru³

¹Reproductive Medicine Center, Affiliated Hospital of Guangdong Medical College, Zhanjiang, Guangdong, China, ²Department of Gynecology and Obstetrics, Affiliated Hospital of Guangdong Medical College, Zhanjiang, Guangdong, China, and ³Laboratory of Minimally Invasive Orthopaedic, Guangdong Medical College, Zhanjiang, Guangdong, China

Correspondence should be addressed to Niu Yan-Ru; Email: matzgmc@163.com

Abstract

Emx2 deletion impairs the growth and maintenance of the genital ridge. However, its role in subsequent germ cell differentiation during embryonic stages is unknown. Using a tamoxifen-inducible Cre-loxP mouse model (*Emx2*^{fllox/flox}, Cre-ERTM, hereafter called as *Emx2* knockdown), we showed that germ cell differentiation was impaired in *Emx2*-knockdown testes. Representative characteristics of male germ cell differentiation, including a reduced ability to form embryonic germ (EG) cell colonies *in vitro*, down-regulation of pluripotency markers and G1/G0 arrest, did not occur in *Emx2*-knockdown testes. Furthermore, FGF9 and NODAL signalling occurred at abnormally high levels in *Emx2*-knockdown testes. Both blocking FGF9 signalling with SU5402 and inhibiting NODAL signalling with SB431542 allowed germ cells from *Emx2*-knockdown testes to differentiate *in vitro*. Therefore, EMX2 in somatic cells is required to trigger germ cell differentiation in XY fetuses, posterior to its previously reported role in the growth and maintenance of the genital ridge.

Reproduction (2016) 151 673–681

Introduction

Primordial germ cells in embryonic gonads respond to molecular signals from the surrounding somatic cells that trigger their differentiation along the spermatogenic or oogenic pathway (Looijenga *et al.* 2011). Retinoic acid (RA) induces the expression of simulated by retinoic acid 8 (STRA8) to initiate meiosis in female embryonic gonads at approximately E13.5 (Baltus *et al.* 2006, Bowles *et al.* 2006, Koubova *et al.* 2006). In a testicular environment, RA is prevented from inducing meiosis by the action of the degrading p450 enzyme CYP26B1 (Bowles *et al.* 2006, Koubova *et al.* 2006). Fibroblast growth factor 9 (FGF9) also suppresses entry into meiosis, maintains expression of pluripotency-related genes and up-regulates markers associated with male germ cell fate, including *Nanos2* (Barrios *et al.* 2010, Bowles *et al.* 2010). Recent studies have suggested that NODAL signalling works directly on male germ cells to induce their differentiation (Souquet *et al.* 2012, Spiller *et al.* 2012, Wu *et al.* 2013). Souquet and coworkers showed that NODAL signalling reinforces the male fate and prevents meiosis in embryonic germ cells (Souquet *et al.* 2012). However, Spiller and coworkers showed

that NODAL maintains the pluripotency of germ cells and delays differentiation, with no effect on meiosis (Spiller *et al.* 2012). Wu *et al.* further suggested that NODAL signalling acts downstream of the somatic cell signal FGF9 to promote male germ cell fate and suppress meiosis (Wu *et al.* 2013).

The balance between pluripotency regulation and cell fate commitment is crucial. Defects in pluripotency result in insufficient spermatogonia for fertility, whereas escape from commitment causes testicular germ cell tumours (Looijenga *et al.* 2011). Germ cell differentiation is associated with down-regulation of pluripotency regulators (POU5F1 (OCT4), SOX2, NANOG and DPPA4), appearance of differentiation indexes (including up-regulation of the male fate marker gene *Nanos2* (Tsuda *et al.* 2003), entry into cell cycle arrest (Western *et al.* 2008) and *de novo* DNA methylation (Kaneda *et al.* 2004, Sasaki & Matsui 2008, Western *et al.* 2010)). *In vivo*, both transcriptional repression and chromatin remodelling block germ cells from acquiring a fully pluripotent stem cell state (Durcova-Hills & Surani 2008). Although the developmental potency of foetal germ cells is restricted to the germ lineage, foetal germ cells at a bipotential stage (before E11.5) can dedifferentiate into

embryonic germ (EG) cells that possess pluripotency similar to embryonic stem (ES) cells *in vitro* (Labosky *et al.* 1994, Kimura *et al.* 2005, 2008) when exposed to exogenous signalling molecules, leukaemia inhibitory factor (LIF), stem cell factor (SCF) and basic fibroblast growth factor (bFGF); however, this ability is lost after differentiation (Matsui *et al.* 1992, Resnick *et al.* 1992, Durcova-Hills & Surani 2008, Matsui & Tokitake 2009).

The homeobox gene *Emx2* is a mouse homologue of the *Drosophila* head gap gene empty spiracles (*ems*). *EMX2* is expressed in the epithelial components of the developing urogenital system and is required for growth and maintenance of the genital ridge (Miyamoto *et al.* 1997). *Emx2* deletion impairs the migration of epithelial cells through the basement membrane to form the genital ridge (Kusaka *et al.* 2010). However, it is unknown whether *Emx2* regulates subsequent germ cell differentiation during the embryonic stage. In this study, we addressed this question by employing a tamoxifen-inducible *Cre-loxP* mouse model (*Emx2^{fllox/flox}*, *Cre-ErTM*, hereafter called as *Emx2* knockdown).

Various aspects of male germ cell differentiation were disrupted in *Emx2*-knockdown testes. First, the germ cells in *Emx2*-knockdown testes showed a high propensity to form pluripotent EG cell lines *in vitro* at E13.5, which is no longer possible for wild-type differentiated germ cells at the same stage. Secondly, the expression of the pluripotent regulators (POU5F1, SOX2, NANOG and DPPA4) was still detected after E15.5. Thirdly, G1/G0 arrest was not observed in *Emx2*-knockdown testes. We used *in vitro* culture models together with inhibitors to demonstrate that these effects were mediated at least in part by abnormally high levels of FGF9 and NODAL signalling. Our findings expand the known developmental roles of *EMX2* by demonstrating its role in the pluripotency–differentiation transition and its interaction with the FGF9/NODAL pathway.

Materials and methods

Mouse breeding

All animal research was approved by the committee on animal care at the Guangdong Medical College (Permit number: SYXK (Guangdong): 2008-0007). All mice were maintained on a C57BL/6;129/SvEv-mixed background. A targeting vector containing *Emx2* exon 2 flanked by a loxP site and a loxP-Neo cassette was constructed and introduced into mouse embryonic stem (ES) cells (AB1, 129/SvEv) by electroporation (Supplementary Fig. S1A, see section on supplementary data given at the end of this article). Next, ES cell clones containing the targeted *Emx2* construct were injected into C57BL/6 (B6) blastocysts to generate chimaeras. The resulting male chimaeras were identified by coat colour and mated with wild-type females to obtain germ line transmission. Tail biopsies of agouti-pigmented F1 animals were genotyped using a primer set specific to the Neo cassette. *Emx2^{fllox/flox}* mice were mated with mice carrying the *Cre-ErTM* (Hayashi & McMahon 2002)

Figure 1 The tamoxifen-inducible *Emx2* knockdown used in this study. (A) Immunohistochemical staining was performed on paraffin sections. Haematoxylin was used for counterstaining. *EMX2* was specifically expressed in the somatic cells of the genital ridge at E10.5 and E11.5 (a and b). *EMX2* was localized to Sertoli cells from E12.5 and E13.5 (c and d). gr, genital ridge; tc, testis cord. Scale bar, 50 μm. (B) Schematic for generating the animals carrying the *Emx2^{fllox}* and the recombined allele in which exon 2 is flanked by loxP sites. (C) To induce Cre recombinase activity, pregnant mice were injected with 12 mg/40 g (body weight) tamoxifen at E9.5, and foetal testes were collected from embryos after E11.5.

transgene to produce *Emx2^{fllox/flox}, Cre-ErTM* offspring. DNA isolated from tail and embryo biopsies was used for genotyping (Fig. S1B). The presence of the floxed allele and *Cre* transgene was determined by PCR amplification. Mice were genotyped by PCR analysis using primers (5'-GAGCCTGAGGACTGCCA-3' and 5'-GGCTCGCTTGTTCACTCT-3') to identify the loxP locus and primers (5'-GCCAGGCCACACTACATC-3' and 5'-GCGGAGAAAAGCTAGC-3') to identify the exon 2-deleted locus for *Emx2* targeting. Tamoxifen (Sigma-Aldrich) was dissolved in corn oil to a final concentration of 20 mg/mL. For detailed characterization of the *Cre* activity, 3, 6, 9, 12, 13 or 15 mg tamoxifen/40 g of body weight was injected intraperitoneally into pregnant mothers at E9.5. A higher concentration of tamoxifen (>12 mg tamoxifen/4 g body weight)

caused embryonic lethality. Accordingly, we chose 12 mg tamoxifen/40 g body weight to induce excision of the floxed *Emx2* allele (Hayashi & McMahon 2002). Tamoxifen-treated *Emx2^{+/flox}, Cre-ErTM* mice served as controls in all experiments.

Germ cell-EG dedifferentiation

Embryonic germ cell culture was performed as described previously with minor modifications (Durcova-Hills & Surani 2008). Gonads containing germ cells from E11.5 and E13.5 embryos were dissociated into single cells by incubation in 0.05% trypsin/0.02% EDTA (Invitrogen) for 5 min in a 37°C water bath, and a cell suspension was obtained by gently pipetting tissues. Germ cells labelled with a C-terminal DDX4/MVH antibody (Abcam, ab13840; 1:100) were sorted by fluorescence-activated cell sorting (FACS) using an Influx Cell Sorter (BD Biosciences, San Jose, CA, USA). Cells were plated

Figure 2 Morphological analysis of *Emx2^{fllox/flox}, Cre-ErTM* gonads. (A) The efficiency of tamoxifen-induced *Cre* recombination was examined by qRT-PCR at E11.5. Compared with wild-type testes, *Emx2* transcription levels were reduced by approximately 70% in *Emx2^{fllox/flox}, Cre-ErTM* testes. * $P < 0.05$; Student's *t*-test. (B) Morphological analysis of *Emx2^{fllox/flox}, Cre-ErTM* gonads at E15.5. DDX4-expressing germ cells were present throughout the gonad, and the basement membrane did not outline testis cords. Scale bar in a and b, 100 µm. Scale bar in c and d, 50 µm.

Figure 3 EMX2 determines germ cell pluripotency to form EG clones *in vitro*. (A) Gonads containing PGCs from E11.5 (a and b) and E13.5 (c and d) control (a and c) or *Emx2* knockdown (b and d) testes were isolated and cultured on feeder cells in the presence of essential survival and growth factors for 10 days. Arrows denote EG clones. Scale bar, 50 µm. (B) Number of EG cell colonies was shown from E11.5 and E13.5 control or *Emx2*-knockdown testes. ** $P < 0.01$; Student's *t*-test.

Figure 4 Germ cells in *Emx2^{flox/flox}*, *Cre-ErTM* testes exhibited prolonged pluripotency. Quantitative analysis of *Pou5f1* (A), *Nanog* (B), *Sox2* (C) and *Dppa3* (D) mRNA levels in wild-type and *Emx2^{flox/flox}*, *Cre-ErTM* testes from E13.5 to E16.5. Expression was normalized to *Ddx4*. Three independent biological replicates were induced for each time point. A *post hoc* multiple comparisons test following ANOVA was used. **P* < 0.05, ***P* < 0.01.

onto mitotically inactivated (treated with 5 µg/mL mitomycin C (Sigma-Aldrich) for 2 h the day before use) SI/SI4-m220 feeder cells in 12-well plates with PGC growth media (Durcova-Hills & Surani 2008). Media were changed daily for 10 days until small colonies were observed. EG cells were identified by the characteristic colony morphology of densely packed cells and by staining for pluripotency markers such as POU5F1 (sc-8629, 1:200; Santa Cruz), NANOG (ab80892, 1:200; Abcam), SOX2 (2683-1, 1:100; Epitomics, Burlingame, CA, USA) and c-MYC (ab32072, 1:200; Abcam).

Collection of foetal gonads and organ culture

Foetal gonads were isolated and cultured as described previously (Livera *et al.* 2006). The gonads of tamoxifen-treated *Emx2^{flox/flox}*, *Cre-ErTM* and *Emx2^{flox/flox}*, *Cre-ErTM* (control) embryos were harvested at E11.5, and foetal sex was determined using PCR amplification of *Sry* (Koopman *et al.* 1991). One gonad per embryo was used as the control and was cultured with the appropriate vehicle. The contralateral gonad was cultured with 20 µM FGFR-specific tyrosine kinase inhibitor SU5402 (Calbiochem) (Wu *et al.* 2013) or 20 µM specific type I ALK 4/5/7 receptor inhibitor SB431542 (Sigma-Aldrich) (Souquet *et al.* 2012) for 72 h before quantitative RT-PCR.

Immunofluorescence and immunohistochemistry

Embryonic gonads were collected from E12.5 to E18.5. Gonads were dissected in PBS and fixed in 4% PFA for up to 24 h, stored in 70% ethanol and embedded in paraffin. Tissue sections (5 µm)

were cut and mounted on glass slides. Immunofluorescence and immunohistochemistry of deparaffinized sections were performed as described previously (Chen *et al.* 2013). Primary antibodies included anti-EMX2 (ab110112, 1:200; Abcam), anti-DDX4 antibody (ab13840, 1:200; Abcam), anti-KI67 (ab15580, 1:400; Abcam), anti-STELLA (sc-67249; Santa Cruz), anti-SOX9 (ab3697, 1:400; Abcam), anti-STR8 (ab49602; Abcam) and anti-DAZL (MCA2336, 1:200; AbD Serotec (San Diego, CA, USA)). FITC or TRITC-conjugated secondary antibodies were from Jackson ImmunoResearch. The sections were counterstained with DAPI (Sigma-Aldrich) to label the nuclei. To determine the percentage of gonocytes that were positive for KI67, we counted at least ten sections for each group.

Western blot

Proteins from isolated foetal testes were electrophoresed under reducing conditions in 12% SDS-PAGE gels and transferred onto nitrocellulose membranes. The blots were blocked in 5% BSA, incubated overnight at 4°C with primary antibody (anti-EMX2; Abcam, ab110112, 1:1000) and incubated with the IRDye 680 or IRDye 800 (LI-COR Bioscience, Lincoln, NE, USA) secondary antibody for 1 h at room temperature. The specific signals and the corresponding band intensities were evaluated using an Odyssey Infrared Imaging system and software (LI-COR Bioscience, Lincoln, NE, USA).

Figure 5 Immunohistochemical staining for POU5F1 protein in sections of wild-type (A, B and C) and *Emx2^{flox/flox}*, *Cre-ErTM* testes (D, E and F). Scale bar, 50 µm.

Figure 6 Germ cells in *Emx2*-knockdown testes did not undergo G1/G0 cell cycle arrest. Germ cells were labelled with DAZL (green) and KI67 (red) labelled proliferating cells. The KI67 signal was absent in most of the germ cells of control testes after E13.5 (A, B and C, arrows). Germ cells in *Emx2*-knockdown testes were still KI67 positive from E13.5 to E15.5 (D, E and F, arrowheads). Nuclei were stained with DAPI (blue). Scale bar, 50 μ m. (G) Percentage of KI67-positive gonocytes in control and *Emx2*-knockdown testes at E13.5, E14.5 and E15.5. ** $P < 0.01$; Student's *t*-test.

Quantitative (q)RT-PCR

Total RNA was extracted from foetal testes using a Qiagen RNeasy kit in accordance with the manufacturer's instructions. To quantify gene expression, real-time SUBR Green assays were performed with the isolated RNA (Tian Gen, Beijing, China). The results were analysed by the $2^{-\Delta\Delta C_t}$ method. Gene

expression was quantified relative to *Gapdh* or *Ddx4* (*Mvh*) expression. Primer sequences are listed in Table S1.

Statistical analysis

The results of three experiments are presented as the mean $\pm$ S.E.M. The data were evaluated for significant differences using Student's *t*-test. When comparing multiple samples, a *post hoc* multiple comparisons test following ANOVA was used. A *P*-value < 0.05 was considered to be significant, and a *P*-value < 0.01 was considered to be highly significant. Bar graphs were plotted in MS Excel.

Results

Tamoxifen-inducible *Emx2* knockdown after E11.5

EMX2 was predominantly expressed in the somatic cells of the genital ridge at E10.5 and E11.5 (Fig. 1A; a and b). Afterwards, EMX2 was specifically localized

Figure 7 FGF9 and NODAL signalling was hyperactivated in *Emx2*-knockdown testes. (A) qRT-PCR analysis of *Fgf9* from E10.5 to E15.5 between control and *Emx2*-knockdown testes. *Gapdh* was used as normalization control. (B, C, D and E) qRT-PCR analysis of *Nodal* (B), *Cripto* (C), *Lefty1* (D) and *Lefty2* (E) from E13.5 to E16.5 control and *Emx2*-knockdown testes. *Ddx4* was used as normalization control. Three independent biological replicates were induced for each time point. * $P < 0.05$; Student's *t*-test.

Figure 8 Blocking FGF9 or NODAL signalling allowed germ cells in *Emx2*-knockdown testes to differentiate. E11.5 *Emx2*-knockdown and control testes were treated with SU5402 (20 μ M) (A) or SB431542 (20 μ M) (B), and *Cripto*, *Lefty1*, *Nanos2*, pluripotency-related genes *Nanog*, *Sox2* and *Pou5f1* as well as the differentiation-related gene *Dnmt3l* were analysed by qRT-PCR after 72 h of culture. *Ddx4* was used as normalization control. Three independent biological replicates were induced for each time point. * $P < 0.05$; Student's *t*-test.

to Sertoli cell nuclei at E12.5 and E13.5 (Fig. 1A; c and d). In addition, we detected a band of the predicted EMX2 size in testes from E11.5 to E15.5 by western blot (Fig. S2A). We performed double staining for EMX2 and SOX9 (a somatic cell marker) and EMX2 and STELLA (a germ cell maker) at E11.5. As expected, EMX2 was specifically expressed in somatic cells but not in gonocytes (Fig. S2B). To investigate the role of EMX2 during embryonic gonad development, we utilized a tamoxifen-inducible *Cre-loxP* mouse model (*Emx2^{fllox/fllox}*, *Cre-ErTM*) (Fig. 1B). To induce Cre recombinase activity, pregnant mice were injected with 12 mg/40 g (body weight) tamoxifen at E9.5, and foetal testes were collected from the embryos after E11.5 (Fig. 1C). The *Emx2* mRNA level was reduced by approximately 70% in *Emx2*-knockdown testes relative to controls at E11.5 (Fig. 2A). EMX2 was successfully deleted in *Emx2*-knockdown testes, as demonstrated by immunohistochemistry (Fig. S2C). Morphological analysis revealed that the basement membrane poorly outlined the cords, and DDX4-expressing germ cells were present throughout the testes in the *Emx2*-knockdown mice at E15.5 (Fig. 2B). *Emx2*-knockdown mice exhibited perinatal lethality.

Germ cells in *Emx2*-knockdown testes maintain pluripotency instead of differentiation

The ability of PGCs to generate EG cell colonies is restricted to the undifferentiated PGCs before E11.5 (Matsui & Tokitake 2009). To examine the differentiation status, XY gonads containing germ cells from E11.5 and E13.5 control or *Emx2*-knockdown embryos were isolated and cultured under EG colony-forming conditions. EG cells were identified by the characteristic colony morphology of densely packed cells and by staining for the pluripotency markers POU5F1, NANOG, SOX2 and c-MYC (Fig. S3). No significant difference in the formation of primary EG cell colonies was observed between control testes and *Emx2*-knockdown testes at E11.5 (Fig. 3A; a and b). After E11.5, PGCs differentiated along the spermatogenic or oogenic pathway. As expected, the efficiency of deriving EG cells from E13.5 germ cells in control testes was vanishingly low (Fig. 3A; c). Significant reduction in EG cell colony formation was observed in E13.5 *Emx2*-knockdown testes relative to control testes (Fig. 3A; d, 3B). Collectively, germ cells maintain pluripotency and the ability to dedifferentiate into EG cells in *Emx2*-knockdown testes.

Next, we examined the expression of pluripotency markers in *Emx2*-knockdown testes from E13.5 to E16.5 by qRT-PCR (Fig. 4). Compared with control testes, germ cells in *Emx2*-knockdown testes continued to express *Sox2*, *Nanog* and *Dppa3* at high levels from E13.5 to E16.5 (Fig. 4A, B and C). Although the *Pou5f1* mRNA level remained unchanged (Fig. 4D), POU5F1 protein levels were significantly reduced after E14.5 in wild-type testes, as demonstrated by staining (Fig. 5A, B and C). We observed that germ cells extended the period of POU5F1 protein production in *Emx2*-knockdown testes (Fig. 5D, E and F).

In wild-type testes, most germ cells were negative for the active cell cycle marker KI67 after E13.5 (Fig. 6A, B and C), indicating that they had entered mitotic arrest. By contrast, the percentage of KI67-positive male gonocytes was significantly increased in *Emx2*-knockdown testes from E13.5 to E15.5 (Fig. 6D, E, F and G).

Hyperactivation of FGF9-NODAL signalling in *Emx2*-knockdown testes

Using qRT-PCR, we observed that the *Fgf9* mRNA level was significantly increased in *Emx2*-knockdown testes compared with control testes from E12.5 to E15.5 (Fig. 7A). FGF9 stimulates *Nanos2* expression in ex vivo gonad cultures (Barrios et al. 2010, Bowles et al. 2010). As expected, the *Nanos2* mRNA level was significantly up-regulated in *Emx2*-knockdown testes, relative to control testes from E12.5 to E16.5 (Fig. S4A). Furthermore, the mRNA levels of *Nodal* and the NODAL downstream target genes *Cripto*, *Lefty1* and *Lefty2* were substantially down-regulated after E13.5 in control testes. However, *Nodal*, *Cripto*, *Lefty1* and *Lefty2* were significantly overexpressed in *Emx2*-knockdown testes, relative to control testes between E14.5 and E16.5 (Fig. 7B, C, D and E).

Blocking either FGF9 or NODAL signalling allows germ cells to differentiate in *Emx2*-knockdown testes

To determine the effects of high levels of FGF9 and NODAL signalling on the arrest of differentiation phenotype, the FGFR tyrosine kinase inhibitor SU5402 (Mohammadi et al. 1997) and the ALK4/5/7 inhibitor SB431542 (Inman et al. 2002) were added to E11.5 control and *Emx2*-knockdown testes respectively (Fig. 8). The expression of NODAL target genes such as *Cripto* and *Lefty1* was significantly decreased in the SU5402 or SB431542 treatment conditions. The mRNA levels of *Nanos2* and pluripotency-related genes such as *Nanog*, *Sox2* and *Pou5f1* were significantly reduced in SU5402-treated control and *Emx2*-knockdown testes after 72 h of ex vivo culture, accompanied by increased expression of the differentiation-related gene *Dnmt3l*, compared with the untreated groups (Fig. 8A). We also observed the reduced expression of pluripotency-related genes

such as *Nanog*, *Sox2* and *Pou5f1*, and, conversely, the increased expression of the differentiation-related gene *Dnmt3l* in control and *Emx2*-knockdown testes after SB431542 treatment (Fig. 8B).

Discussion

EMX2 is required for embryonic gonad development, as *Emx2* deletion leads to urogenital development defects (Miyamoto et al. 1997, Kusaka et al. 2010). In this study, we used a conditional knockdown strategy (*Emx2*^{flox/flox}, *Cre-Er*TM) to knock down *Emx2* at approximately E11.5 (after genital ridge formation) and then examined the consequences of this deletion on male germ cell differentiation. We showed that various aspects of male germ cell differentiation were blocked in these knockdown mice. In tamoxifen-treated *Emx2*^{flox/flox}, *Cre-Er* foetal testes, germ cells expressed pluripotency genes, including *Sox2*, *Nanog*, and *Pou5f1*, at high levels after E14.5, when their expression is normally down-regulated to allow spermatogenic differentiation to proceed. As a result, germ cells in *Emx2*-knockdown foetal testes were still proliferating at E13.5, whereas germ cells in control testes had become mitotically quiescent at this stage. Consistently, germ cells in *Emx2*-knockdown testes retained the ability to form EG colonies in vitro at E13.5, when control germ cells have already lost this ability.

Interestingly, although germ cells in *Emx2*-knockdown testes failed to complete male differentiation and extended the period of pluripotency, they did not adopt a female fate. The expression of female marker genes such as *Stra8*, *Foxl2*, *Rspo1*, *Ctnnb1*, *Bmp4*, *Nr0b1* and *Fst* did not differ between control testes and *Emx2*-knockdown testes (Fig. S5A). We did not observe male germ cell meiosis in inhibitor-treated *Emx2*-knockdown testes (Fig. S5B). Among the candidate signals analysed, FGF9 and NODAL signalling occurred at abnormally high levels in *Emx2*-knockdown testes. We hypothesize that the failure of germ cells in *Emx2* knockdown testes to undergo spermatogenic differentiation is caused by persistently high levels of FGF9 secreted by Sertoli cells and its targets, the NODAL signalling genes mainly expressed in germ cells. This hypothesis is supported by rescue experiments performed on ex vivo cultured foetal gonad, in which treatment with chemical inhibitors of either FGF9 or NODAL signalling pathway down-regulated the pluripotency genes (*Nanog*, *Sox2* and *Pou5f1*) and restored the expression of the differentiation marker gene *Dnmt3l* in *Emx2*-knockdown testes. We also examined the mRNA levels of other Sertoli cell marker genes at E12.5 (Fig. S6). The mRNA levels of *Cyp26b1*, *Ptgds*, *Dmrt1*, *Sox9*, *Amh*, *Wt1* and *Gata4* in *Emx2*-knockdown testes were not different from those in control testes.

Our finding that EMX2 suppresses *Fgf9* expression in Sertoli cells suggests a possible link between EMX2 and

FGF signalling. However, the underlying mechanism by which EMX2 regulates *Fgf9* (directly or indirectly) requires further investigation. *Nanos2* expression was up-regulated in *Emx2*-knockdown testes from E12.5 to E16.5 compared with the control group (Fig. S4A). This result is consistent with previous studies that FGF9 stimulates *Nanos2* expression (Barrios *et al.* 2010, Bowles *et al.* 2010). Unexpectedly, the *Dnmt3l* mRNA level was reduced in *Emx2*-knockdown testes after E13.5 (Fig. S4B), which is inconsistent with a previous report that FGF9 stimulates *Dnmt3l* expression (Bowles *et al.* 2010). We suggest that other yet-to-be-identified signals in addition to FGF9 are involved in the regulation of *Dnmt3l* expression. Increased expression of *Dnmt3l* is observed in *Nodal* knockdown gonads at E14.5 (Spiller *et al.* 2012). In our study, we observed that *Dnmt3l* expression was significantly up-regulated after addition of the NODAL signalling inhibitor SB431542. Thus, we suggest that Nodal signalling suppresses the expression of *Dnmt3l*. Given the FGF9 and NODAL signalling up-regulation in *Emx2*-knockdown testes and their opposite roles in regulating *Dnmt3l* expression, *Dnmt3l* was ultimately down-regulated in the *Emx2* conditional knockdown testes.

After E12.5, the expression of NODAL pathway components is reduced to allow normal male germ cell differentiation (Spiller *et al.* 2012). By contrast, germ cells in *Emx2*-knockdown testes expressed high levels of *Nodal*, *Cripto* and *Lefty1/2*, which restrict differentiation and contribute to pluripotency maintenance. Our conclusions regarding NODAL signalling function are complementary with the study demonstrating that *Nodal* knockdown leads to premature differentiation, reduced pluripotency marker expression and reduced ability to form embryonic germ (EG) cell colonies *in vitro* (Spiller *et al.* 2012). Wu *et al.* suggested that the 50% inhibitory concentration of SU5402 is 10–20 μ M (Wu *et al.* 2013). Our inhibitor experiments (20 μ M SU5402) reduced the abnormally high level of NODAL signalling in *Emx2*-knockdown testes to an almost 'normal' level.

SB431542 and SU5402 inhibit TGFB1 and FGF signalling respectively (Mohammadi *et al.* 1997, Inman *et al.* 2002). The TGFB family comprises many members, including TGFB1–3, ACTIVIN and NODAL that signal through the activin-like kinases (ALK)-4, -5 and -7 to regulate target gene transcription (Young *et al.* 2015). The chemical inhibitor SB431542 blocks ALK-4, -5 and -7 and is not a specific inhibitor of NODAL signalling, as it also blocks TGFB1–3 and ACTIVIN signalling. In our inhibitor experiments, loss of *Lefty* expression confirmed that NODAL signalling was repressed; however, other TGFB1 signalling pathways may also have been inhibited. Similarly, FGF9 signalling is inhibited in company with other branches of the FGF family by the general FGFR tyrosine kinase inhibitor SU5402.

The close relationship between pluripotency and other characteristics of male germ cell differentiation has been evaluated in recent studies (Western *et al.* 2008, 2010, Nettersheim *et al.* 2011). Following the mitotic arrest of male germ cells between E12.5 and E13.5, transcriptional repression of pluripotency regulators is initiated between E13.5 and E14.5 (Western *et al.* 2010). Persistent expression of pluripotency regulators in germ cells is always associated with continuance of the mitotic cell cycle in the dysregulated mouse models (Krentz *et al.* 2009, Pesce & Scholer 2001). Control of pluripotency in male germ cells normally involves the establishment of DNA methylation within the functional promoters of *Nanog* and *Sox2* (Western *et al.* 2010). Therefore, it is particularly interesting to investigate the functional relationship between these physiological events.

In summary, this study reveals that male germ cell differentiation is arrested and FGF9/NODAL signalling occurs at abnormally high levels in *Emx2*-knockdown testes. Our study expands the known developmental roles of EMX2 by demonstrating its role in the pluripotency–differentiation transition and linking FGF9/NODAL pathway dysregulation with arrest of germ cell differentiation.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-16-0022>.

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.

Funding

This research was supported by the National Nature Science Foundation of China (81300484), Special Competitive Allocation Project of Science and Technology Special Financial Funding of Zhanjiang (no. 2013A1006) and The PhD Start-up Fund of Guangdong Medical College (B2012027).

Author contribution statement

YR-N conceived and designed the experiments. TZ-M, B-C and Y-Z performed the experiments. X-J, CL-P, YS-Z and MW-H analysed the data. TZ-M and YR-N wrote the paper.

References

- Baltus AE, Menke DB, Hu YC, Goodheart ML, Carpenter AE, de Rooij DG & Page DC 2006 In germ cells of mouse embryonic ovaries, the decision to enter meiosis precedes premeiotic DNA replication. *Nature Genetics* **38** 1430–1434. (doi:10.1038/ng1919)

- Barrios F, Filippini D, Pellegrini M, Paronetto MP, Di Siena S, Geremia R, Rossi P, De Felici M, Jannini EA & Dolci S 2010 Opposing effects of retinoic acid and FGF9 on Nanos2 expression and meiotic entry of mouse germ cells. *Journal of Cell Science* **123** 871–880. (doi:10.1242/jcs.057968)
- Bowles J, Knight D, Smith C, Wilhelm D, Richman J, Mamiya S, Yashiro K, Chawengsaksophak K, Wilson MJ, Rossant J *et al.* 2006 Retinoid signaling determines germ cell fate in mice. *Science* **312** 596–600. (doi:10.1126/science.1125691)
- Bowles J, Feng CW, Spiller C, Davidson TL, Jackson A & Koopman P 2010 FGF9 suppresses meiosis and promotes male germ cell fate in mice. *Developmental Cell* **19** 440–449. (doi:10.1016/j.devcel.2010.08.010)
- Chen SR, Chen M, Wang XN, Zhang J, Wen Q, Ji SY, Zheng QS, Gao F & Liu YX 2013 The Wilms tumor gene, Wt1, maintains testicular cord integrity by regulating the expression of Col4a1 and Col4a2. *Biology of Reproduction* **88** 56. (doi:10.1095/biolreprod.112.105379)
- Durcova-Hills G & Surani A 2008 Reprogramming primordial germ cells (PGC) to embryonic germ (EG) cells. *Current Protocols in Stem Cell Biology* Chapter 1 Unit1A.3. (doi:10.1002/9780470151808.sc01a03s5)
- Hayashi S & McMahon AP 2002 Efficient recombination in diverse tissues by a tamoxifen-inducible form of Cre: a tool for temporally regulated gene activation/inactivation in the mouse. *Developmental Biology* **244** 305–318. (doi:10.1006/dbio.2002.0597)
- Inman GJ, Nicolas FJ, Callahan JF, Harling JD, Gaster LM, Reith AD, Laping NJ & Hill CS 2002 SB-431542 is a potent and specific inhibitor of transforming growth factor-beta superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. *Molecular Pharmacology* **62** 65–74. (doi:10.1124/mol.62.1.65)
- Kaneda M, Okano M, Hata K, Sado T, Tsujimoto N, Li E & Sasaki H 2004 Essential role for de novo DNA methyltransferase Dnmt3a in paternal and maternal imprinting. *Nature* **429** 900–903. (doi:10.1038/nature02633)
- Kimura T, Murayama K, Nakamura T, Watanabe S, Umehara H, Tomooka M & Nakano T 2005 Testicular teratomas: back to pluripotent stem cells. *Progress in Molecular and Subcellular Biology* **40** 133–150. (doi:10.1007/3-540-27671-8)
- Kimura T, Tomooka M, Yamano N, Murayama K, Matoba S, Umehara H, Kanai Y & Nakano T 2008 AKT signaling promotes derivation of embryonic germ cells from primordial germ cells. *Development* **135** 869–879. (doi:10.1242/dev.013474)
- Koopman P, Gubbay J, Vivian N, Goodfellow P & Lovell-Badge R 1991 Male development of chromosomally female mice transgenic for Sry. *Nature* **351** 117–121. (doi:10.1038/351117a0)
- Koubova J, Menke DB, Zhou Q, Capel B, Griswold MD & Page DC 2006 Retinoic acid regulates sex-specific timing of meiotic initiation in mice. *PNAS* **103** 2474–2479. (doi:10.1073/pnas.0510813103)
- Krentz AD, Murphy MW, Kim S, Cook MS, Capel B, Zhu R, Matin A, Sarver AL, Parker KL, Griswold MD *et al.* 2009 The DM domain protein DMRT1 is a dose-sensitive regulator of fetal germ cell proliferation and pluripotency. *PNAS* **106** 22323–22328. (doi:10.1073/pnas.0905431106)
- Kusaka M, Katoh-Fukui Y, Ogawa H, Miyabayashi K, Baba T, Shima Y, Sugiyama N, Sugimoto Y, Okuno Y, Kodama R *et al.* 2010 Abnormal epithelial cell polarity and ectopic epidermal growth factor receptor (EGFR) expression induced in Emx2 KO embryonic gonads. *Endocrinology* **151** 5893–5904. (doi:10.1210/en.2010-0915)
- Labosky PA, Barlow DP & Hogan BL 1994 Mouse embryonic germ (EG) cell lines: transmission through the germline and differences in the methylation imprint of insulin-like growth factor 2 receptor (Igf2r) gene compared with embryonic stem (ES) cell lines. *Development* **120** 3197–3204.
- Livera G, Delbes G, Pairault C, Rouiller-Fabre V & Habert R 2006 Organotypic culture, a powerful model for studying rat and mouse fetal testis development. *Cell and Tissue Research* **324** 507–521. (doi:10.1007/s00441-006-0167-7)
- Looijenga LH, Gillis AJ, Stoop H, Biermann K & Oosterhuis JW 2011 Dissecting the molecular pathways of (testicular) germ cell tumour pathogenesis; from initiation to treatment-resistance. *International Journal of Andrology* **34** e234–e251. (doi:10.1111/j.1365-605.2011.01157.x)
- Matsui Y & Tokitake Y 2009 Primordial germ cells contain subpopulations that have greater ability to develop into pluripotent stem cells. *Development Growth and Differentiation* **51** 657–667. (doi:10.1111/j.1440-169X.2009.01125.x)
- Matsui Y, Zsebo K & Hogan BL 1992 Derivation of pluripotent embryonic stem cells from murine primordial germ cells in culture. *Cell* **70** 841–847. (doi:10.1016/0092-8674(92)90317-6)
- Miyamoto N, Yoshida M, Kuratani S, Matsuo I & Aizawa S 1997 Defects of urogenital development in mice lacking Emx2. *Development* **124** 1653–1664.
- Mohammadi M, McMahon G, Sun L, Tang C, Hirth P, Yeh BK, Hubbard SR & Schlessinger J 1997 Structures of the tyrosine kinase domain of fibroblast growth factor receptor in complex with inhibitors. *Science* **276** 955–960. (doi:10.1126/science.276.5314.955)
- Nettersheim D, Biermann K, Gillis AJ, Steger K, Looijenga LH & Schorle H 2011 NANOG promoter methylation and expression correlation during normal and malignant human germ cell development. *Epigenetics* **6** 114–122. (doi:10.4161/epi.6.1.13433)
- Pesce M & Scholer HR 2001 Oct-4: gatekeeper in the beginnings of mammalian development. *Stem Cells* **19** 271–278. (doi:10.1634/stemcells.19-4-271)
- Resnick JL, Bixler LS, Cheng L & Donovan PJ 1992 Long-term proliferation of mouse primordial germ cells in culture. *Nature* **359** 550–551. (doi:10.1038/359550a0)
- Sasaki H & Matsui Y 2008 Epigenetic events in mammalian germ-cell development: reprogramming and beyond. *Nature Reviews Genetics* **9** 129–140. (doi:10.1038/nrg2295)
- Souquet B, Tourpin S, Messiaen S, Moison D, Habert R & Livera G 2012 Nodal signaling regulates the entry into meiosis in fetal germ cells. *Endocrinology* **153** 2466–2473. (doi:10.1210/en.2011-2056)
- Spiller CM, Feng CW, Jackson A, Gillis AJ, Rolland AD, Looijenga LH, Koopman P & Bowles J 2012 Endogenous Nodal signaling regulates germ cell potency during mammalian testis development. *Development* **139** 4123–4132. (doi:10.1242/dev.083006)
- Tsuda M, Sasaoka Y, Kiso M, Abe K, Haraguchi S, Kobayashi S & Saga Y 2003 Conserved role of nanos proteins in germ cell development. *Science* **301** 1239–1241. (doi:10.1126/science.1085222)
- Western PS, Miles DC, van den Bergen JA, Burton M & Sinclair AH 2008 Dynamic regulation of mitotic arrest in fetal male germ cells. *Stem Cells* **26** 339–347. (doi:10.1634/stemcells.2007-0622)
- Western PS, van den Bergen JA, Miles DC & Sinclair AH 2010 Male fetal germ cell differentiation involves complex repression of the regulatory network controlling pluripotency. *FASEB Journal* **24** 3026–3035. (doi:10.1096/fj.09-151555)
- Wu Q, Kanata K, Saba R, Deng CX, Hamada H & Saga Y 2013 Nodal/activin signaling promotes male germ cell fate and suppresses female programming in somatic cells. *Development* **140** 291–300. (doi:10.1242/dev.087882)
- Young JC, Wakitani S & Loveland KL 2015 TGF-beta superfamily signaling in testis formation and early male germline development. *Seminars in Cell and Developmental Biology* **45** 94–103. (doi:10.1016/j.semcdb.2015.10.029)

Received 14 January 2016

First decision 1 February 2016

Revised manuscript received 8 March 2016

Accepted 21 March 2016