Developmental Biology 389 (2014) 173–181

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

CYP26B1 promotes male germ cell differentiation by suppressing STRA8-dependent meiotic and STRA8-independent mitotic pathways

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ARTICLE INFO

Article history:
Received 30 June 2013
Received in revised form
31 January 2014
Accepted 17 February 2014
Available online 24 February 2014

Keywords:
Retinoic acid
Germ cell
Cyp26b1
Stra8
Kit
Meiosis
Mitosis

ABSTRACT

Germ cell sex is defined by factors derived from somatic cells. CYP26B1 is known to be a male sex-promoting factor that inactivates retinoic acid (RA) in somatic cells. In CYP26B1-null XY gonads, germ cells are exposed to a higher level of RA than in normal XY gonads and this activates *Stra8* to induce meiosis while male-specific gene expression is suppressed. However, it is unknown whether meiotic entry by an elevated level of RA is responsible for the suppression of male-type gene expression. To address this question, we have generated *Cyp26b1/Stra8* double knockout (dKO) embryos. We successfully suppressed the induction of meiosis in CYP26B1-null XY germ cells by removing the *Stra8* gene. Concomitantly, we found that the male genetic program represented by the expression of NANOS2 and DNMT3L was totally rescued in about half of dKO germ cells, indicating that meiotic entry causes the suppression of male differentiation. However, half of the germ cells still failed to enter the appropriate male pathway in the dKO condition. Using microarray analyses together with immunohistochemistry, we found that KIT expression was accompanied by mitotic activation, but was canceled by inhibition of the RA signaling pathway. Taken together, we conclude that inhibition of RA is one of the essential factors to promote male germ cell differentiation, and that CYP26B1 suppresses two distinct genetic programs induced by RA: a *Stra8*-dependent meiotic pathway, and a *Stra8*-independent mitotic pathway.

Introduction

To differentiate into eggs or spermatozoa is a critical choice of pathways for germ cells. Primordial germ cells (PGCs) generated in the early mouse embryo have the ability to become both spermatozoa and eggs. After migrating to and colonizing the genital ridge, germ cells start sexual differentiation upon responding to signals supplied by the somatic cells surrounding them (Bowles et al., 2010; Ottolenghi et al., 2007).

Retinoic acid (RA) signaling is implicated in the sex determination of mouse germ cells. In the embryonic XX gonad, RA supplied by the mesonephros activates the RA-responsive gene *Stra8* in germ cells (Bowles et al., 2006; Koubova et al., 2006). This instructs them to progress to meiotic prophase I. The important function of the STRA8 protein in the meiotic initiation of XX germ cells is proven by analysis of the *Stra8* knockout (KO) mouse, in which germ cells fail to undergo premeiotic DNA replication (Baltus et al., 2006). On the other hand, *Stra8* expression in germ cells is greatly suppressed in the XY gonad even though RA is similarly supplied from the mesonephros, because cytochrome P450 26B1 (CYP26B1), an enzyme that inactivates RA, is expressed in Sertoli cells (Bowles et al., 2006; Maclean et al., 2007). In this condition, XY germ cells express *Nanos2*, which promotes the male-type genetic program (Suzuki and Saga, 2008; Tsuda et al., 2003). Downstream of the action of NANOS2, paternal genomic imprinting is executed by the function of DNMT3L during late embryogenesis (Bourc'his et al., 2001; Suzuki and Saga, 2008).

Cyp26b1 works downstream of SRY, which initiates masculinization in the embryonic gonads by upregulating male-specific genes such as *Sox9* and *Fgf9* in somatic cells (McClelland et al., 2012). In the absence of *Cyp26b1*, XY germ cells start to express *Stra8* probably in response to the elevated level of RA (Bowles et al., 2006; Maclean et al., 2007) and enter the meiotic pathway in a manner similar to XX germ cells in female gonads, indicating that such XY germ cells might have changed their sex to being female. Alternatively, it is possible that XY germ cells initiate

Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; Cyp26b1, cytochrome P450 b261; dKO, double knockout; E, embryonic day; ES, embryonic stem; Fgf9, fibroblast growth factor 9; IHC, immunohistochemistry; KO, knockout; RA, retinoic acid; RAR, retinoic acid receptor; PBS, phosphate-buffered saline; PI3K, phosphatidylinositide 3-kinase; PGC, primordial germ cell; SD, standard deviation

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http://dx.doi.org/10.1016/j.ydbio.2014.02.013
0012-1606

meiosis prematurely while retaining their maleness. This is because of the expression of fibroblast growth factor (FGF) 9, which is reported to have an antagonistic effect on the induction of meiosis, as its downstream *Sox9* expression is unaffected even in the CYP26B1-null condition (Bowles et al., 2010). Thus, it is proposed that FGF9 promotes male genetic program by making XY germ cells less responsive to RA, suggesting that RA signaling in CYP26B1-null gonads might compete with FGF9 signaling. However, there is no direct evidence indicating that the male genetic program is maintained in such gonads.

It has also been shown that the administration of RA to mouse embryonic day (E) E11.5–E13.5 XY gonads prevents germ cells from entering mitotic arrest, and this is accompanied by upregulation of *Stra8* leading to the induction of meiosis. In an organ culture system, RA effectively induced meiosis when it was added at E11.5 but not E13.5 (Bowles et al., 2006; Koubova et al., 2006; Trautmann et al., 2008). Moreover, the conditional KO of *Cyp26b1* in Sertoli cells after the entry of germ cells to mitotic arrest resulted in the promotion of XY germ cells to exit from the G0 phase, re-enter the mitotic cell cycle and initiate meiosis (Trautmann et al., 2008). These results indicate that a high level of RA can induce mitosis and also meiosis. However, it is unknown how RA signal promotes, mitosis or meiosis.

Here we investigated germ cell properties under a high RA condition by simultaneous disruption of *Stra8* and *Cyp26b1*. We addressed the following two questions: (1) whether the initiation of meiosis via *Stra8* expression is responsible for suppression of the male pathway; and (2) whether higher RA itself affects the intrinsic male-type gene program via a *Stra8*-independent pathway. From analyses of *Cyp26b1*/*Stra8* double knockout (dKO) mice, we clarified the dual roles of RA on male germ cell differentiation through *Stra8*-dependent meiotic and *Stra8*-independent mitotic pathways.

Materials and methods

Mice

The *Stra8*-targeted mouse line was established in our laboratory as described (Saba et al., 2014). The *Cyp26b1*-targeted mouse line (Yashiro et al., 2004) was provided by H. Hamada (Osaka University). *Nanos2*-targeted, *CAG-floxed-CAT-3xFlag-Nanos2* and *Pou5f1/Oct4-CreERT2* transgenic mouse lines are established in our laboratory (Tsuda et al., 2003; Suzuki and Saga, 2008; Wu et al., 2013). *Rosa-CreERT2* mice were purchased from Artemis Pharmaceuticals GmbH (Cologne, Germany). These mice are maintained in the genetic background of ICR or MCH (a closed ICR colony established at CLEA Japan Inc., Tokyo, Japan). All mouse experiments were carried out with the permission of the Animal Experimentation Committee at the National Institute of Genetics.

Transcript analysis by microarray

Total RNA was prepared from embryonic gonads from E12.5 to E14.5 using an RNeasy mini kit (Qiagen, Hilden, Germany). For a single hybridization assay, 200 ng of total RNA was labeled with Cy3 and hybridized to a Whole Mouse Genome Oligo Microarray (G4122F, Agilent Technologies, Santa Clara, CA, USA) in accordance with the manufacturer's protocol for the Low RNA Input Linear Amplification Kit and One color (Agilent Technologies) and Gene Expression Hybridization Kit (Agilent Technologies). Arrays were scanned using a Microarray Scanner System (G2565BA, Agilent Technologies) and the generated images were processed with Feature Extraction, version 9.1 (Agilent Technologies) software to generate signal values and evaluate the presence or absence of calls for each probe set. Two independent datasets were obtained for each collation. Processed data were analyzed with Genespring GX software, version 7.3.1 (Agilent Technologies). The following normalization steps were applied to each dataset: measurements of <5 set to 5 for data transformation, per chip normalization to the 50th percentile and per gene normalization to the median. Microarray data reported in this study were registered in the Gene Expression Omnibus (GEO) database (GSE38317; http://www.ncbi.nlm.nih.gov/geo/).

Transcript analysis by quantitative PCR

cDNAs were synthesized from the total RNA of E12.5–E14.5 mouse fetal gonads using Superscript III (Invitrogen, Carlsbad, CA, USA) and oligo dT_{18} primers; qPCR analysis was then performed using a KAPA SYBR FAST qPCR Kit (Kapa Biosystems Inc., Woburn, MA, USA) and Thermal Cycler Dice (TaKaRa Bio Inc., Shiga, Japan). The primer sets are listed in the Supplementary material, Table S1.

Organ culture of embryonic mouse gonads

Embryonic male gonads at E12.5 were dissected in ice-cold phosphate buffered saline (PBS) and cultured as described (Hiramatsu et al., 2003). Gonads were placed onto an ISOPORE membrane filter (pore size 5.0 μm; Millipore, Bedford, MA, USA), floated on Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% horse serum, penicillin/streptomycin with or without 2 μM RA (Sigma-Aldrich) or 5 μM AGN193109 (RAR antagonist, Santa Cruz Biotechnology, Dallas, TX, USA) under 5% CO_2 at 37 °C for 48–72 h.

Immunohistochemistry

Mouse embryonic XY gonads were dissected in ice-cold PBS, fixed with 4% paraformaldehyde in PBS at 4 °C for 2 h. 5-Bromo-2′-deoxyuridine (BrdU) was administered to pregnant mice at 100 μg/g body weight 2 h before dissection. Fixed embryos were embedded in OCT compound (Sakura Finetek Japan, Tokyo, Japan), frozen and sectioned at 8 μm using a cryostat. Frozen sections were incubated with the following primary and secondary antibodies: rat anti-TRA98 (a gift from Y. Nishimune, Osaka University); rabbit anti-DNMT3L (a gift from S. Yamanaka, Kyoto University), rabbit anti-SYCP3 (a gift from S. Chuma, Kyoto University); Armenian hamster anti-KIT (gift of T. Hirata, National Institute of Genetics); rabbit anti-H2AFX/γH2A.X (Abcam, Cambridge, MA, USA); rabbit anti-KI67 (Neomarkers, Thermo Scientific, Waltham, MA, USA); rabbit anti-NANOS2 (Suzuki et al., 2007); rabbit anti-PLZF (Santa Cruz Biotechnology, Dallas, TX, USA); mouse anti-BrdU (3D4, BD, Franklin Lakes, NJ, USA); mouse anti-FLAG (M2, Sigma-Aldrich); donkey anti-rat, rabbit and mouse IgG conjugated with Alexa Fluor 594 (Invitrogen); donkey anti-goat, rabbit and mouse IgG conjugated with Alexa Fluor 488 (Invitrogen); goat anti-rat IgG conjugated with Alexa Fluor 633 (Invitrogen). DNA counterstaining was done using Hoechst 33342 (Calbiochem, San Diego, CA, USA).

Result

Stra8 is responsible for the initiation of meiosis in CYP26B1-null XY gonads

As a factor other than RA might be responsible for the induction of *Stra8* in XY germ cells in CYP26B1-null embryos (Kumar et al., 2011), we first tested this possibility by treating such gonads with a retinoic acid receptor (RAR) antagonist