SOHLH1 and SOHLH2 coordinate spermatogonial differentiation

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ABSTRACT

Spermatogonial self-renewal and differentiation are essential for male fertility and reproduction. We discovered that germ cell specific genes Sohlh1 and Sohlh2, encode basic helix-loop-helix (bHLH) transcriptional regulators that are essential in spermatogonial differentiation. Sohlh1 and Sohlh2 individual mouse knockouts show remarkably similar phenotypes. Here we show that SOHLH1 and SOHLH2 proteins are co-expressed in the entire spermatogonial population except in the GFRA1− spermatogonia, which includes spermatogonial stem cells (SSCs). SOHLH1 and SOHLH2 are expressed in both KIT negative and KIT positive spermatogonia, and overlap Ngn3/EGFP and Sox3 expression. SOHLH1 and SOHLH2 heterodimerize with each other in vivo, as well as homodimerize. The Sohlh1/Sohlh2 double mutant phenocopies single mutants, i.e., spermatogonial continue to proliferate but do not differentiate properly. Further analysis revealed that GFRA1− population was increased, while meiosis commenced prematurely in both single and double knockouts. Sohlh1 and Sohlh2 double deficiency has a synergistic effect on gene expression patterns as compared to the single knockouts. SOHLH proteins affect spermatogonial development by directly regulating Gfra1, Sox3 and Kit gene expression. SOHLH1 and SOHLH2 suppress genes involved in SSC maintenance, and induce genes important for spermatogonial differentiation.

Article history:
Received for publication 23 July 2011
Revised 12 October 2011
Accepted 20 October 2011
Available online 26 October 2011
Keywords:
Spermatogonia
Sohlh1
Sohlh2
Differentiation
Stem cell

Introduction

Sperm are produced via spermatogenesis, a male-specific process that involves continual self-renewal and proliferation of spermatogonial stem cells followed by differentiation. Much interest has been devoted to understanding spermatogonial self-renewal and its “stemness”, yet much remains to be learned about how spermatogonia choose to differentiate, as well as the nature of spermatogonia-specific regulators that affect such critical transition.

In the adult mouse testes, spermatogonia, including spermatogonial stem cells (SSCs), are localized in the seminiferous tubules along the peripheral basement membrane, and the differentiating offspring of spermatogonia is arranged in a sequential order towards the center of the tubule. The cycle of the seminiferous epithelium is divided into 12 stages (stages I–XII) in mice (de Rooij, 1998, 2001; de Rooij and Russell, 2000). The spermatogonial stem cell function resides in the undifferentiated type A spermatogonia, which comprise the most primitive set of spermatogonia including A-single (A1: isolated spermatogonia), A-paired (A2: 2-chained cells), and A-aligned (A3: 4 to 16 or occasionally 32-chained cells). In addition to their self-renewal capabilities as a population of cells, undifferentiated spermatogonia generate A1, A2, A3, and A4 intermediate (In) and B differentiating spermatogonia. Differentiating spermatogonia enter meiosis around postnatal day 10 to form meiotic spermatocytes, which in turn give rise to haploid spermatids and spermatozoa.

Extensive work on molecular mechanisms that control spermatogenesis has elucidated marker proteins that distinguish spermatogonial cell types. Gial cell line derived neurotrophic factor (GDNF) is secreted by the Sertoli cells and acts via GDNF receptors on undifferentiated spermatogonia to exert paracrine regulation of spermatogonial self-renewal. A1 to A4 and a portion of A5,6 undifferentiated spermatogonia express GDNF family receptor alpha 1 (GFRα1), as well as a receptor tyrosine kinase (RET), which is part of the GDNF receptor complex (Buagaw et al., 2005; Jijiwa et al., 2008; Naughton et al., 2006; Tokuda et al., 2007). Ngn3/EGFP transgenic mice express the EGFP gene under the control of the Neurog3 promoter, and mark mainly undifferentiated and differentiating spermatogonia (Yoshida et al., 2004). Undifferentiated type-A spermatogonia show functional and molecular heterogeneity, and GFRα1 and Ngn3/EGFP mark these subsets. GFRα1 is expressed in most primitive undifferentiated spermatogonia, and Ngn3/EGFP is predominantly expressed in GFRα1-negative cells, which likely represent transit amplifying spermatogonia (Nakagawa et al., 2010; Suzuki et al., 2009; Tokuda et al., 2007; Zheng et al., 2009). The transcriptional repressor ZBTB16 (also known as PLZF) and a homophilic cell–cell adhesion molecule,
SOHLH1 and SOHLH2 are co-expressed in GFRA1-negative spermatogonia

Although Sohlh1 and Sohlh2 knockout phenotypes and expression patterns are similar to each other (Ballow et al., 2006a; Hao et al., 2008; Pangas et al., 2006; Toyoda et al., 2009), co-expression and genetic interaction between these two transcriptional regulators have not been studied in vivo. We performed whole mount immunofluorescence with adult seminiferous tubules using anti-SOHLH1 and anti-SOHLH2 antibodies. SOHLH1 and SOHLH2 were co-expressed in the vast majority of spermatogonia in the adult testis (Fig. 1). These results are not surprising given previous observations of the SOHLH1 and SOHLH2 expression patterns, and suggest functional interrelationship.

To classify SOHLH1 and SOHLH2-expressing spermatogonial cell types in adult testis, we performed multiple immunofluorescence analysis of whole mount seminiferous tubules with anti-SOHLH1, anti-SOHLH2 and markers known to be expressed in undifferentiated spermatogonia such as CDH1, ZBTB16, GFRA1, and Ngn3/EGFP, and differentiating spermatogonia as determined by KIT expression. In our hands, CDH1 (Fig. S1A, B) and ZBTB16 (Fig. 2A and D–G) were also detected in differentiating spermatogonia (Suzuki et al., 2009). We combined the triple immunofluorescence data with the stage of the seminiferous epithelial cycle to better define spermatogonial populations that express SOHLH proteins (Kotaja et al., 2004). The tubules were grouped into four stages; I–VI, V–VIII, IX–XI and X–III respectively (see Supplementary information). SOHLH1 and SOHLH2 expressing spermatogonia co-express CDH1 and ZBTB16 in undifferentiated spermatogonia (A, A<sub>nr</sub>, A<sub>nr-a</sub>, A<sub>nr-b</sub>) but a subset of CDH1 or ZBTB16 positive cells expressed neither SOHLH1 nor SOHLH2 (Figs. 1A, B and 2 yellow arrowheads). These findings suggest that SOHLH proteins are not expressed in the most undifferentiated spermatogonial population.

We also investigated the expression patterns of SOHLH1 and SOHLH2, relative to GFRA1 and Ngn3/EGFP in the adult testis. GFRA1 is expressed in the most primitive undifferentiated spermatogonia, and Ngn3/EGFP is predominantly expressed in GFRA1-negative cells and likely marks more differentiated spermatogonia. The heterogeneous expression of GFRA1 and Ngn3/EGFP in the undifferentiated type-A spermatogonial compartment underlies the complexity of the spermatogonial stem cells, as well as their functional hierarchy (Nakagawa et al., 2010; Suzuki et al., 2009; Zheng et al., 2009). SOHLH1 and SOHLH2 proteins were predominantly expressed in the GFRA1-negative population (Fig. 1C) (Suzuki et al., 2009), and only a small subset of spermatogonia co-expressed GFRA1 with SOHLH1 and SOHLH2 (Fig. 1C, white arrowhead). Staining for SOHLH1 and EGFP in tests of Ngn3/EGFP transgenic mice revealed that SOHLH1 was expressed by most Ngn3/EGFP-positive spermatogonia. However, a small portion of the SOHLH1-positive population was Ngn3/EGFP-negative, suggesting that SOHLH1 expression precedes Ngn3/EGFP (Fig. S1C and D). The transcription factor SOX3 (Sry-related HMG-box 3) is expressed in spermatogonia, and a germ cell specific Sox3 knockout male mouse showed similar defects in spermatogonial differentiation as Sohlh1/Sohlh2 knockouts (Laronda and Jameson, 2011; Raverot et al., 2005). Interestingly, the expression pattern of SOX3 in undifferentiated spermatogonia was similar to SOHLH1, and Sox3 was predominantly expressed in the GFRA1-negative population (Fig. 1D).

We also examined the expression of SOHLH1 and SOHLH2 in the differentiating spermatogonia. KIT is expressed in differentiating spermatogonial types A<sub>i</sub> to B, as well as leptotene spermatocytes. KIT expression is first visualized in spermatogonia at stages VI–VII; following that, undifferentiated spermatogonia transform to differentiating A<sub>i</sub> spermatogonia (de Rooij, 1998; Schrans-Stassen et al., 1999). SOHLH1 expression was observed in both KIT-negative
undifferentiated spermatogonia (Fig. 2, white arrowheads) and KIT-
positive differentiating spermatogonia (Fig. 2, magenta arrowheads).
SOHLH1 expression in KIT-positive differentiating spermatogonia
gradually disappeared during stages IV–VI when intermediate and
type-B spermatogonia were observed (Fig. 2B and C). Our observa-
tions indicate that SOHLH proteins disappear during differentiation
of intermediate to type-B spermatogonia, and are not expressed in
spermatocytes. SOHLH1 and SOHLH2 expression shows that
SOHLH1 and SOHLH2 are not expressed in the most primitive sper-
matogonia but do precede Ngn3/EGFP and KIT expression. The ex-
pression patterns of SOHLH1 and SOHLH2 relative to known
spermatogonial markers are summarized in Fig. S1E.

SOHLH1 and SOHLH2 can form homodimers and heterodimers

Basic helix-loop-helix transcription factors are known to dimerize
via their HLH domains. SOHLH1 and SOHLH2 have almost identical
patterns of expression and co-localize in the spermatogonial nuclei,
raising the possibility that SOHLH1 and SOHLH2 interact with each
other in vivo. We investigated whether SOHLH1 and SOHLH2 co-
immunoprecipitate in the 1-week-old testis, at a time when the vast
majority of spermatogonia co-express SOHLH1 and SOHLH2 (Fig. S1F).
We used anti-SOHLH1 and anti-SOHLH2 antibodies on wild type testes
to determine whether SOHLH2 co-immunoprecipitates with SOHLH1
and vice versa. These antibodies were developed against the highly di-
vergent SOHLH COOH regions (Ballow et al., 2006b; Pangas et al.,
2006) and do not cross react. Both anti-SOHLH1 and anti-SOHLH2 anti-
bodies immunoprecipitated SOHLH2 and SOHLH1 proteins respectively,
as shown by Western blots on immunoprecipitated products (Fig. 3A).
Our in vivo data suggests that SOHLH1 and SOHLH2 can heterodimerize
in spermatogonia.

We also examined SOHLH1 or SOHLH2 homodimer formation.
Homodimers may differ from heterodimers in binding affinity and spec-
ificity, and may affect expression of different genes. We co-expressed
MYC-tagged SOHLH1 and FLAG-tagged SOHLH2 in the 293T cell line
and immunoprecipitated SOHLH1 from extracts with anti-FLAG anti-
body, to determine if homodimers were present. We conducted identi-
cal studies with SOHLH2. Our results indicate that SOHLH1 and SOHLH2
can homodimerize (Fig. 3B, highlighted lanes). We repeated identical
experiments with anti-MYC antibody and the results were consistent.
with the anti-FLAG data (data not shown). SOHLH1 and SOHLH2 can therefore exist both as heterodimers and homodimers.

**SOHLH1 and SOHLH2 functions in spermatogonial differentiation and repression of precocious meiosis**

At 7 weeks of age, most of the seminiferous tubules in the Sohlh1−/−/Sohlh2−/− mice retained only spermatogonia and Sertoli cells (Fig. S3E–H). Whole mount immunostaining of Sohlh1−/−/Sohlh2−/− seminiferous tubules showed occasional large clusters of CDH1+/ZBTB16+/MVH+/Kit− cells, which is a typical protein expression pattern of undifferentiated spermatogonia (Fig. S3J–L). The morphology of these mutant spermatogonia was abnormal, with tightly packed cells, diminished cytoplasm, and lack of fine processes (filopodia) that emanate when wild type spermatogonia are stained with anti-CDH1 (compare Figs. 1A, B and S3J–O). Even in the region of the tubule where spermatogonial density was not as high, Aal-4 and longer chains did not show the normal filopodia-staining pattern seen with CDH1 (Fig. S3J–O). Confocal microscopy revealed that intercellular bridges in a subset of single and double mutant spermatogonia were misshapen and wider than in the wild type (Fig. S3M–O). The wild type spermatogonia in experimentally induced cryptorchid testis did not show abnormal intercellular bridges and filopodia, indicating that these are not caused by the diminished space in the tubules or disrupted spermatogenesis (data not shown). Our observations show that undifferentiated spermatogonia were retained in the tubules. SOHLH1 and SOHLH2 functions in spermatogonial differentiation and repression of precocious meiosis.
spermatogonia in single and double Sohlh1/Sohlh2 knockouts are morphologically abnormal, but can proliferate.

We observed that a portion of cells showed thread-like chromosome condensations, characteristic of the leptotene spermatocytes (Fig. S3E–H). We assessed whether Sohlh1 and/or Sohlh2 congenital deficiency affected expression of Hormad1, a germ cell specific component of the synaptonemal complex that is essential for early meiosis (Shin et al., 2010; Wojtasz et al., 2009). Whole mount immunostaining revealed that a portion of germ cells (MVH+/CDH1+) in adult mutants (either single or double knockout) expressed Hormad1. Hormad1 expressing cells were always located near the CDH1+ germ cell cluster (Figs. S3L and S4A), suggesting that Hormad1+ cells emerged from such clusters. No spermatids were observed in mutant testes, and these meiotic-like germ cells were eliminated by apoptosis (Fig. S3H). We also observed Hormad1 positive cells in 1-week-old testis, with thread-like chromosome condensations in a few seminiferous tubules of either single or double Sohlh1/Sohlh2 mutants. Hormad1 positive cells were not observed in the 1-week-old wild type testis (Fig. 4M–P). These observations suggest that spermatogonia in Sohlh1/Sohlh2 mutants undergo precocious meiosis.

Transcriptional regulator Dmrt1 represses spermatogonial commitment to meiosis, and Dmrt1 induces Sohlh1 expression and suppresses Stra8 expression in spermatagonia (Matson et al., 2010). It is therefore plausible that SOHLHs deficiency leads to precocious meiosis. Transcription factor Stra8 is one of several factors required for the initiation of meiosis (Anderson et al., 2008; Baltus et al., 2006). In adult testis of either single or double Sohlh1/Sohlh2 mutants, a portion of spermatogonias expressed Stra8, and the frequency of STRA8+ tubules was considerably increased (Fig. S4D), although Dmrt1 expression was still present in CDH1+ spermatogonia (Fig. S4E–F). Our data suggest that Sohlh1 and/or Sohlh2 deficiency causes a subset of cells to precociously commit to meiosis.

SOHLH1 and SOHLH2 double deficiency has a synergistic effect on gene expression

We performed microarray analysis on 1-week-old testes to determine molecular perturbations that precede a frank pathology in double knockout versus single knockout mouse testes. SOHLH1 and SOHLH2 are co-expressed in the same spermatogonial population at this stage (Fig. S1F). Moreover, Sohlh1 expression persists in the Sohlh2 knockout and vice versa, and we hypothesized that Sohlh1−/−/Sohlh2−/− deficiency will have a synergistic effect on molecular pathology. We analyzed the mRNA expression pattern of wild type, Sohlh1−/−, Sohlh2−/− and Sohlh1−/−/Sohlh2−/− testes at 1 week, and focused on genes which showed a statistically significant fold change equal to or greater than 2, as compared with the wild type. In Sohlh1−/− and Sohlh2−/−, nearly identical genetic pathways were altered, as very few genes were differentially expressed between the two (Figs. 5A and S5). In Sohlh1−/−/Sohlh2−/−, however, the expression of 421 genes was disrupted; that is, nearly 4 times as many genes were affected in the double mutant as compared to the single mutants: 114 genes in Sohlh1−/− and 109 genes in Sohlh2−/− (Fig. 5B, Tables S3–S5). Among all of the genes with altered expression, there were 71 that were commonly altered between the three mutants: 47 genes were down-regulated and 24 genes were up-regulated (Fig. 5B and Table S6). When we compared double and single knockout gene lists, 123 genes were specifically altered in Sohlh1−/−/Sohlh2−/− double mutant (≧2-fold change as compared to single mutants). Of the 123 genes that were only altered in Sohlh1−/−/Sohlh2−/− mutants, only 3 of the genes were down-regulated, while 120 genes were up-regulated (Table S7).

We confirmed the microarray data for a select group of genes using quantitative real-time PCR from 1-week-old testes. Genes known to be involved in spermatogonial differentiation such as Kit and Sox3 were significantly down-regulated in single and double

Fig. 3. SOHLH1 and SOHLH2 can form homodimers and heterodimers. (A) Co-immunoprecipitation analyses with guinea pig anti-SOHLH1 (α-S1) and guinea pig anti-SOHLH2 (α-S2) antibodies using 1-week-old testes extracts from wild type, Sohlh1−/− and Sohlh2−/− mice. Each antibody used for western blot analysis (W.B.) is indicated. SOHLH2 was co-immunoprecipitated with SOHLH1 and vice versa (I.P.), indicating that SOHLH1 and SOHLH2 heterodimerize. Input: pre-immunoprecipitation testes lysate (1%), IgG: normal rabbit IgG or normal guinea pig IgG. (B) Co-immunoprecipitation analyses with anti-FLAG antibody using 293T cells over-expressing MYC- or FLAG-tagged SOHLH1 or SOHLH2 proteins. Each combination of transfected vectors and each antibody used for western blot analysis are indicated. The lanes highlighted by yellow indicate that SOHLH1 and SOHLH2 can form homodimers. 3xMYC-SOHLH1 co-immunoprecipitated with 3xFLAG-SOHLH1, and 3xMYC-SOHLH2 co-immunoprecipitated with 3xFLAG-SOHLH2, indicating that both SOHLH1 and SOHLH2 are capable of forming homodimers. Input: pre-immunoprecipitation cell lysate (1%), Mock: pcDNA-3.1(−)–3FLAG.
mutants (Fig. 5C). Interestingly, Sohlh2 expression in Sohlh1−/− testis was up-regulated, whereas Sohlh1 expression in Sohlh2−/− was down-regulated (Fig. 5C). Therefore, Sohlh1 may repress Sohlh2 expression, while Sohlh2 may act to stimulate Sohlh1 expression, via direct or indirect feedback mechanisms.

A number of genes involved in spermatogonial stem cell maintenance such as Ret, Gfra1, Nanos2 and Pou5f1 also showed altered expression levels in single and double mutants. Ret and Gfra1 encode GDNF receptors and are mainly expressed in SOHLH1 and/or SOHLH2-negative undifferentiated spermatogonia. Both were up-regulated in single and double mutants, while their ligand, Gdnf, was not significantly altered in any of the mutants (Fig. 5D). Nanos2 is a translational regulator expressed in GFRA1− spermato- gonias including stem cells (Sada et al., 2009), and Nanos2 was up-regulated in both single and double mutants. Pou5f1 is a homeobox-containing transcription factor that plays critical roles in pluripotency and self-renewal of SSCs. Pou5f1 protein expression is lower in portions of male germline stem cells that express high levels of SOHLH1 in a culture system (Dann et al., 2008). Pou5f1 transcript expression is significantly up-regulated in single and double knockouts (Fig. 5D). One interpretation is that gradual expression of SOHLH1 and SOHLH2 during spermatogonial differentiation suppresses expression of genes that are involved in SSC maintenance/self-renewal, such as Ret, Gfra1, Nanos2 and Pou5f1, and promotes spermatogonial differentiation.

We also examined the effects of single and double Sohlh deficiencies on the expression of RNA binding proteins that are critically involved at various stages of spermatogenesis. Several RNA binding proteins are highly expressed in germ cells and localize to nuage, an electron-dense structure in the cytoplasm including ribonucleoprotein (RNP) complexes such as P-bodies, stress granules and chromato- toid bodies. NANOS2, which is essential for SSC maintenance in the mouse testis, localizes to P-bodies (Sada et al., 2009; Suzuki et al., 2009). NANOS3, which is essential for the development of primordial germ cells (PGCs), is similarly localized in P-bodies and stress granules, although its function in the testis is unknown (Kedersha et al., 1999; Tsuda et al., 2003; Yamaji et al., 2010). NANOS2 is expressed mainly in GFRA1−/Ngn3EGFP− spermatogonia, while NANOS3 is predominantly expressed in GFRA1−/Ngn3EGFP+ spermatogonia, just like SOHLHs (Suzuki et al., 2009). In Sohlh1/Sohlh2 single and double mutants, both Nanos2 and Nanos3 expression was remarkably up-regulated (Fig. 5D and E). Tia1 is another RNA binding protein localized to the stress granules, and Tia1 expression was significantly up-regulated in the double knockout as compared to the single knockouts (Fig. 5E). LIN28A is also localized to P-bodies and stress granules, and specifically expressed in undifferentiated spermatogonia in the mouse testis. Expression pattern of LIN28A is similar to that of ZBTB16 (Balzer and Moss, 2007; Zheng et al., 2009). The functional role of LIN28A in the testes is currently unknown. Decapping enzyme, DCP2, co-localizes to P-bodies with LIN28A (Liu et al.,...
Both Lin28a and Dcp2 are significantly down-regulated in Sohlh single and double mutants (Fig. 5E). Other genes involved in RNA metabolism also show altered expression patterns in Sohlh mutants (Tables S3 and S4). These results suggest that SOHLH1 and SOHLH2 directly or indirectly affect critical RNA processing pathways during spermatogonial development.

Genes that are important for epigenetic modification were also altered in single and double mutants. PIWI-like proteins interact with short RNA molecules, piRNAs, and regulate various aspects of spermatogenesis (Chuma et al., 2009; Deng and Lin, 2002); Piwil1/Miwi is important for spermiogenesis and Piwil4/Miwi2 is essential for retrotransposon silencing as well as meiosis progression (Carmell et al., 2007; Deng and Lin, 2002; Kuramochi-Miyagawa et al., 2004). Piwil1 and Piwil4 were significantly up-regulated in 1-week-old single and double Sohlh mutants (Fig. 5F). On the other hand, DNMT3B, which is essential for DNA methylation, was significantly down-regulated (Fig. 5F and Table S3) (Gowher et al., 2005; Okano et al., 1999). RNA expression arrays also show premature expression of mRNA corresponding to Dmrtc2/Dmrt7, Spo11 and Rec8 at 1 week, which are essential for meiosis (Fig. 5F). Dmrtc2 appears to play a role in the sex chromatin transformation that occurs between diplo-nema and pachynema, while Spo11 and Rec8 are involved in DNA...
double strand break formation and homologous recombination during meiosis (Kawamata and Nishimori, 2006; Kawamata et al., 2007; Romanienko and Camerini-Otero, 2000; Xu et al., 2005). The aberrant expression pattern of these genes may lead to abnormal meiosis and apoptosis in the mutants.

Currently there are very few proteins identified as markers for undifferentiated spermatogonial spermatogenesis. The array expression analysis on Sohlh1/Sohlh2 mutants revealed that numerous genes were affected, including testis specific genes with unknown functions during spermatogenesis such as Nmt2, Ly6k, 4732415M23Rik, Dmrtb1/Dmrt6, Gml, Dzip3, Tbx22, and Gas5 (Fig. S6). Further studies will be necessary to examine their expression pattern and functions during spermatogonial differentiation.

SOHLH1 and SOHLH2 bind chromatin upstream of genes essential for SSC maintenance and spermatogonial differentiation.

We conducted chromatin immunoprecipitation (ChIP) with anti-SOHLH1 and anti-SOHLH2 antibodies to determine whether SOHLH proteins directly regulate genes critical for SSC maintenance and spermatogonial differentiation, such as Gfra1, Sox3, Kit, Sohlh1 and Sohlh2. We used 1-week-old testes to examine whether Sohlh1 or Sohlh2 bind conserved E-boxes (CANNTG) in a promoter region of each gene. E-boxes are known as DNA elements, which are bound by transcription factors associated with certain development processes such as stem cell maintenance, cell fate determination, and cell proliferation.

These results were consistent with the qRT-PCR data showing higher expression in mutant spermatogonia is partially deregulated. In the adult Sohlh mutant testes, the proportion of Gfra1$^+$ cells in ZBTB16$^+$ spermatogonia were higher in mutants as compared to the wild type (Fig. 7A). These results were consistent with the qRT-PCR data showing higher levels of Gfra1 in mutant testes (Fig. 5D), and suggest that Gfra1 expression in mutant spermatogonial spermatogenesis is partially deregulated. In the adult Sohlh mutant testes, the proportion of Gfra1$^+$ chains greater than A$_{1.8}$ was increased when compared to the average in wild

Fig. 6. SOHLH1 and SOHLH2 bind chromatin upstream of genes essential for SSC maintenance and spermatogonial differentiation. Chromatin immunoprecipitation (ChIP) assay. Anti-SOHLH1 (αS1) and anti-SOHLH2 (αS2) antibodies precipitate genomic DNA containing conserved E-boxes surrounding Gfra1 (A), Sohlh1 (B), Sohlh2 (C) Sox3 (D) and Kit (E) genes. Kit position −397/388 relative to the transcriptional initiation site is an E-box that was not immunoprecipitated significantly with anti-SOHLH1 antibodies (E), and Kit position −988 is an E-box that was not immunoprecipitated with both anti-SOHLH1 and anti-SOHLH2 antibodies. One-week-old testes from the wild type (WT), Sohlh1$^{+/−}$ (S1$^{+−}$) and Sohlh2$^{+/−}$ (S2$^{+−}$) mice were used for ChIP. “Input” is the PCR product from chromatin pellets before immunoprecipitation (1% of the total volume used for IP). Numbers at the left of each figure indicate the E-box location from the transcription start site.

SOHLH1 and SOHLH2 are important for differentiation from the most primitive set of undifferentiated spermatogonial spermatogenesis.H. Suzuki et al. / Developmental Biology 361 (2012) 301–312
type (Fig. 7B). In adult wild type testes, the profile of GFRA1-positive cells depended on the stage of the epithelial cycle (Fig. 7C). In Sohlh single and double mutants, the stage of the seminiferous epithelial cycle could not be determined since spermatogenesis was disrupted. The average morphological profile of GFRA1+ cells in either single or double mutants (\(A_1\): 39.3–43.7\%, \(A_{2+}\): 30.4–35.6\%) and 3 or more...
in the chain: 25.0–26.0% was similar to the profile of stages X–III in wild type (A.; 39.7%, Ap.; 34.2% and 3 or more in the chain: 26.1%) (Fig. 7C). Interestingly, we often observed the GFRA1 + cells to be part of chains of more than 8 cells in the mutant (Fig. 7E), which was very rare in wild type even during stages X–III (Fig. 7C and D). The longer GFRA1 + chains observed in Sohlh mutants are not solely due to the block in spermatogenesis since we did not observe such long chains in the experimentally induced cryptorchidism in wild type testes (Fig. 7F–G).

We did observe large clusters of cells showing typical protein expression patterns of undifferentiated spermatogonia, CDH1+/ZBTB16+/MHV+/Kit+, that were GFRA1− (Figs. 7E (yellow arrowhead) and S3J–L). SOHLH protein deficiency is therefore not sufficient to de-repress GFRA1 expression, and additional factors must regulate GFRA1 expression.

Discussion

Spermatogenesis is a dynamic process that transforms diploid spermatogonial stem cells into highly differentiated haploid spermatozoa. Several transcriptional regulators preferentially expressed in spermatogenesis are known to affect various steps of spermatogonial development. Different stages of spermatogenesis require different transcriptional regulators. Pou5f1, Taf4b and Zbtb16/Plflb are important in spermatogonial proliferation and self-renewal. Few spermatogonia-specific transcriptional regulators are known to be critical in spermatogonia differentiation. Sox3, a member of the HMG group of transcriptional regulators, is important in brain development as well as spermatogonial differentiation. Others and we recently discovered that Sohlh1 and Sohlh2 are tissue-specific bHLH transcriptional regulators that play critical roles during spermatogonial differentiation (Ballow et al., 2006a; Hao et al., 2008; Toyoda et al., 2009). Basic helix-loop-helix transcriptional regulators are essential in the differentiation of many cell types. Often, multiple tissue-specific bHLH factors are necessary for tissue differentiation, and double deficiencies have shown synergistic effects and more profound phenotypic changes as compared to the single knockouts (Kruger et al., 2006; Zhou and Anderson, 2002). Our current study indicates that Sohlh1 and Sohlh2 are co-expressed in undifferentiated and differentiating spermatogonia, and interact as heterodimers and homodimers. This is different from previous studies suggesting that Sohlh2 is expressed earlier than Sohlh1 (Ballow et al., 2006a; Toyoda et al., 2009). In adult testis, the vast majority of SOHLH1 and SOHLH2 are not expressed in GFRA1-positive spermatogonia, which are the most primitive set of spermatogonia and include spermatogonial stem cells. Our result is consistent with a previous report showing that SOHLH2 was not expressed in a subset of CDH1 + spermatogonia (Toyoda et al., 2009). In addition, we showed that SOHLH1 and SOHLH2 directly regulate the transcription of Gfra1, Sox3, Sohlh1, Sohlh2 and Kit genes that are essential for spermatogonial development and differentiation. We have also noticed that SOHLH1 and SOHLH2 deletion induces expression of meiosis related genes such as Dmrt1/Dmrt7, Piwil1/Plzf and HORMAD1. Moreover, the frequency of tubules containing STRA8 + spermatogonia without Kit expression increased in mutants and a subset of remaining germ cells showed HORMAD1 + thread-like chromosome condensation approximating leptotene to zygotene stage of meiosis. STRA8 associates with the onset of meiosis and HORMAD1 is a critical component of the synaptonemal complex (Anderson et al., 2008; Baltus et al., 2006; Shin et al., 2010; Wojtasz et al., 2009). Interestingly, Dmrt1, one of the doublesex-related transcription factors, overlaps SOHLH1 expression (Fig. S4), and Dmrt1 deficiency partially affects spermatogonial differentiation as well as induces uncontrolled meiosis in Dmrt1 mutant germ cells (Matson et al., 2010). Although Dmrt1 has been implicated in differentiation, its phenotype is leakier than the Sohlh1 knockout phenotype, with differentiating spermatogonia as well as spermatocytes and spermatids still observed in Dmrt1 mutants (Matson et al., 2010). This may or may not be due to the conditional nature of the deletion. Nevertheless, Sohlh1 and Dmrt1 results suggest that disruption in differentiation leads to aberrant entry along the meiotic pathway. Moreover, our findings are consistent with the interpretation that proper differentiation is necessary for complete meiosis, as we did not observe pachytene stage spermatocytes.

Redundancy of SOHLH1 and SOHLH2

Although Sohlh2 was identified as a homologue of Sohlh1, their limited homology is confined to the bHLH domains (~50%). Because of the limited homology and detectable SOHLH1/SOHLH2 heterodimers, we hypothesized that these two proteins play independent roles, and that double deficiency will produce synergistic effects. The onset and extent of pathology in mice lacking both Sohlh1 and Sohlh2 is identical to single knockouts. These results suggest that Sohlh1 and Sohlh2 share a common pathway that is enabled at a specific point during spermatogonial development. Gene expression array analyses on Sohlh1 and Sohlh2 mutants show little differences and also support the notion that Sohlh1 and Sohlh2 regulate common pathways. The molecular explanation for the lack of a more severe pathology in double mutant mice could be due to the essential roles of the SOHLH1/SOHLH2 heterodimers in spermatogonial differentiation. At the gene expression level, however, we did notice synergism in the double mutants. Almost four-fold more genes were affected in the double mutant as compared to the single knockouts, yet the phenotype is practically the same. These results suggest that either SOHLH1 or SOHLH2 homodimers, or hetero-complexes with other proteins, regulate genes involved in later steps of spermatogonial development rather than earlier steps of development, which are regulated by SOHLH1/SOHLH2 heterodimers.

Sohlh1 and Sohlh2 deficiency and long GFRA1 + chains

Maintenance of spermatogenesis requires active proliferation of spermatogonia and their stem cells. Several transcription factors have been implicated in spermatogonial proliferation and include Zbtb16 and Taf4b, but single and double Sohlh1/Sohlh2 mutants spermatogonia can proliferate (Fig. 4I–L) (Ballow et al., 2006a; Hao et al., 2008). Moreover, many long GFRA1 + chains were observed in single and double Sohlh1/Sohlh2 mutant spermatogonia whereas those chains are rarely visible in wild type testes. Interestingly, long GFRA1 + chains are also frequently observed in busulfan-treated testes undergoing regeneration (Nakagawa et al., 2010). Such chains may accumulate as a result of perturbed balance between differentiated and undifferentiated spermatogonia and a generalized stress response. Sohlh1 and/or Sohlh2 deficient spermatogonia lack characteristic filopodia, and a subset of mutant spermatogonia has misshapen intercellular bridges. These morphologic changes in Sohlh mutants were not observed in the wild type experimentally induced cryptorchid testes, and indicate important roles for SOHLH proteins at the onset of spermatogonial differentiation and the transition from GFRA1 + to GFRA1 − spermatogonia.

Upstream regulators of Sohlh1 and Sohlh2

Recent data indicates that transcriptional regulator Dmrt1 directly regulates Sohlh1 and Stra8 expression in spermatogonia (Matson et al., 2010). Dmrt1 mutant spermatogonia precociously exit the spermatogenic program and Sohlh1 expression was extinguished in Dmrt1 mutants. Dmrt1 appears to regulate Sohlh1 gene expression directly, as chromatin immunoprecipitation studies show Dmrt1 binding to the Sohlh1 promoter (Matson et al., 2010). The bound region shares similarity to the Dmrt1 known DNA binding consensus site. Sohlh1 expression during spermatogenesis overlaps Dmrt1 expression, and these data together suggest that Dmrt1 directly regulates Sohlh1. Our immunofluorescence data indicates that Dmrt1 is
expressed in the double Sohlh1/Sohlh2 knockout, and is therefore likely to be upstream of Sohlh1. However, the role of DMRT1 in regulating Sohlh2 is unknown. Moreover, DMRT1 is unlikely to be the only regulator of Sohlh1, as DMRT1 conditional knockouts retain KIT expression and complete spermatogenesis. Sohlh mutants do not express KIT in adult spermatogonia, and meiotic-like cells eventually die. It is therefore likely that a sufficient quantity of SOHLH1 and SOHLH2 proteins remain in the Dmrt1 mutant. Further studies are necessary to determine the actions of Dmrt1 on Sohlh2, as well as to identify other direct regulators of Sohlh1 and Sohlh2.

It is likely that both germline and somatic factors are involved in the regulation of Sohlh1 and Sohlh2 genes in testis. Sohlh2 was discovered during the search for Bmp4 inducible genes in embryonic stem cells (Hao et al., 2008). BMP4 is expressed in Sertoli cells during the first week of life, and also in spermatogonia and early spermatocytes in adult mouse testes (Baleato et al., 2005; Pellegrini et al., 2003). Bmp4 homozygous deletion causes embryonic lethality (Winner et al., 1995), and heterozygous males on a C57BL/6 background show compromised fertility due to degeneration of germ cells (Hu et al., 2004). SMAD proteins, transcriptional regulators that translocate to the nucleus upon ligand stimulation, relay BMP signals. SMAD1, SMAD4 and SMAD5 are expressed in spermatogonia in mouse testes, and are likely involved in BMP4-mediated induction of KIT expression in spermatogonial cultures from postnatal day 5 testes (Itman and Loveland, 2008; Pellegrini et al., 2003). In Sohlh1/Sohlh2 single and double mutants, Bmp4 transcripts were not significantly altered compared to the wild type. USP9X/FAM, a deubiquitinizing enzyme which promotes SMAD4 translocation to the nucleus in human cell lines (Dupont et al., 2009), was up-regulated in single and double Sohlh mutants, suggesting that BMP signaling is activated (Fig. S7). Future studies are necessary to determine if SMAD4 is involved in the regulation of Sohlh1 and Sohlh2 genes. We have summarized our current thinking on the regulators of Sohlh1/Sohlh2 and downstream effectors of Sohlh1/Sohlh2 in Fig. 8.

SOHLH1 and SOHLH2 are key regulators of spermatogonial differentiation, and two of the few known germ cell-specific transcriptional regulators. Our study implicates SOHL1 and SOHL2 in the regulation of genes critical for spermatogonial development, such as Gfra1, Sox3, Sohlh1, Sohlh2 and Kit. Moreover, our study clearly shows the stage-specific nature of spermatogonial differentiation. Future studies will be essential to determine signaling pathways and regulators that activate SOHLH1 and SOHLH2 expression and downstream pathways.

Supplementary materials related to this article can be found online at doi:10.1016/j.ydbio.2011.10.027.

Acknowledgment

Anti-KIT (H2C7) antibody and anti-SOX3 antibody were kindly provided by Dr. Tasumi Hirata and Dr. Klymkowsky, respectively. Ngn3/GFP mice were generated and generously provided by Dr. Shosei Yohida. We thank Julia Pascarella in Dr. Kyle Orwig’s laboratory for technical support. We would also like to thank Megan McGuire and Metz Allison for general assistance. This study was supported by R01HD056351 and March of Dimes grant #6-FY08-313 (to A.R.) and Uehara Memorial Foundation Research Fellowship, which supports H. Suzuki.

References


Fig. 8. SOHLH1 and SOHLH2 regulation of spermatogonial development. In this figure, DMRT1 is shown as a direct regulator of SOHLH1. Current data do not support direct regulation of SOHLH2 by DMRT1 (Matson et al., 2010). DMRT1 is unlikely to be the sole regulator of SOHLH1 as spermatogonial differentiation proceeds in the DMRT1 conditional mutants (Matson et al., 2010). SOHLH1 and SOHLH2 bind the promoters of key spermatogonial molecules Gfra1, Sox3, Sohlh1, Sohlh2 and Kit. Solid lines indicate presumed direct transcriptional control, and dashed lines with question marks indicate undefined regulatory interactions.