Expression and Function of the Testis-Predominant Protein LYAR in Mice

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Mammalian spermatogenesis is a complex process involving an intrinsic genetic program of germ cell-specific and -predominant genes. In the present study, we analyzed the Ly-1 reactive clone (Lyar) gene in the mouse. Lyar, which is known to be expressed abundantly in the testis, encodes a nucleolar protein that contains a LYAR-type C2HC zinc finger motif and three nuclear localization signals. We herein confirmed that Lyar is expressed predominantly in the testis, and further showed that this expression is specific to germ cells. Protein analyses with an anti-LYAR antibody demonstrated that the LYAR protein is present in spermatocytes and spermatids, but not in sperm. To assess the functional role of LYAR in vivo, we used a gene-trap mutagenesis approach to establish a LYAR-null mouse model. Male mutant mice were born live and developed normally. Male mutant mice lacking LYAR were fully fertile and showed intact spermatogenesis. Taken together, our results demonstrate that LYAR is strongly preferred in male germ cells, but has a dispensable role in spermatogenesis and fertility.

INTRODUCTION

Spermatogenesis (i.e., male germ cell development) involves the continuous mitotic proliferation of spermatogonial stem cells, the meiotic division of spermatocytes, and dramatic morphological changes from haploid spermatids to highly specialized sperm (spermiogenesis). The tightly regulated nature of mitotic progression, meiotic progression and spermiogenesis suggests the presence of a highly organized network of genes specifically expressed in germ cells during spermatogenesis. The regulation of gene expression during spermatogenesis occurs at three levels: intrinsic, interactive, and extrinsic (Eddy, 2002). The intrinsic program determines which genes are utilized and when the genes are expressed in germ cells. The interactive process between germ cells and somatic cells is necessary for germ cell proliferation and progression. The extrinsic program regulates the interactive process via external influences. Notably, the intrinsic program involves germ cell- and stage-specific gene expression patterns that constitute the unique features of male reproduction.

The nucleolus, which is a noticeable compartment of the nucleus, plays a crucial role in ribosome biogenesis, which includes the processing of precursor rRNA, the transcription of ribosomal DNA, and pre-ribosome assembly (Ginisty et al., 1999; Lo et al., 2006; Melese and Xue, 1995; Shaw and Jordan, 1995). The rate of rRNA synthesis correlates with cell proliferation and varies depending upon the proliferative status of the cell. Nucleolar proteins have been implicated in the control of cell proliferation and growth (Chen et al., 1991; Grisendi et al., 2006). The rate of ribosomal RNA synthesis is dramatically altered during spermatogenesis and the generation of various types of germ cells. A previous autoradiographic study found that spermatogonia show very active rRNA synthesis, which peaks at the midpachytene stage in spermatocytes and then decreases in late spermatids, coinciding with changes in chromatin structure (Kierszenbaum and Tres, 1978). Therefore, the regulation of nucleolar functions in the testis could be important for the proper production of male germ cells.

During the course of our studies into unknown or unexplored genes with testis-specific or -predominant expression, we investigated the Ly-1 antibody reactive clone (Lyar) gene in mice. Lyar encodes a nucleolar protein that consists of 388 amino acid residues and has a LYAR-type C2HC zinc finger motif along with three nuclear localization signals. Lyar was first identified from a mouse T-cell leukemia line, and was shown to be induced during oncogenic transformation, suggesting that it may function as a novel cell growth-regulating nucleolar oncoprotein (Su et al., 1993). A microarray study showed that Lyar is highly upregulated in undifferentiated human embryonic stem cells (ESCs), and its expression is dramatically downregulated upon differentiation (Cai et al., 2006). In addition, Lyar was found to be overexpressed in human medulloblastoma (MB), the most frequent childhood brain tumor (Swartling et al., 2010). Recently LYAR was known to regulate the self-renewal and differentiation of ESCs (Li et al., 2009).

Previous studies have indicated that Lyar is abundantly expressed in the testis (Su et al., 1993). Considering the known functions of LYAR in various cell types, this suggests that it...
might be involved in male germ cell proliferation and differentiation. However, the expression patterns and functions of Lyar in germ cells are unknown. In the present study, we report our investigation of Lyar in mice, providing comprehensive information on its expression in male germ cells and its function in reproduction. In particular, we generated mice carrying a gene-trap mutation in Lyar and found that the loss of LYAR in the testis did not affect spermatogenesis or fertility.

MATERIALS AND METHODS

Reverse transcription polymerase chain reaction

The tissue-specific expression of the Lyar gene was examined by reverse transcription-polymerase chain reaction (RT-PCR) analysis of cDNAs from nine different mouse tissues (testis, ovary, brain, heart, kidney, liver, lung, spleen and ES cells), as well as germ cell-lacking testes from W/Wv (c-kit) mutant mice. Total RNA was extracted using the TRIzol reagent (Molecular Research Center, USA) according to the manufacturer’s protocol, and cDNA was synthesized using Omniscript reverse transcriptase (Qiagen, Netherlands). A specific region of the Lyar transcript was amplified with the primers [5′-GGT GGA TCC CCG GAG GCA AAG GCT AT-3′ (forward) and 5′-CCG GAA TTC TCA GGC CTC GGT TTG CTC TT-3′ (reverse)]. Glyceraldehyde-3-phosphate dehydrogenase (Gapdh), which was used as a control, was amplified using the primers [5′-TGA AGG TCG GAG TCA ACG GAT TTG GT-3′ (forward) and 5′-CAT GTG GGC CAT GAG GTC CAC CAC-3′ (reverse)]. Stage-specific expression during spermatogenesis was examined using total RNA obtained from the testes of prepubertal and adult male mice (ages: 8, 10, 12, 14, 16, 20, 30, and 84 days).

Antibodies and immunoblot analysis

The hydrophilic region of LYAR (amino acids 190-286) was PCR amplified, digested, and ligated into the pGEX-5X-2 vector (GE Healthcare, UK). Then, anti-LYAR antibody was generated by immunizing rabbits with the recombinant protein and purified by protein A affinity chromatography. The hydrophilic region of LYAR (amino acids 190-286) was PCR amplified, digested, and ligated into the pGEX-5X-2 vector (GE Healthcare, UK). Then, anti-LYAR antibody was generated from a mouse testis cDNA using the appropriate primers (5′-TTC GAA TTC GCA AGA GTT CAA-3′ and 5′-GTT GGA TCC CCT TCA GAA GCT TTA C-3′) and inserted into the pEGFP-N2 vector (BD Clontech, USA). HEK293T cells were transiently transfected with the Lyar-EGFP construct using the Lipofectamine LTX reagent (Invitrogen, USA) according to the manufacturer’s instructions. At 24 h after transfection, cells were fixed with formaldehyde, stained with Hoechst 33342 (Sigma-Aldrich), and analyzed under a fluorescent microscope.

Generation of mutant mice

An RRG292 embryonic stem (ES) cell line carrying a Lyar gene-trap allele was purchased from BayGenomics. The ES cells (which were derived from 129-strain mice) were injected into C57BL/6 blastocysts using standard procedures, and chimeric mice were generated. Chimeric males were mated with C57BL/6 females and germ-line transmission in pups was confirmed. The Lyarflox line was obtained by crossing heterozygotes. Mice were genotyped to discriminate between wild-type and mutant alleles using the following primers: 5′-CAC AAG ATC TGA CGC-3′ (forward) and 5′-GTC ATG CTC AAC TGG-3′ (reverse) for the wild-type allele; and 5′-GGTCCG CTG ACA-3′ (forward) and 5′-AGT ATC GGC CTC AGG AAG ATC G-3′ (reverse) for the gene-trap allele.

Phenotypic analyses of mutant mice

To test the fertility of Lyar-mutant males, Lyar-mutant and wild-type (WT) males (8-wk-old) were mated with C57BL/6 females and the fertility rate was calculated as described in (Lee et al., 2011). To determine sperm counts, sperm samples were collected from the cauda epididymis and vas deferens of 8-wk-old Lyar-mutant and WT males, and sperm cells were determined using a hemocytometer under a light microscope. To analyze testicular integrity, testes from WT and Lyar-mutant mice were fixed by immersion in Bouin’s fixative for 24 h, embedded in paraffin, and sectioned using standard protocols. After being deparaffinized, the sections were stained with hematoxylin and eosin and the testicular morphologies were observed under a light microscope.

Statistics

Results are presented as means ± standard errors of the mean (SEMs) or standard deviations (SDs). The statistical significance of between-mean differences was determined using a two-tailed Student’s t-test.

RESULTS

LYAR expression

We first analyzed the expression profiles of LYAR at the transcriptional and protein levels. The tissue distribution of Lyar transcripts was examined by RT-PCR using cDNAs from different adult mouse tissues. We found that Lyar was expressed strongly in the testis and very weakly in the other tissues (Fig. 1A). Since Lyar is known to be expressed in undifferentiated ES cells (Li et al., 2009), we also compared Lyar expression between the testis and undifferentiated ES cells. Consistent with the prior report, undifferentiated ES cells were found to express Lyar; however, the expression level was greatly lower than that of the testis. To investigate which cells in the testis transcribed Lyar, we performed RT-PCR with cDNA from the testes of W/Wv (c-kit) mutant mice, which lack germ cells. We found that Lyar transcripts were absent from the testes of the mutant mice (Fig. 1B), suggesting that the Lyar mRNA is expressed in germ cells. RT-PCR was additionally performed to