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Prolyl isomerase Pin1 is required sperm production by promoting mitosis progression of spermatogonial stem cells

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ABSTRACT

A prolyl isomerase Pin1 deficient ($Pin1^{-/-}$) male mice had severe testicular atrophy. We investigated the function of Pin1 in spermatogenesis by analyzing the $Pin1^{-/-}$ mice at reproductive age. $Pin1^{-/-}$ mice had lessαPLZF positive spermatogonia (undifferentiated spermatogonia) than wild type (WT). Nevertheless, the $Pin1^{-/-}$ testis contained approximately the same number of GFRα1 positive spermatogonia (SSCs in steady state) as the WT testis. Furthermore, degeneration of the spermatogenia appeared in seminiferous tubules of 10 months old $Pin1^{-/-}$ mouse testis, and abnormal shape GFRα1 positive spermatogonia were observed. In $Pin1^{-/-}$ spermatogonia, the ratio of the phospho-histone H3 positive cells (mitotic cells) in GFRα1-positive spermatogonia was higher than that of WT. These results suggest that Pin1 promotes the progression of the mitotic cell cycle of SSC in steady-state, which is required for the sperm production from SSCs.

1. Introduction

A peptidyl prolyl *cis*/*trans* isomerase Pin1 specifically binds the phosphorylated Ser/Thr-Pro motifs and catalyzes the *cis*/*trans* isomerization of the peptide bond. Pin1, a eukaryotic PPIase, and its yeast homologue, Ess1, have been reported to be required for the progression of mitosis in HeLa cells and yeast respectively [1]. Nonetheless, depletion of Pin1 was not fatal in mammals [2]. Currently, analysis of $Pin1^{-/-}$ mice has revealed Pin1 is involved in various diseases [3]. One of the most prominent phenotypes of $Pin1^{-/-}$ mice is infertility. $Pin1^{-/-}$ mice with the original 129/Sv and the C57BL/6j produced by backcross became infertile due to fewer male and female germ cells [2,4,5]. The severity of infertility was slightly different, depending on the level of other prolyl isomerases [6–9]. In this report, in order to clarify the role of Pin1 in spermatogenesis, $Pin1^{-/-}$ testes at reproductive age were examined in detail.

Spermatozoa are produced from spermatogenic stem cells (SSCs) in adult mammalian testis. Undifferentiated spermatogonia, which are found as singly isolated cells (A_s) or syncytia consisting mainly of 2 (A_{pr}), 4 ($A_{al\text{-}4}$), 8 ($A_{al\text{-}8}$), or 16 ($A_{al\text{-}16}$) cells localize on basement membrane of seminiferous tubules [10,11]. Within undifferentiated spermatogonial compartment, all or subset of GFRα1 (glial cell derived neurotrophic factor family receptor α1)-expressing undifferentiated spermatogonia, mainly comprise of A_s and A_{pr}, are thought to act as SSCs, because GFRα1 is a receptor for GDNF (glial cell derived neurotrophic factor), which is essential factor for SSCs maintenance *in vivo* and *in vitro* [12–16].

$Pin1^{-/-}$ testis of old mouse contained several abnormal seminiferous tubules. Since Pin1 is a regulatory molecule of cell cycle G1/S and G2/M progression [2,17–20], we speculated that the incomplete cell cycle progression of SSC causes testicular atrophy into $Pin1^{-/-}$ mice. $Pin1^{-/-}$ mice showed the similar phenotypes as shown in cyclin D1 depleted mice, and their expression levels are correlated with each other [21,22]. Testicular atrophy of $Pin1^{-/-}$ mice was not found in the cyclin D1 depleted mice, but it was seen in cyclin D2 depleted mice [23] or cyclin D dependent kinase inhibitor treated mice [24]. These results also support the hypothesis that Pin1 affects sperm production by controlling cell cycle

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Abbreviation

PGC	Primordial germ cells
SSC	spermatogonial stem cell
GDNF	glial-cell derived neurotrophic factor
GFRα1	GDNF family receptor α1
PLZF	promyelocytic leukaemia zinc finger protein
WT	wild type
Pin1$^{-/-}$	Pin1-deficient

progression of SSCs.

In mammalian testis, spermatozoa are produced from SSCs, but the molecular regulation of the stem cell population is largely unclear [10,14]. In this paper, we showed that Pin1 is involved in proliferation of undifferentiated spermatogonia and suggested that Pin1 is an important molecule that promotes mitotic cell cycle progression in undifferentiated spermatogonia.

2. Materials and methods

2.1. Animals

Pin1$^{-/-}$ mice were generated and bred according to our previous report [2]. Our study was approved by Tohoku University animal use and care committee and all investigations were conducted according to the principles of the Declaration of Helsinki. Genotypes of the mice bred by mating Pin1$^{+/-}$ mice were examined by PCR. Primers: WILD1.2A (5′-AAG GGA TTA GAA GCA AGA TTC G-3′), 2L (5′-AGC ACC CGA TCC TGT TCT GCA A-3′) and Start2 (5′-CAG AGG CCA CTT GTG TA-3′) [2].

2.2. Whole-mount immunostaining of seminiferous tubules

Immunostaining of whole-mount seminiferous tubes with anti-GFRα1 antibody (1:800 dilution; R&D Systems) and staining of nuclei with Hoechst 33342 (1:5000, Millipore) were performed as previously papers [16,26]. Analysis was performed with a fluorescent microscope (BX63; Olympus Co).

2.3. Immunohistochemistry

Testes were fixed with 4% paraformaldehyde in a phosphate-buffered solution (Wako) for overnight at 4 °C and embed the tissues in paraffin. Five-micrometer-thick testis sections were cut from paraffin blocks and placed on MAS-coated slides (Matsunami). After the slides were deparaffinized, antigen retrieval was performed by microwave methods (10 min microwaves 700 W, followed by cooling for 40 min at room temperature), or using Histo VT One (Nacalai Tesque), and washed for 10 min at 3 times by TBS. Inactivation of endogenous peroxidase was performed by treating sections with 0.3% (v/v) H_2O_2 in methanol for 20 min. Anti-αPLZF (promyelocytic leukaemia zinc finger protein) antibody (1:200, Santa Cruz) [25,26] or anti-GFRα1 antibody (1:800, Neuromics) [16,27] diluted in the blocking solution were reacted with the sections for overnight at 4 °C. After wash, sections were incubated with the biotinylated anti-mouse or anti-goat secondary antibody for 1 h at room temperature, and incubated with ABC solution (VECTOR Laboratories) for 30 min at room temperature and stained with 3,3′-Diaminobenzidine solution.

For the immunofluorescence analysis, the secondary antibody employed was either Alexa Fluor 594-labeled or Alexa Fluor 488-labeled (1:400). After staining cell nucleus with Hoechst 33342 (1:5000, Millipore), the sections were analyzed with a fluorescence microscope. Spermatogonia were judged as belonging to a syncytium when, based on a continuous GFRα1 staining using a 60× water immersion objective lens, the cell-cell connection was visually detected.

2.4. Analysis

The circumference length of seminiferous tubes cross section and the positive cell unit of seminiferous tubules cross section were measured simultaneously with BTFHRm (Mio Tools Inc.). The total circumference length of seminiferous tubes in the total number of positive cells around 30 seminiferous tubes per a testis section was calculated.

3. Results

3.1. Comparison of Pin1$^{-/-}$ and WT testes

Testis atrophy of Pin1$^{-/-}$ mice was observed at 38 weeks of age (Fig. 1A). The testes weight of WT mice and Pin1$^{-/-}$ mice increased postnatally, and there was no significant testicular weight difference between WT mice and Pin1$^{-/-}$ mice up to about 16 weeks. Thereafter, the testicular weight of WT did not change, but the

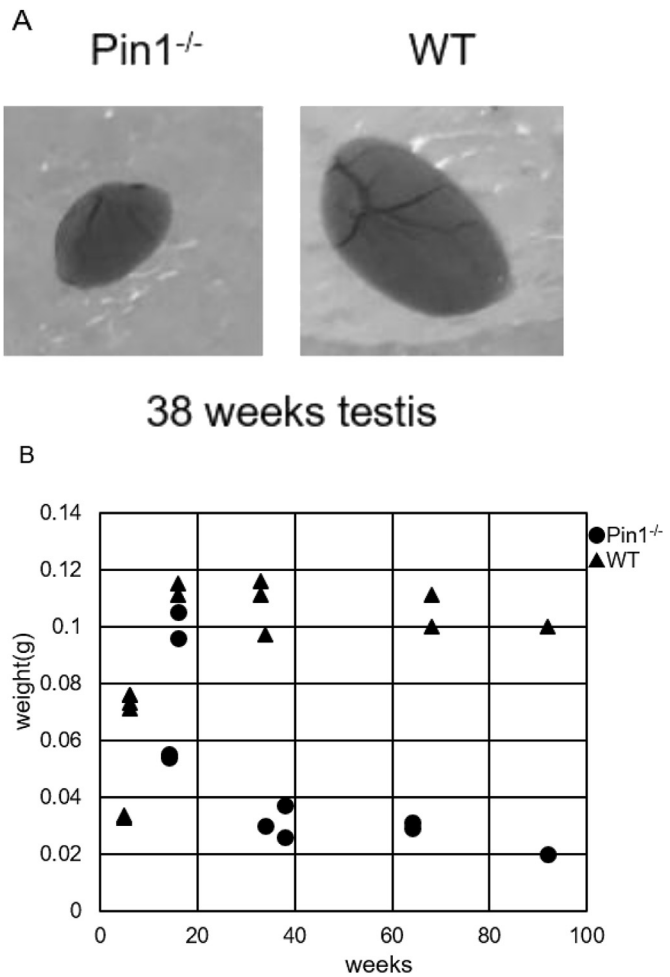

Fig. 1. Testicular atrophy of Pin1$^{-/-}$ mice.
(A) Testes of 38 weeks old Pin1$^{-/-}$ and WT mice. (B) Weights of WT (triangle) at 5, 6, 16, 33, 34, 68 and 92 weeks and Pin1$^{-/-}$ testes (circle) at 14, 16, 34, 38, 64 and 92 weeks.

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