Pelota mediates gonocyte maturation and maintenance of spermatogonial stem cells in mouse testes

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Abstract

Pelota (Pelo) is an evolutionarily conserved gene, and its deficiency in Drosophila affects both male and female fertility. In mice, genetic ablation of Pelo leads to embryonic lethality at the early implantation stage as a result of the impaired development of extra-embryonic endoderm (ExEn). To define the consequences of Pelo deletion on male germ cells, we temporally induced deletion of the gene at both embryonic and postnatal stages. Deletion of Pelo in adult mice resulted in a complete loss of whole-germ cell lineages after 45 days of deletion. The absence of newly emerging spermatogenic cycles in mutants confirmed that spermatogonial stem cells (SSCs) were unable to maintain spermatogenesis in the absence of PELO protein. However, germ cells beyond the undifferentiated SSC stage were capable of completing spermatogenesis and producing spermatozoa, even in the absence of PELO. Following the deletion of Pelo during embryonic development, we found that although PELO is dispensable for maintaining gonocytes, it is necessary for the transition of gonocytes to SSCs. Immunohistological and protein analyses revealed the attenuation of FOXO1 transcriptional activity, which induces the expression of many SSC self-renewal genes. The decreased transcriptional activity of FOXO1 in mutant testes was due to enhanced activity of the PI3K/AKT signaling pathway, which led to phosphorylation and cytoplasmic sequestration of FOXO1. These results suggest that PELO negatively regulates the PI3K/AKT pathway and that the enhanced activity of PI3K/AKT and subsequent FOXO1 inhibition are responsible for the impaired development of SSCs in mutant testes.


Introduction

After sexual differentiation, primordial germ cells (PGCs) in male fetal gonads become gonocytes that proliferate until embryonic day 15.5 (E15.5) and remain mitotically quiescent until shortly after birth (Culty 2009). The gonocytes are the precursors of spermatogonial stem cells (SSCs) and give rise to the first wave of spermatogenesis, whereas subsequent waves are derived from SSCs (Kluin & de Rooij 1981, Yoshida et al. 2006). Maintenance of male germ cell production throughout the life time of an individual is the result of tightly controlled balance between SSC self-renewal and differentiation (Oatley et al. 2006). Self-renewal of SSCs requires complex crosstalk between extrinsic signals from Sertoli cells and cellular intrinsic regulators of SSCs. One of the key growth factors secreted by Sertoli cells is glial cell line-derived neurotrophic factor (GDNF), which exerts its biological effects by binding to its receptor components GFRα1 and c-Ret and activating the PI3K/AKT signaling pathway in SSCs (Meng et al. 2000, Sariola & Saarma 2003, Naughton et al. 2006). The PI3K/AKT signaling pathway regulates the activity of many transcription factors, and, in turn, their targeted proteins control SSC self-renewal (Lee et al. 2007). The transcriptional activity of the FOXO family of fork-head transcription factors is negatively regulated via the PI3K/AKT signaling pathway. Phosphorylation of FOXO proteins by AKT leads to its cytoplasmic translocation and inactivation (Brunet et al. 1999, Kops et al. 1999). Therefore, the balanced activation of FOXO proteins via PI3K/AKT critically regulates SSC self-renewal and differentiation. Analyses of conditional FOXO1-knockout mice revealed that FOXO1 is not only critical for SSC self-renewal, but also for the transition of gonocytes to SSCs. During this transition, which occurs in the first week of postnatal development, the AKT-dependent phosphorylation of FOXO1 prevents its nuclear translocation, resulting in the inhibition of its transcriptional activity (Goertz et al. 2011).
Pelota (Pelo) is an evolutionarily conserved gene that has been identified in diverse species from archaeabacteria to humans (Ragan et al. 1996, Shamsadin et al. 2000, 2002). The Pelo protein contains an RNA-binding domain similar to that found in eukaryotic release factor 1 (eRF1), which is involved in the terminal step of protein synthesis (Davis & Engelbrecht 1998). The biological role of Pelo was originally identified in Drosophila melanogaster, in which Pelo-null mutants exhibited spermatogenic arrest at the G2/M boundary of the first meiotic division (Éberhart & Wasserman 1995). Subsequently, the impaired fertility of mutant females was not found to be a defect during meiotic division but rather the result of a disrupted balance between the self-renewal and differentiation of germ line stem cells (GSCs). This study revealed a critical role for Pelo in controlling GSC self-renewal, which is mediated by repressing the differentiation pathway in GSCs (Xi et al. 2005).

The function of Pelo at the molecular level has been extensively investigated in yeast, in which the Pelo ortholog Dom34 and its interacting partner Hsb1 were found to participate in an RNA quality control mechanism called No-Go decay (NGD). NGD recognizes mRNAs on which the ribosome is stalled at a stable stem-loop, rare codon, or pseudoknot, triggering the endonucleolytic cleavage of these mRNAs (Doma & Parker 2006, Graille et al. 2008, Chen et al. 2010). Further experiments revealed that Dom34 recycles stalled ribosomes from mRNAs lacking stop codons, truncated mRNAs, or the noncoding regions of many cellular mRNAs (Shoemaker & Green 2011, Tsuboi et al. 2012, Shao et al. 2013, Guydosh & Green 2014). Failure to recycle stalled ribosomes would ultimately lead to a critical default of the translational machinery (Guydosh & Green 2014). Despite the significant role of Dom34 in RNA quality control and translational machinery, it is dispensable for yeast survival (Carr-Schmid et al. 2002). In contrast, depletion of Pelo leads to embryonic lethality at an early post-implantation stage in mice (Adham et al. 2003). To determine the role of Pelo during embryonic and postnatal life, we generated a conditional knockout mouse model (Nyamsuren et al. 2014). Characterization of Pelo-null embryonic stem cells (ESCs) revealed that a Pelo deficiency did not markedly affect the self-renewal of ESCs, but impaired their differentiation into extra-embryonic endoderm (ExEn). Furthermore, Pelo depletion led to failure in the reprogramming of somatic cells toward induced pluripotent stem cells due to inactive BMP signaling and an impaired mesenchymal-to-epithelial transition (Nyamsuren et al. 2014).

To date, the consequences of Pelo deficiency on the development of male germ cells in mice have not been investigated. In this study, we found that Pelo is required for SSC self-renewal and maintenance, but is dispensable for the development of later stages of spermatogenesis and sperm function. In addition, we showed that the transition of gonocytes to SSCs is impaired in the absence of Pelo. At the molecular level, we showed that the exhaustion of SSCs and impaired conversion of gonocytes to SSCs are the result of elevated PI3K/AKT activity and decreased transcriptional activity of FOXO1 that induces the expression of genes that maintain the balance between SSC self-renewal and differentiation.

Materials and methods

Mouse strains, treatments, and sample collection

The generation of conditional Pelo-knockout (PeloF/FCreERT2) mice was described previously (Nyamsuren et al. 2014). To conditionally delete Pelo in PeloF/FCreERT2 mice, 8-week-old male mice were injected intraperitoneally (i.p.) with 1 mg tamoxifen (Tam) for 5 consecutive days. To investigate the progression of spermatogenesis after Tam application, males were killed at different time points after Tam-treatment. Immediately following this step, testes were weighed; one testis was fixed in Bouin’s solution for histological analyses, and the other was frozen for molecular studies. To inactivate Pelo at neonatal stages, pregnant PeloF/FCreERT2 mice were treated i.p. with Tam at E17.5 as before. After birth, the offspring were genotyped, and testes were collected at postnatal days 1 (P1), P7, and P14. Testes from Tam-treated PeloF/F mice were prepared at the same time points as controls to assess any unspecific effects of Tam. To detect Cre-mediated Pelo recombination, which deletes floxed exons 2 and 3, a piece of frozen testis was genotyped by PCR as described previously (Nyamsuren et al. 2014). All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Göttingen.

Fertility test

To investigate the fertility of Tam-treated PeloF/FCreERT2 and PeloF/F males, the animals were each intercrossed with three WT CD1 females after 15, 45, and 60 days of Tam treatment. Females were checked daily for the presence of vaginal plugs (VP), and VP-positive females were placed in separate cages to give birth. Offspring of each male were genotyped to determine the CreERT2-mediated deletion of the floxed Pelo alleles and the fertility of mutant males.

Tissue processing and immunohistochemistry

Bouin’s fixed testes were embedded in paraffin and cut into 5-μm sections for immunohistochemistry. The sections were deparaffinized in xylene, rehydrated through a graded ethanol series, and either stained with hematoxylin and eosin (H&E) or subjected to immunohistochemistry. For immunohistochemistry, sections were boiled in 10 mM sodium citrate antigen retrieval buffer for 6 min and cooled on ice for 10 min. To eliminate endogenous peroxidase activity, sections were immersed in 3% hydrogen peroxide in methanol for 15 min at room temperature (RT). The following procedures were performed using the R.T.U. Vectastain Universal Quick Kit (Vector Laboratories, Burlingame, CA, USA) according to the
manufacturer’s instructions. Briefly, after washing with PBS containing 0.1% Tween 20 (PBST), sections were blocked and incubated overnight with anti-SALL4 (#ab29112, Abcam, Cambridge, UK), anti-FOXO1 (#2880, Cell Signaling Technology, Leiden, The Netherlands), anti-HSPA4 (#sc-6240, Santa Cruz Biotechnology), or anti-GCNA1 antibodies at 1:100 dilutions. After washing with PBST, sections were incubated with secondary antibody for 10 min at RT, followed by incubation with streptavidin conjugated to HRP for 5 min. The sections were then incubated in peroxidase substrate solution until the development of desired stain intensity and counterstained with hematoxylin, rinsed, mounted with Roti Mount Aqua (Carl Roth, Karlsruhe, Germany) and imaged using an Olympus BX60 microscope (Olympus, Karlsruhe, Germany).

**Western blotting**

Total cellular proteins were extracted by homogenizing testes in RIPA lysis buffer (Millipore, Schwalbach, Germany) supplemented with a proteinase inhibitor cocktail (Roche Diagnostics) and phosphatase inhibitor (SERVA, Heidelberg, Germany) and incubated on ice for 1 h. The homogenates were sonicated and then centrifuged at 13,000 g for 20 min at 4 °C. The concentration of proteins was estimated using a Bio-Rad protein assay kit (Bio-Rad Laboratories). To determine the activity of the PI3K/AKT signaling pathway, testes were isolated from control and mutant P7 or adult mice and de-capsulated, and seminiferous tubules (STs) were incubated at 37 °C in StemPro-34FSM Medium (Life Technologies) supplemented with 1% FCS in the presence or absence of 100 ng/ml GDNF (Life Technologies) and/or 30 μM LY294002 inhibitor (Sigma–Aldrich). After 1 h of treatment, STs were collected by centrifugation, washed, and subjected to protein extraction. The protein samples were separated by 4–12% SDS–PAGE and transferred onto nitrocellulose membranes (Amersham Biosciences). The membranes were then blocked for 1 h with 5% nonfat milk in PBST. The blots were probed at 4 °C overnight with antibodies against pAKT, AKT, FOXO1, PARP (Cell Signaling Technologies) at 1:2000, or PELO (Burnicka-Turek et al., 2010) at 1:100 dilutions. After washing with PBST, sections were incubated with secondary antibody for 10 min at RT, followed by incubation with streptavidin conjugated to HRP for 5 min. The sections were then incubated in peroxidase substrate solution until the development of desired stain intensity and counterstained with hematoxylin, rinsed, mounted with Roti Mount Aqua (Carl Roth, Karlsruhe, Germany) and imaged using an Olympus BX60 microscope (Olympus, Karlsruhe, Germany).

**Preparation of nuclear protein fractions**

Nuclear and cytoplasmic protein extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific) according to the manufacturer’s instructions. Briefly, testes were collected, washed, and homogenized in ice-cold CER I buffer. After incubation on ice for 10 min, ice-cold CER II was added to the testis suspension, mixed, and incubated for 1 min on ice. The cytoplasmic fractions were collected after centrifugation at 16,000 g for 5 min, and the nuclear pellets were re-suspended in ice-cold NER, and incubated for 40 min with vortexing for 15 s every 10 min. The nuclear extracts were collected after centrifugation (16,000 g for 10 min at 4 °C). The prepared nuclear fractions were then processed for western blotting as described previously.

**Quantitative real-time PCR**

Total RNA was extracted using the TRIzol reagent following the manufacturer’s instructions (Life Technologies, Darmstadt, Germany). Five micrograms of total RNA were used for cDNA synthesis using the SuperScript II System (Life Technologies). To avoid the genomic DNA contamination, total RNAs were treated with RNA-free DNaseI (Sigma–Aldrich) for 15 min at 37 °C. Quantitative real-time PCR (qRT-PCR) was performed using the QuantFast SYBR Green PCR Master Mix following the manufacturer’s instructions (Qiagen). The reactions were performed in triplicate and run in an ABI 7900HT Real-Time PCR System (Applied Biosystems). Hypoxanthine-guanine-phosphoribosyl transferase (Hprt) or succinate dehydrogenase (Sdhα) expression levels were used for normalization and for analysis of relative changes in gene expression. The primers for PCR and qRT-PCR are as follows: Pelo, 5′-CGGA CAATGAAGTGCTCCTTG-3′ (forward) and 5′-GCTGCGCT TTGTG TCAGAAAAG-3′ (reverse); Egr4, 5′-GAGCGCCCTTC TCAAG-3′ (forward) and 5′-CTCAAACCCAGCTCAAGAA-3′ (reverse); Ret, 5′-GGCTGAAGCTGATTITGCT-3′ (forward) and 5′-CATAGAGCAGAGGTGTGCCA-3′ (reverse); Lhx1, 5′-AATTGAAATGCAACCTGACC-3′ (forward) and 5′-AAC CAGATCGTGGAGAGA-3′ (reverse); Sall4, 5′-AGGACAT CACTGGGAGAGG-3′ (forward) and 5′-GACTAAAGAATCT GGCACAGC-3′ (reverse); Hprt, 5′-AGCCCCAAAAATGTTAA GGTTGC-3′ (forward) and 5′-TTTGAATCACTTCGTC AT-3′ (reverse); Sdhα, 5′-GCTTGGCGAGCTGCATTGGG-3′ (forward) and 5′-CATCTCCAGTTGTCTCTTCA-3′ (reverse).

**Statistical analyses**

Paired comparisons of the number of marker-positive cells per tubule in control and mutant testes were performed using Student’s t-tests. A P value <0.05 was considered to be statistically significant. All statistical analyses were performed using the Statistica 9 software package (StatSoft, Inc., Tulsa, OK, USA). Data are shown as mean ± s.d.

**Results**

**Deletion of Pelo disrupts fertility in adult male mice**

We studied the consequence of Pelo deficiency on male fertility by the temporal deletion of the gene in Pelo<sup>F<sup>F</sup></sup>CreERT2 mice, which have floxed Pelo alleles and a knock-in allele containing Tam-induced Cre recombinase driven by the ubiquitously expressed Rosa26 promoter (Nyamsuren et al., 2014). Excision of floxed exons 2 and 3 of Pelo was induced by i.p. administration of Tam for 5 consecutive days. Time points from the last Tam injection were expressed as days.
post-injection (DPI). Control animals were Tam-treated Pelo<sup>Δ/Δ</sup> mice lacking the CreERT2 allele, which are phenotypically equivalent to animals with the WT allele. To assay the CreERT2-mediated deletion of the floxed Pelo allele in the testes of Tam-treated Pelo<sup>Δ/Δ</sup>CreERT2 males (thereafter referred to as Pelo mutant or Pelo<sup>Δ/Δ</sup>), PCR genotyping, western blotting, and qRT-PCR analyses were performed on testicular DNA, RNA, and proteins, respectively, isolated from mice at 5 DPI. The Cre-mediated deletion of the floxed allele generated a null allele as verified by PCR (Fig. 1A). Protein and qRT-PCR analyses revealed that the expression of PELO was diminished to <85% in testes of mutants compared with that of control Pelo<sup>Δ/Δ</sup> mice (Fig. 1C and D).

Upon Pelo deletion, mutant mice at 45 DPI showed a significant decrease in testicular size when compared with control mice (Fig. 1E and F). We then defined the progression of male infertility by breeding mutant and control mice with WT females after 15, 45, and 60 DPI. After 15 DPI, mutant males were fertile and produced litter of normal size (14.3 ± 2.1 pups, n=5 vs control 13.8 ± 2.1 pups, n=5). PCR analysis revealed that the mutant allele (Pelo<sup>Δ</sup>) is transmitted to ~93% of offspring (Fig. 1B), suggesting that Pelo deletion did not disrupt the progression of meiotic and post-meiotic cells to functional sperm. In contrast, all mutant males were infertile after 45 and 60 DPI.

**Pelo is required for SSCs maintenance but is dispensable for later spermatogenic stages**

To determine the cause of male infertility, histological analyses and expression patterns of different germ cell markers were studied in testicular sections derived from mutant and control animals at 5, 15, 25, and 45 DPI. Verification of H&E-stained tubular sections at 5 DPI showed no marked differences in the diameters of STs or in the presence of different types of germ cells between control and mutant testes (Fig. 2A, B, C, and D). However, by 15 and 25 DPI, most STs were devoid of pre-meiotic cells and retained only post-meiotic germ cells that were closely located at the peripheral layers of the STs (Fig. 2E, F, G, and H). By 45 DPI, 87% of STs were devoid of most germ cells in the mutant testes (Fig. 2I and J). The absence of new waves of meiotic germ cells in ~88% of mutant STs after 15 days of Pelo deletion indicates that the early stages of undifferentiated spermatogonia are affected by PELO deficiency.

To confirm the disruption of early stages of spermatogenesis, we estimated the numbers of GCNA1- and SALL4-positive germ cells in the cross-sections of control and mutant testes. GCNA1 is expressed in undifferentiated spermatogonia to the preleptotene spermatocyte stage, whereas the expression of SALL4 in testes is restricted to undifferentiated spermatogonia (A<sub>d</sub> to A<sub>al</sub>), a subset of which is considered to be SSCs (Hobbs et al. 2012). No significant differences were detected in the number of GCNA1-positive cells per tubule in mutant testes compared with that in control by 5 DPI, but this number sharply dropped in mutant testes by 15 DPI (Fig. 3A and B). The numbers of SALL4-positive spermatogonia were not significantly different between mutant and controls at 5 DPI and were slightly increased at 15 DPI, but were absent in 90% of mutant STs by 25 DPI (Fig. 3A and C). In mice, progression of spermatogonia to sperm takes about 35 days, whereas the...
PELO regulates SSC development

PELO deficiency leads to spermatogenic failure. Testes sections from adult control (A and B) and mutant mice at 5 (C and D), 15 (E and F), 25 (G and H), and 35 DPI (I and J) were stained with H&E. Compared with control testes (A and B), all pre-meiotic (blue arrowheads), meiotic (white arrowheads), and post-meiotic (black arrowheads) germ cells were recognized in STs of mutant mice at 5 DPI (C and D). Most STs of mutant mice at 15 (E and F) and 25 DPI (G and H) were devoid of pre-meiotic cells and contained round and elongated spermatids located at the periphery. Very few STs at 15 DPI contained meiotic cells. In most STs of mutant testes at 45 DPI, only Sertoli cells (yellow arrowheads) could be recognized at basement membrane, and vacuoles were present in the region lacking germ cells (I and J). Scale bar (A, C, E, G, and I) = 20 μm; (B, D, F, and J) = 10 μm.

Figure 2 PELO deficiency leads to spermatogenic failure. Testes sections from control and mutant mice at 5, 15, 25, and 45 DPI were probed with antisera against GCNA1 (A) and Sall4 (A). Black arrowheads mark SALL4-positive cells. Counterstaining was done with hematoxylin to stain the nucleus. (B and C) Scale bar graphs represent the mean ± s.d. of GCNA1-positive (B) and SALL4-positive (C) cells per tubule of mutant mice after different time points of Tam treatment. *Significantly different from controls; P < 0.05. n = 3 animals.

(D) Immunoblotting analysis for the expression of NGN3 in the testes of control (F/F) and mutant mice (∆/∆) at 2 and 5 DPI (left panel). In the histogram presented in the right panel, the expression levels of NGN3 were normalized to that of α-tubulin (TUB). Value is presented as mean and s.d. AU, indicates arbitrary units. *Significantly different from controls; P < 0.05. (E) Protein blotting analysis for the expression of full-length PARP (PARP) and cleaved PARP (Cl. PARP) in the testes of control (F/F) and mutant mice at 2, 5, 10, and 15 DPI. (F) Histological sections from control and mutant adult mice at 45 DPI were probed with anti-GATA4 antibody. No marked changes in the number of GATA4-positive Sertoli cell nuclei in mutant testes compared with controls were observed. Scale bars (A and D) = 20 μm.

Figure 3 PELO is required for maintenance of SSCs. Testis tissue sections from control and mutant mice at 5, 15, 25, and 45 DPI were probed with antisera against GCNA1 (A) and Sall4 (A). Black arrowheads mark SALL4-positive cells. Counterstaining was done with hematoxylin to stain the nucleus. (B and C) Scale bar graphs represent the mean ± s.d. of GCNA1-positive (B) and SALL4-positive (C) cells per tubule of mutant mice after different time points of Tam treatment. *Significantly different from controls; P < 0.05. n = 3 animals.

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In undifferentiated spermatagonia that are destined for differentiation (Nakagawa et al. 2010). As shown in Fig. 3D, the expression levels of NGN3 were significantly reduced in Pelo-null testes after 2 and 5 DPI, suggesting that arrested spermatogenesis in Pelo-deficient testes is a result of failed differentiation of spermatogonia.

In order to investigate whether these undifferentiated spermatagonia undergo apoptosis, in the absence of differentiation, we checked the expression levels of cleaved PARP, a specific marker of apoptosis, in testes after different time point of Pelo deletion. As shown in Fig. 3E, no significant differences in expression levels of cleaved PARP in mutant testes after different time point

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of Pelo deletion were observed. These results suggest that the degeneration of arrested spermatogonia occurred not via apoptotic pathway. Furthermore, to analyze whether Pelo deletion affects Sertoli cells, we performed an immunohistological analysis of GATA4, a Sertoli cell marker. This analysis revealed the presence of comparable numbers of cells stained for GATA4 in both genotypes at 45 DPI, suggesting that Sertoli cells are unaffected by Pelo deletion (Fig. 3D).

**Gonocytes are unaffected by Pelo deficiency, but their transition to SSCs is disrupted**

After the homing of PGCs in the gonads of mouse embryos, PGCs proliferate and become gonocytes, which undergo G1/G0 cell cycle arrest from E15.5 until birth. Between P3 and P7, gonocytes migrate toward the periphery of the STs; when they reach the basement membrane, they develop into SSCs (Culty 2009). To determine the effect of Pelo deletion on the development of germ cells, pregnant females were injected i.p. with Tam at E17.5. Later, the testes of the pups were isolated at P1, P7, and P14 and subjected to further analyses. Genotyping PCR showed the successful recombination of the floxed allele in mutant pups (Fig. 1A). RNA and protein analyses revealed that the expression levels of Pelo in mutant testes were reduced by more than 75% compared with controls (Fig. 1C and D).

Effects of Pelo deficiency on gonocytes were elucidated by the expression analyses of gonocyte marker HSPA4, which is highly enriched in gonocytes and subsequently downregulated in SSCs (Held et al. 2011). The numbers of HSPA4-positive gonocytes in Pelo-deficient testes at P1 were not significantly different from those of controls, indicating that Pelo deficiency does not disrupt gonocytes (Fig. 4A and B). The immunohistochemical analysis of mutant testes at P7 and P14 showed a dramatic decrease in number of GCNA1-positive germ cells (Fig. 4C and D). These finding suggested that either the maturation of gonocytes or the development of SSCs is affected by the absence of Pelo. The severe development of atopic dermatitis and high lethality of mutant mice beyond the second week of birth restricted us to follow the progression of the first and subsequent waves of spermatogenesis in later postnatal development of Pelo-deficient mice.

**Attenuation of transcriptional activity of FOXO1 impairs the transition of Pelo-deficient gonocytes to SSCs**

Previous studies have demonstrated that the PI3K/AKT signaling pathway regulates the development and maintenance of SSCs through the control of transcriptional activity of FOXO1. Interestingly, gonocyte maturation to SSCs is demarcated by the translocation of FOXO1 from the cytoplasm to the nucleus (Goertz et al. 2011). They showed that FOXO1 is localized in the cytoplasm of all gonocytes in testes at P1–P3 and undergoes a translocation from cytoplasm to nucleus at P3–P7. This translocation demarcates the transition of gonocytes to SSCs, where the FOXO1 is predominantly nuclear. Upon nuclear export in SSCs, the FOXO1 is degraded quickly and so often not detectable in cytoplasm. Their finding shows that gonocytes that retain nuclear localization of FOXO1 are mature while those that retain cytoplasmic FOXO1 around P7 are rather immature. To investigate whether the impaired maturation of gonocytes in Pelo-deficient testes is due to the inactivation of FOXO1, we determined the sub-cellular localization of FOXO1 in the testicular cells of mutant and control P7 and P14 mice by immunohistochemistry (Fig. 5A). At P7, a significantly increased number of germ cells with a nuclear distribution of FOXO1 were present in controls compared with mutant testes (Fig. 5A and B). By P14, most Pelo-deficient germ cells localized at the peripheral layer of STs and showed a cytoplasmic distribution of FOXO1, whereas FOXO1 localization was detected mainly in nuclei of corresponding cells in control P14 tubules (Fig. 5A and B). To confirm these results, we performed western blotting analyses using nuclear protein fractions prepared from mutant and control P7 testes. As shown in Fig. 6A, the levels of nuclear FOXO1 were significantly reduced in mutants.
controls, and that GDNF induction further enhanced AKT activity only in mutants but not in controls (Fig. 6C). To further confirm these results, we treated STs with LY294002, a specific inhibitor of PI3K. As shown in Fig. 6C, basal and GDNF-induced phosphorylation of AKT in mutant testes was markedly reduced after PI3K inhibition. These results suggest that the enhanced activity of the PI3K/AKT pathway in mutant testes is responsible for the impaired development of SSCs. The elevated induction of PI3K/AKT activity in STs after treatment with GDNF in the absence of Pelo confirmed these results, showing that Pelo negatively regulates the PI3K/AKT pathway (Pedersen et al. 2014).

Discussion

In this report, we have investigated the biological function of Pelo in male germ cell development through the temporal deletion of Pelo at different

Figure 5 Sub-cellular localization of FOXO1 in control and mutant germ cells. (A) Testis sections from control and mutant mice at P7 and P14 were probed with anti-FOXO1 antibody. High magnification inserts show the localization of FOXO1. (B) Quantitative analysis of nuclear (left panel) or cytoplasmic FOXO1-positive cells per tubule (right panel) in control and mutant testes of P7 and P14 mice. Data represent mean ± s.d. *Significantly different from controls; \( P<0.05 \), \( n=3 \) per group. Scale bar (A) = 20 μm.

Figure 6 Enhanced activation of PI3K/AKT signaling attenuates FOXO1 transcriptional activity in Pelo-deficient testes. (A) Western blotting with nuclear protein fractions derived from the testes of P7 control Pelo\(^{-/-}\) and Pelo\(^{+/+}\) mice were probed with anti-FOXO1 antibody. The blots were stripped and reprobed with histone (H3) as a protein loading control (left panel). The histogram shows the relative intensity of FOXO1 to that of H3 (right panel). Protein levels of control testes were expressed as 1.0. *Significantly different from controls; \( P<0.05 \). AU, indicates arbitrary units. (B) RNA isolated from testes of P7 and P14 control and mutant mice was used to determine the expression levels of Lhx1, Ret, Sall4, and Dppa3 by qRT-PCR. Values of expression levels normalized to Hprt or Sdha are presented as mean ± s.d. Transcript levels in control testes were expressed as 1.0. *Significantly different from controls; \( P<0.05 \). \( n=3 \) per age and genotype. (C) STs from P7 control and mutant testes were incubated with or without 100 ng/ml GDNF and/or PI3K inhibitor LY294002 (30 μM) for 60 min. The blots of protein extracts were probed with pAKT antibody and subsequently with anti-AKT antibody that recognized total AKT protein as a protein loading control.
PELO deficiency in adult mice results in the depletion of all germ cells after 45 days of gene deletion. The absence of new emerging spermatogenic cycles in the mutants confirmed that the SSCs were unable to maintain spermatogenesis in the absence of PELO. However, germ cells that entered the spermatogenic cycle are capable of completing spermatogenesis and producing spermatooza. The gradual loss of SALL4-positive undifferentiated and GCNA1-positive spermatogonia in the Pelo mutants suggests the exhaustion of the SSC pool and the loss of undifferentiated spermatogonia. Moreover, the absence of a new wave of spermatogenesis showed that self-renewal of SSCs is impaired in the absence of PELO. The dramatic disruption of spermatogenesis in Pelo-deficient mice is similar to that observed in mice lacking the Plzf, Ev5, Foxo1, or Shp2 genes, which regulate the self-renewal of SSCs (Costoya et al. 2004, Simon et al. 2007, Goertz et al. 2011, Puri et al. 2014).

Despite the fact that PELO is essential for the maintenance of SSCs, their precursors were not sensitive to PELO depletion. However, gonocyte derivatives were differentially affected by PELO depletion. Although a subset of gonocytes differentiated and gave rise to the first wave of spermatogenesis, as indicated by the presence of meiotic cells in 2-week-old mutant testes, the maturation of mutant gonocytes to SSCs was impaired. These results indicate that PELO is not required for gonocyte survival or differentiation of their derivatives during the first wave of spermatogenesis. Previous studies demonstrated that the transcription factor FOXO1 plays an essential role in the developmental conversion of gonocytes to SSCs via the induction of many genes, whose encoded proteins are required for the development and maintenance of SSCs (Goertz et al. 2011, Ngo et al. 2013). Our investigation of the sub-cellular localization of FOXO1 showed a clear impairment in the transition of gonocytes to SSCs, as most of the gonocytes retained FOXO1 in the cytoplasm and were unable to transform to SSCs. Thus, there were a reduced number of GCNA1-positive spermatogonia in the absence of PELO during postnatal development. Further the absence of spermatogenesis confirms the impairment of these cells to maintain spermatogenesis in the absence of PELO. The phosphorylation of FOXO1 is mediated by the activation of the PI3K/AKT signaling pathway. The GDNF-mediated PI3K/AKT pathway is known to play an essential role in SSC self-renewal as its deficiency leads to progressive germ cell loss phenotype (Braydich-Stolle et al. 2007). Our results showed that both basal and GDNF-induced AKT phosphorylation is enhanced in Pelo-deficient testes, suggesting that PELO negatively regulates GDNF-mediated PI3K/AKT activation. The AKT-dependent phosphorylation of FOXO1 triggers its rapid nuclear export and subsequent degradation via the ubiquitin-proteosome degradation pathway (Huang & Tindall 2011). A recent study has shown that PELO antagonists direct the binding of the p84 regulatory subunit of PI3K to active HER2 and the epidermal growth factor receptor in tumor cell lines (Pedersen et al. 2014). Therefore, it is likely that PELO regulates PI3K/AKT and its mediator FOXO1 in the regulation of SSC development and maintenance. This hypothesis is supported by our results, which show higher levels of pAKT, the downstream mediator of PI3K, in mutants compared with WTs. The higher levels of pAKT in Pelo mutant testes in turn phosphorylate FOXO1, resulting in its cytoplasmic localization and inactivation. The reduction in the levels of pAKT upon the addition of PI3K inhibitor further confirmed that the PI3K/AKT signaling cascade was enhanced in the absence of PELO. Consistent with our findings, SSC depletion was also accompanied by a persistent increase in PI3K/AKT activity in mice lacking PTEN, which normally antagonizes the PI3K/AKT pathway (Goertz et al. 2011). On the other hand, the nuclear retention of FOXO1 in the absence of GILZ resulted in the accumulation of SSCs, as their differentiation potential was impaired (Ngo et al. 2013).

In conclusion, we have shown the evidence that PELO indirectly regulates the sub-cellular localization of FOXO1, as PI3K/AKT signaling is highly activated in the absence of PELO. This in turn affects SSC pool formation, disrupts the balance between SSC self-renewal and differentiation, and contributes to a loss of spermatogenesis. Thus, PELO is essential for finely regulating the signals required for the maintenance of spermatogenesis.

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.

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