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

Priyadharsini Raju*, Gunsmaa Nyamsuren*, Manar Elkenani, Aleksandra Kata, Erdenechimeg Tsagaan, Wolfgang Engel and Ibrahim M Adham

Institute of Human Genetics, University Medical Center of Göttingen, Heinrich-Düker-Weg 12, 37073 Göttingen, Germany

Correspondence should be addressed to I M Adham; Email: iadham@gwdg.de

*(P Raju and G Nyamsuren contributed equally to this work)

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 I (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 (Èberhart & 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 germline 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 (Pelo<sup>F<sub>F</sub></sup>CreERT2) mice was described previously (Nyamsuren et al. 2014). To conditionally delete Pelo in Pelo<sup>F<sub>F</sub></sup>CreERT2 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 Pelo<sup>F<sub>F</sub></sup>CreERT2 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 Pelo<sup>F<sub>F</sub></sup> 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 Pelo<sup>F<sub>F</sub></sup>CreERT2 and Pelo<sup>F<sub>F</sub></sup> 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


www.reproduction-online.org

Downloaded from Bioscientifica.com at 02/14/2019 11:56:45PM
via free access
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 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.

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>fl</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 after Tam for 5 consecutive days. Time points from the last Tam injection were expressed as days after 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$^{FF}$ 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$^{FF}$CreERT2 males (thereafter referred to as Pelo mutant or Pelo$^{DD}$), 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$^{FF}$ 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 $\pm$ 2.1 pups, n = 5 vs control 13.8 $\pm$ 2.1 pups, n = 5). PCR analysis revealed that the mutant allele (Pelo$^{D}$) is transmitted to $\sim 93\%$ 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 $\sim 88\%$ 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 spermatogonia, 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 (A0, to Aal), 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 progression of meiotic and post-meiotic cells to functional sperm is required for later spermatogenic stages.

**Figure 1** Conditional deletion of Pelo affects male fertility. (A) Genotyping PCR analysis using primers specifically amplifying the genomic fragments of WT (+), floxed (F), and recombined (Δ) alleles of Pelo was performed on genomic DNA isolated from the testes of control Pelo$^{FF}$ and mutant Pelo$^{DD}$P7 or adult mice at 5 DPI. The presence of an amplified 455-bp fragment of Pelo$^{D}$ allele and the loss of a 376-bp of Pelo$^{D}$ allele in mutant testes demonstrated successful Cre-mediated recombination. (B) Genotyping PCR was performed on genomic DNA isolated from tail of pups obtained from the breeding of mutant males at 15 DPI with WT females. Transmission of the Pelo$^{D}$ allele to offspring indicated that Pelo deletion did not affect the progression of spermatogenic cells to spermatozoa with fertilizing capability. (C) Western blotting of testicular proteins extracted from adult Pelo$^{FF}$ and Pelo$^{DD}$ mice at 5 DPI or from P7 mutant mice using anti-PELO antibodies. The blot was stripped and reprobed for α-tubulin (TUB). (D) Expression levels of Pelo in the testes of adult Pelo$^{DD}$ and Pelo$^{DD}$ mice after 5 DPI, or from P7 Pelo$^{DD}$ and Pelo$^{DD}$ mice, were determined by qRT-PCR. Values of expression levels normalized to Hprt and are presented as mean ± S.D. of three animals. Transcript levels of control adult mice were expressed as 1.0. *Significantly different from controls; P < 0.05. (E) Representative image of the relative size of testes from adult Pelo$^{FF}$ and Pelo$^{DD}$ mice at 45 DPI. (F) Comparison of testis weights from adult control and mutant mice at 45 DPI. Results are shown as mean ± S.D. of six animals from each genotype. ***Significantly different from controls; P < 0.05.
PELO regulates SSC development

in undifferentiated spermatogonia 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 spermatogonia 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

![Figure 2 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 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 hemotoxylin 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. ALI, 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.

www.reproduction-online.org
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 1–2 days after 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 PEOLO 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). 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 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

**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 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
compared with controls. We then investigated the expression levels of Lhx1, Ret, Sall4, and Dppa3 by qRT-PCR, whose expression levels were significantly attenuated in the testes of Foxo1-null mice (Goertz et al. 2011). The expression levels of Sall4 and Dppa3 were significantly lower in mutant P7 and P14 testes compared with controls, whereas no significant differences were detected in the expression levels of Lhx1 and Ret between control and mutant testes (Fig. 6B). Collectively, these data suggest that the impaired transition of gonocytes to SSCs in mutant postnatal testes is due to the decreased transcriptional activity of FOXO1.

The proper balance of PI3K/AKT activity is critical for the development and maintenance of SSCs via FOXO1 (Singh et al. 2011); hence, we were prompted to examine whether the excessive activity of the PI3K/AKT signaling pathway is the cause of the impaired maturation of gonocytes in mutant testes. AKT kinase is activated by phosphorylation downstream of PI3K, and serves as an indirect measurement of the activity of PI3K/AKT signaling. To define whether Pelo deficiency affects PI3K/AKT activity, tests from control and mutant P7 mice were incubated for 1 h with or without GDNF. Western blotting analysis using anti-phospho-AKT (pAKT) showed that the levels of basal pAKT were markedly higher in mutant testes compared with 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
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 derivates 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 derivates 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-proteasome 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.

Funding

This work was supported in part by University Medical Center of Goettingen. P Raju is supported by Indian Council of Agricultural Research (ICAR) International fellowship. M Elkenani is supported by fellowship from Egyptian Government.

Acknowledgements

The authors are grateful to Dr D V K Pantakani for critically reading the manuscript. They thank G C Enders (Kansas University, Medical Center, Kansas City, USA) for providing the GCNA1 antibody.

References

PELO regulates SSC development


Davis L & Engelbrecht J 1998 Yeast dom34 mutants are defective in multiple developmental pathways and exhibit decreased levels of polyribosomes. Genetics 149 45–56.


Received 1 August 2014
First decision 30 August 2014
Revised manuscript received 4 November 2014
Accepted 2 December 2014