Title

MEK/ERK signaling directly and indirectly contributes to the cyclical self-renewal of spermatogonial stem cells

Authors

Hasegawa, Kazuteru
Namekawa, Satoshi H
Saga, Yumiko

Peer reviewed

MEK/ERK signaling directly and indirectly contributes to the cyclical self-renewal of spermatogonial stem cells

Kazuteru Hasegawa[1,2], **Satoshi H. Namekawa**[2], and **Yumiko Saga**[1,3,*]

[1]Division of Mammalian Development, National Institute of Genetics, 1111 Yata, Mishima, Shizuoka 411-8540, Japan

[2]Division of Reproductive Sciences, Perinatal Institute, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio 45229, USA

[3]Department of Genetics, SOKENDAI, 1111 Yata, Mishima, Shizuoka 411-8540, Japan

Abstract

Coordination of stem cell fate is regulated by extrinsic niche signals and stem cell intrinsic factors. In mammalian testes, spermatogonial stem cells maintain constant production of abundant spermatozoa by alternating between self-renewal and differentiation at regular intervals according to a periodical program known as the seminiferous epithelial cycle. Although retinoic acid (RA) signaling has been suggested to direct the cyclical differentiation of spermatogonial stem cells, it remains largely unclear how their cycle-dependent self-renewal/proliferation is regulated. Here, we show that MEK/ERK signaling contributes to the cyclical activity of spermatogonial stem cells. We found that ERK1/2 are periodically activated in Sertoli cells during the stem cell self-renewal/proliferation phase, and that MEK/ERK signaling is required for the stage-related expression of the critical niche factor *GDNF*. In addition, ERK1/2 are activated in GFR 1-positive spermatogonial stem cells under the control of GDNF and prevent them from being differentiated. These results suggest that MEK/ERK signaling directly and indirectly maintains spermatogonial stem cells by mediating a signal that promotes their periodical self-renewal/proliferation. Conversely, RA signaling directly and indirectly induces differentiation of spermatogonial stem cells. We propose that temporally-regulated activations of RA signaling and a signal regulating MEK/ERK antagonistically coordinates the cycle-related activity of spermatogonial stem cells.

Keywords

Germline; Tissue-specific stem cells; Stem-cell microenvironment interactions; MAPK

Introduction

Stem cells maintain tissue homeostasis by changing their potential to either self-renew or differentiate in response to extrinsic niche signals and stem cell intrinsic factors. In mammalian testes, stem cell function resides in the undifferentiated spermatogonia, which

are classified into three types according to their morphology, marker gene expression and function. Asingle (As; isolated single cell) and Apaired (Apr; two interconnected cells) cells, a large part of which are known to express NANOS2 and GDNF family receptor -1 (GFR 1), are more likely to exhibit self-renewal ability. Conversely, Aaligned (Aal; interconnected 4, 8, 16 or 32 cells) cells, which are predominantly positive for NANOS3 and Neurogenin 3 (NGN3), have an inclination to differentiate into c-KIT-positive differentiating spermatogonia [1-4], although they also contribute to reproducing stem cells by reverting to GFR 1-positive cells [2]. Intriguingly, proliferation and differentiation of undifferentiated spermatogonia take place under the control of a periodical program known as the seminiferous epithelial cycle [5, 6]. Proliferation of undifferentiated spermatogonia occurs during stages I-III and X-XII, while differentiation is initiated around stages VII-VIII [7]. Although this cycle-associated change of stem cell activity is essential for constant production of abundant spermatozoa, it remains largely unclear how cycle-dependent stem cell activity is regulated.

Sertoli cells retain functionally and morphologically specialized roles to support spermatogenesis, and periodically change their gene expression in a coordinated manner with the seminiferous epithelial cycle. We previously identified stage-dependent genes in Sertoli cells by comprehensive expression analyses [8]. In that study, we found that retinoic acid (RA) signaling, which is known to be important for differentiation of spermatogenic cells, contributes to cyclical gene expression changes by regulating RA-responsive genes, although involvement of other signaling pathway(s) has also been suggested [8]. The cyclical activities of Sertoli cells provide a stage-specific microenvironment for spermatogonial stem cells. One important cyclical niche signal is glial cell line-derived neurotrophic factor (GDNF), which is secreted from Sertoli cells and promotes self-renewal of spermatogonial stem cells via GFR 1 and RET co-receptors [9]. Reduction in GDNF expression results in a gradual loss of stem cells, whereas overexpression of GDNF blocks their differentiation and causes abnormal accumulation of undifferentiated spermatogonia [10]. Regarding the proliferation and differentiation stages of undifferentiated spermatogonia, GDNF shows higher expression around stage I, with lower expression around stage VII. Furthermore, distribution of GFR 1-positive cells is also associated with the local abundance of GDNF [11-13], suggesting involvement of stage-dependent GDNF expression in Sertoli cells in cycle-related behavior of spermatogonial stem cells. Although the fibroblast growth factor 2 (FGF2)/ MAP kinase/Erk kinase (MEK) pathway has been reported to stimulate GDNF expression in cultured Sertoli cells [14], the molecular mechanisms underlying the cyclical expression change of GDNF remain poorly understood.

GDNF is known to stimulate proliferation and suppress differentiation of spermatogonial stem cells. In cultured germline stem (GS) cells, GDNF signaling activates phosphoinositide 3-kinase (PI3K)/AKT signaling [15, 16], which is important for the proliferation of GS cells, while hyperactivation of the signal increases c-KIT expression [17]. Moreover hypoactivation of PI3K/AKT signaling in undifferentiated spermatogonia causes a differentiation block rather than their depletion [13]. This *in vivo* phenotype is contrastive to the premature differentiation and stem cell-loss phenotype observed in GDNF signaling mutant mice [10, 18]. In addition to PI3K/AKT3 signaling, MEK/ extracellular signal-regulated kinase (ERK) signaling is also activated by GDNF in spermatogonial stem cells and has been suggested to be involved in proliferation and suppression of differentiation [15, 19, 20]. However, it is still unknown how GDNF signaling maintains spermatogonial stem cells in an undifferentiated state.

In the current study, we found that MEK/ERK signaling contributes to the cycle-related behavior of spermatogonial stem cells. MEK/ERK signaling in Sertoli cells is activated during the proliferation phase of undifferentiated spermatogonia and increases *GDNF*

expression. In addition, MEK/ERK signaling in spermatogonial stem cells promotes and suppresses gene expression associated with self-renewal and differentiation, respectively. Our results offer new insight into how spermatogenic cycle-associated differentiation and proliferation of spermatogonial stem cells are regulated.

Materials & Methods

Animals

NANOS3$^{-/-}$ mice, *ERK1$^{-/-}$* mice, *ERK2-floxed* mice, *GFRa1-floxed* mice and *Cre-ERT2* mice have been previously described [18, 21-23]. *Amh-cre* mice and C57BL6/j mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and CLEA Japan, respectively. Generation of vitamin A-deficient (VAD) mice and administration of retinol were performed as previously described [8]. All animals were maintained in accordance with the National Institute of Genetics (NIG) guidelines, and all animal procedures were carried out with approval from the Committee for Animal Care and Use at NIG.

Testicular injection

PD0325901 (Wako, Osaka, Japan) was dissolved in dimethyl sulfoxide at 10 mM and diluted with Hank's balanced salt solution at 100 μM for injection into adult testes. PD0325901, LV-VENUS and LV-dnRAR were prepared and injected into 6–8-week testes as previously described [8]. Stage-specific tubules were isolated as previously reported [24].

Culture of primary Sertoli cells and GS cells

Primary Sertoli cells were isolated and cultured as previously described [25]. Culture medium was changed at days 2 and 4, and Sertoli cells were stimulated with 1 μM RA (Sigma, St. Louis, MO, USA), 20 ng/ml bFGF (Invitrogen, Carlsbad, CA, USA) or 10 μM PD0325901 at day 5 for 24 h.

GS cells were cultured as previously reported [26]. After withdrawal of growth factors for 24 h, GS cells were incubated with 40 ng GDNF (R&D systems, Minneapolis, MN, USA), 10 μM PD0325901 or 30 μM LY294002 (Wako) for 20 min prior to protein extraction for western blotting and 24 h prior to cell harvesting for gene expression analysis. For RA treatment, GS cells were cultured with 100 nM RA and 10 μM PD0325901 or 30 μM LY294002 for 12 h.

Real-time RT-PCR

Total RNAs were purified using an RNeasy kit (Qiagen, Tokyo, Japan), and cDNA was synthesized using oligo(dT) primers and SuperScript III (Invitrogen) in accordance with the manufacturer's instructions. Real-time RT-PCR was then performed using SYBR Premix Ex Taq™ II (Takara, Otsu, Japan) and an MJ Mini Thermal Cycler (Bio-Rad, Hercules, CA, USA). Signals were normalized against *GAPDH* expression. The primer pairs used in these experiments are listed in Supplemental Table 1.

Microarray

Microarray analysis was performed as previously described [27]. Our microarray data are deposited in the Gene Expression Omnibus (GEO) under accession number GSE41645.

Histological analysis

Immunohistochemistry was conducted as previously described [8] using the following antibodies: chick anti-GFP (Aves), goat anti-gata4 (Santa Cruz, CA, USA), rabbit anti-phospho-ERK1/2 (Cell Signaling, Danvers, MA, USA), goat anti-GFR 1 (Neuromics,

Edina, MN, USA), rabbit anti-PLZF (Santa Cruz), rabbit anti-phospho-Histone H3 (Ser10; Cell Signaling) and rabbit anti-Nanos3 [3]. For the detection of phospho-ERK1/2, GFR 1 and Nanos3, Can Get Signal immunostain (TOYOBO, Osaka, Japan) was used. The resulting signals were detected by incubation with Alexa488- or Alexa594-conjugated IgG antibodies (Molecular Probes, Grand Island, NY, USA). For detection of phospho-ERK1/2, Envision+ anti-rabbit (DAKO, Carpinteria, CA, USA) and Tyramid Signal Detection Reagent (Perkin Elmer, Waltham, MA, USA) were used.

In situ hybridization was performed as previously described [25]. *RET* was subcloned from testis cDNA by RT-PCR. Digoxigenin (DIG)-labeled cRNA probes were synthesized with RNA labeling mix (Roche, Basel, Switzerland). Paraffin sections were hybridized with each DIG-labeled probe, and incubated with horseradish peroxidase (HRP)-conjugated anti-DIG Fab fragments (Roche). Signals were detected using Cyanin 3-Tyramid Signal Detection Reagent (Perkin Elmer). Co-immunostaining for PLZF was then performed as described above.

Western blotting

Cell extracts were resolved on SDS-PAGE gels and electroblotted onto nitrocellulose membranes (Pall Corporation, Port Washington, NY, USA). Membranes were incubated with the following primary antibodies: rabbit anti-phospho-ERK1/2 (Cell Signaling), rabbit anti-ERK1/2 (Cell Signaling), rabbit anti-phospho-AKT (Cell Signaling), rabbit anti-AKT (Cell Signaling), rabbit anti-GDNF (Santa Cruz) or mouse anti-TUBULIN (Sigma). This was followed by incubation with a secondary antibody conjugated with HRP. Signals were detected with ECL Prime Western Blotting Detection Reagent (GE Healthcare, Pittsburgh, PA, USA) according to the manufacturer's instructions. Signal intensities were quantified with ImageJ.

Statistical analysis

All statistical analyses in this study were performed using the Student's *t*-test. Statistical significance was defined as $P < 0.05$.

Results

ERK1/2 are activated in a cycle-dependent manner in Sertoli cells and spermatogonial stem cells

It has been reported that GDNF shows a stage-dependent expression pattern[11-13] and is activated by the bFGF-MEK1/2 pathway in cultured Sertoli cells [14]. To examine the involvement of MEK1/2 signaling in the cyclical regulation of GDNF expression and spermatogonial stem cells, we first investigated the activation patterns of ERK1/2 in adult testes. ERK1/2 are known to be activated via phosphorylation by MEK1/2 [28]. Phosphorylated ERK1/2 were detectable in Gata4-positive Sertoli cells and exhibited stage-dependent distribution in seminiferous tubules, showing higher and lower activation during stages I-III and VII-VIII, respectively (Fig. 1A,B and Supplemental Fig. S1). In addition to Sertoli cells, we also noticed that approximately 30% of cells expressing PLZF, a marker for undifferentiated spermatogonia, were positive for phosphorylated ERK1/2 (Fig. 1C,F). Because undifferentiated spermatogonia exhibit heterogeneity, we further examined ERK1/2 phosphorylation in GFR 1- and NANOS3-positive cells and found that 82.8% and 16.1% showed ERK1/2 activation, respectively (Fig. 1D-F). Moreover, the phosphorylated ERK1/2 in GFRa1-positive cells showed higher and lower activation during stages I-III and VII-VIII, respectively (Fig. 1G and Supplemental Fig. S2). Cycle-related activation of ERK1/2 was also confirmed by western blotting using stage-specific seminiferous tubules (Fig. 1H,I).

These results indicate that MEK/ERK signaling is activated in a stage-dependent manner in Sertoli cells and in GFRα1-positive undifferentiated spermatogonia.

MEK/ERK signaling contributes to stage-dependent GDNF expression in Sertoli cells

To evaluate the significance of ERK1/2 for GDNF expression and the maintenance of spermatogonial stem cells *in vivo*, we crossed *ERK1$^{-/-}$; ERK2$^{flox/flox}$* mice with *Amh-Cre* mice, in which Cre expression starts in Sertoli cells around embryonic day 14.5. We first confirmed the absence of phospho-ERK1/2 in Sertoli cells from *ERK1$^{-/-}$; ERK2$^{flox/flox}$; Amh-Cre (ERK1/2$^{Ser-/-}$)* mice (Supplemental Fig. S3). We then examined the expression level of *GDNF* in *ERK1/2$^{Ser-/-}$* testes at 6 weeks. Interestingly, we found that GDNF expression was significantly reduced in *ERK1/2$^{Ser-/-}$* mice (Fig. 2A); in accordance with this, the number of PLZF-positive spermatogonia also decreased (Fig. 2B-D). These results suggest that ERK1/2 are required for GDNF expression and for the maintenance of spermatogonial stem cells.

Because the removal of ERK2 occurs in embryonic Sertoli cells of *ERK1/2$^{Ser-/-}$* mice, it is difficult to evaluate the implication of MEK/ERK signaling in the stage-related expression of GDNF in adult testes. To examine more directly whether MEK/ERK signaling contributes to the stage-related GDNF expression, we injected the pharmacological MEK1/2 inhibitor, PD0325901, into adult testes. The inhibitor effectively suppressed the phosphorylation of ERK1/2 by 6 h after injection (Fig. 2E-G), but phosphorylation was quickly recovered by 24 h (Supplemental Fig. S4). Accordingly, we isolated stage-specific tubules 6 h after injection of PD0325901 and investigated the stage-related *GDNF* expression change. Interestingly, the suppression of MEK/ERK signaling resulted in decreased *GDNF* expression, especially in stages I-III, hence it disturbed the cyclical expression pattern of *GDNF* (Fig. 2H). This result suggests that the activation of MEK/ERK signaling is required for the spermatogenic cycle-related *GDNF* expression in Sertoli cells.

We next asked whether GDNF signaling is involved in the cycle-related proliferation of spermatogonial stem cells. For this purpose, we crossed GFRα1-conditional knockout (CKO) mice, in which the GFP reporter is knocked-in at the GFRα1 locus resulting in expression after removal of GFRα1, with Cre-ERT2 mice that ubiquitously express tamoxifen-inducible Cre [18]. We injected tamoxifen into 6-week-old mutant mice (*GFRα1$^{flox/flox}$;Cre-ERT2*) and examined the proliferation of GFP-positive cells 5 days after injection. In control mice, the proliferation marker phospho-Histone H3 was predominantly detected in GFP-positive spermatogonia during stages I-III and IX-XII, as expected. However, deletion of GFRα1 dramatically reduced the proportion of proliferating GFP-positive cells (Fig. 2I-K), indicating that GDNF signaling is a critical regulator of stem cell proliferation. Considering that MEK/ERK are signal mediators, MEK/ERK signaling in Sertoli cells indirectly contributes to cycle-related proliferation/self-renewal of spermatogonial stem cells through mediating a signal to induce GDNF expression.

RA signaling facilitates differentiation of undifferentiated spermatogonia by suppressing GDNF expression

We have previously reported 419 genes regulated in a stage-dependent manner in Sertoli cells, among which genes showing lower expression during stages VII-XII tended to be suppressed by RA signaling [8]. We therefore hypothesized that activation of RA signaling in Sertoli cells decreases *GDNF* expression from stage VII. To examine this, we first utilized cultured Sertoli cells and VAD mice, in which RA synthesis is largely suppressed [29]. Administration of RA in both cultured Sertoli cells and VAD mice resulted in a reduction in *GDNF* expression (Fig. 3A,B). Next, we injected lentivirus containing dominant-negative retinoic acid receptor α (RARα) followed by IRES-Venus (LV-dnRARα) into adult testes,

which is known to induce Sertoli cell-specific suppression of RA signaling [8]. At day 5 after injection, we found that overexpression of dnRAR in Sertoli cells induced up-regulation of *GDNF* during stages VII-XII, when RA signaling is typically activated in normal spermatogenesis (Fig. 3C). We next asked whether overexpression of dnRAR in Sertoli cells is sufficient for accumulation of GFR 1-positive undifferentiated spermatogonia. In tubules at stages VII-XII, the number of GFR 1-positive cells significantly increased compared with the control (Fig. 3D-F). These results indicate that RA signaling in Sertoli cells negatively regulates *GDNF* expression and indirectly promotes differentiation of undifferentiated spermatogonia. Taken together, the antagonistic action of RA signaling and the signal mediated by MEK/ERK may determine the expression level of *GDNF* in Sertoli cells and thereby indirectly regulate self-renewal and differentiation of undifferentiated spermatogonia in a stage-dependent manner.

MEK/ERK and RA signaling are independently regulated in Sertoli cells

Because our results suggest that MEK/ERK signaling is involved in the stage-dependent gene expression of *GDNF*, we then asked whether MEK/ERK signaling also contributes to the regulation of other stage-dependent genes in Sertoli cells. It has been reported that bFGF activates MEK/ERK signaling in cultured Sertoli cells [14]. Therefore, to address this question, we first treated cultured Sertoli cells with bFGF or PD0325901 and confirmed that they effectively activated or suppressed ERK1/2 phosphorylation, respectively (Supplemental Fig. S5A,B). Then, using microarray, we assessed how these treatments change the expression of the 419 stage-dependent genes. Interestingly, approximately 40% and 30% of the stage-dependent genes that peaked during stages I-III and VII-VIII, respectively, were up- or down-regulated more than 1.5-fold in bFGF-treated cultured Sertoli cells compared with the PD0325901-treated cells (Supplemental Fig. S5C). This result was confirmed by quantitative RT-PCR (Supplemental Fig. S5D,E). In addition, this tendency of gene expression change was also observed in adult *ERK1/2*$^{Ser-/-}$ testes (Supplemental Fig. S5F,G). Because many genes activated or suppressed by MEK/ERK signaling showed peak expression during stages in which the ERK1/2 activation level was highest (I-III) and lowest (VII-VIII), respectively, the responsiveness of these genes to MEK/ERK signaling correlated well with their expression patterns in testes. These results suggest that MEK/ERK are important mediators of a signal regulating cyclicity in Sertoli cells.

We next asked how the activation of ERK1/2 in Sertoli cells is regulated *in vivo*. It has been reported that germ cells control the periodicity of Sertoli cells, although Sertoli cells themselves may have an intrinsic cycle [30-32]. To test the dependency on germ cells, we investigated the activation pattern of ERK1/2 in NANOS3-deficient testes, which are devoid of germ cells. The activation pattern of ERK1/2 was variable even in the same tubules, although weak tubule-dependency was recognized (Fig. 4A), indicating that synchronized activation of ERK1/2 in Sertoli cells depends on germ cells.

Next, we examined the relationship between RA and MEK/ERK signaling pathways in terms of their spermatogenic cycle-dependent activation. For this purpose, we fist investigated the phosphorylation patterns of ERK1/2 in VAD mice. It has been reported that stage-dependent genes in Sertoli cells showed variable expression patterns in VAD mice and administration of RA induced their synchronized expression [32]. We found that ERK1/2 were activated in a sporadic manner in VAD mice in a similar pattern to the reported stage-dependent genes (Fig. 4B). However, administration of vitamin A to VAD mice did not affect either the distribution of phosphorylated ERK1/2 or the expression of the ERK1/2 signaling responsive genes, *SPRY1* and *PTGR1*, while *STRA6*, a well-known target of RA signaling, was activated (Fig. 4C-E). Furthermore, injection of LV-dnRAR into adult testes did not change the stage-dependent activation of ERK1/2 or the expression of its

downstream targets (Fig. 4F-J). Finally, we examined the effect of ERK1/2 suppression on the expression pattern of RA-responsive genes by injecting PD0325901 into testes and found that their stage-dependent expressions remained unchanged (Supplemental Fig. S6). These results suggest that the MEK/ERK and RA signaling pathways are independently regulated in Sertoli cells.

MEK/ERK signaling is predominantly activated by GDNF signaling in spermatogonial stem cells

We next investigated the significance of MEK/ERK signaling in spermatogonial stem cells. Because ERK1/2 is predominantly activated in GFR 1-positive cells (Fig. 1C-F) and GDNF is known to activate ERK1/2, we asked whether activation of ERK1/2 in spermatogonial stem cells depends on GDNF signaling *in vivo*. To address this question, we injected tamoxifen into 6-week-old GFR 1 CKO (*GFR 1$^{flox/flox}$; Cre-ERT2*) mice and examined the activation of ERK1/2 in GFP-positive cells at day 5 after injection. In GFR 1-null spermatogonia, ERK1/2 phosphorylation dramatically decreased (Fig. 5A-G), suggesting that GDNF signaling is required for maintenance and/or activation of ERK1/2.

To investigate the role of GDNF signaling in ERK1/2 activation more directly, we examined the phosphorylation pattern of ERK1/2 in GDNF-overexpressing testes. Overexpression of GDNF in Sertoli cells was achieved by injecting lentivirus containing GDNF (LV-GDNF) into 3-week-old wild type mice (Supplemental Fig. S7A). Four weeks after injection, GFR 1-positive undifferentiated spermatogonia highly accumulated in the testes (Supplemental Fig. S7B-E) and were positive for phosphorylated ERK1/2 (Fig. 5H-K). These results suggest that overexpression of GDNF is sufficient for accumulation of phospho-ERK1/2-positive cells.

MEK/ERK signaling maintains the undifferentiated state of spermatogonial stem cells

Because GDNF signaling strongly suppresses differentiation of spermatogonial stem cells, we hypothesized that GDNF signaling may maintain the undifferentiated stem cell state via activation of MEK/ERK signaling. To address this question, we used GS cells to examine changes in expression of genes associated with spermatogonial stem cell state upon suppression of MEK/ERK signaling. We also considered the interplay with the PI3K/AKT signaling, which is known to be activated under the control of GDNF signaling, in regulating GS cell differentiation. We first confirmed that GDNF promotes phosphorylation of both ERK1/2 and AKT, and that PD0325901 and LY294002 (a specific AKT inhibitor) effectively suppress ERK1/2 and AKT phosphorylation, respectively (Fig. 6A). Interestingly, suppression of MEK/ERK signaling decreased the expression of the GDNF co-receptors, *GFR 1* and *RET*, but promoted the expression of *NGN3, NANOS3* and the spermatogonia differentiation markers, *STRA8* and *c-KIT* (Fig. 6B). Conversely, suppression of PI3K/AKT signaling promoted *GFR 1* and *RET* expression and suppressed the differentiation markers (Fig. 6B). These results suggest that activation of MEK/ERK, but not PI3K/AKT, is required for maintaining gene expression characteristics of As and Apr cells and therefore the undifferentiated state of GS cells.

It has been suggested that RA promotes differentiation of undifferentiated spermatogonia *in vivo* [32]. We therefore examined the interplay between RA and MEK/ERK signaling in spermatogonial differentiation of GS cells. RA treatment resulted in a pronounced reduction in marker genes' expression in undifferentiated spermatogonia and in turn induced the differentiation markers, *c-KIT* and *STRA8*, as expected (Fig. 6C-E). In the presence of RA, suppression of MEK/ERK signaling further decreased the expression of *GFR 1* and *RET*, and increased *c-KIT* and *STRA8* expression (Fig. 6C-E). This indicates that suppression of MEK/ERK signaling accelerates RA-induced differentiation of GS cells. In contrast, PI3K

inhibitor treatment drastically suppressed the RA-induced activation of *c-KIT* and *STRA8* (Fig. 6E), suggesting that PI3K signaling is required for induction of differentiation rather than suppression. Taken together, MEK/ERK signaling in GS cells is important for maintaining their undifferentiated state and preventing RA-induced differentiation.

Considering the role of MEK/ERK signaling in the maintenance of the undifferentiated state of GS cells, this signaling may play an essential role *in vivo* by suppressing differentiation of undifferentiated spermatogonia. To address this possibility, we injected PD0325901 into the seminiferous tubules and examined gene expression changes in undifferentiated spermatogonia. Six hours after injection, the expression of *RET* mRNA was reduced in PLZF-positive cells (Fig. 6F-K), and quantitative RT-PCR, using whole testes, revealed a decrease in *GFR 1* and *RET* expression (Fig. 6L). Moreover, *NGN3* expression increased, although the up-regulation of *NANOS3* was not statistically significant (Fig. 6M). Because GDNF protein abundance in the testes was unchanged at this time point (Supplemental Fig. S8), the changes in gene expression were not caused by reduction in GDNF. Collectively, these results suggest that MEK/ERK signaling in GFR 1-positive cells is also critical for maintaining their undifferentiated state *in vivo*.

Discussion

Seminiferous epithelial cycle-dependent coordination of spermatogonial stem cell activity

In this study, we identified MEK/ERK as factors involved in the cyclicity of spermatogenesis and revealed that MEK/ERK signaling directly and indirectly contributes to the maintenance of spermatogonial stem cells. Considering that MEK/ERK are signal mediators, upstream receptors may determine the expression level of GDNF through regulating the MEK/ERK signaling activity. Combined with our results regarding RA signaling, we propose a model that explains how cycle-associated activity of stem cells is regulated during spermatogenesis (Fig. 7). Transient activation of RA signaling in the tubule from stage VII, triggers differentiation of undifferentiated spermatogonia by inducing RA-responsive genes in these cells and decreasing *GDNF* expression in Sertoli cells. After the transient reduction in the spermatogonial stem cell population because of differentiation, MEK/ERK signaling is activated and induces *GDNF* expression in Sertoli cells, which promotes recovery of the stem cell pool from stage IX. Furthermore, activation of ERK1/2 in GFR 1-positive cells under the control of GDNF signaling suppresses differentiation-associated genes and maintains the expression of GDNF receptors. This positive feedback loop stabilizes the undifferentiated state of spermatogonial stem cells in a GDNF-dependent manner. Thus, temporal changes in RA signaling and a signal regulating MEK/ERK would ensure production of differentiating spermatogenic cells at regular intervals without depletion of stem cells.

Recent findings indicate that spermatogonial stem cells have a flexible feature, in which their turnover is rapid during steady state spermatogenesis. Mathematical analysis suggests spermatogonial stem cells are stochastically replaced within 2 weeks on average [33]. Because one cycle of mouse spermatogenesis is 8.6 days, cycle-dependent changes in the stem cell niche would contribute to the rapid replacement. Given that niche size determines the number of functional stem cells, a transient decrease in niche signal would promote release of spermatogonial stem cells from their niche. Conversely, a temporal increase in niche signal would lead to recruitment of new stem cells from the pool of undifferentiated spermatogonia [2, 34]. Therefore, GDNF expression change during the seminiferous epithelial cycle would accelerate the stem cell replacement. Stochastic turnover of stem cells has also been suggested to occur in mammalian epithelium and intestine [35-37]. It is an interesting possibility that temporal changes in niche signal, like those observed during spermatogenesis, are involved in the regulation of stem cells in other tissues.

Antagonistic regulation of stem cell niche by RA signaling and the signal regulating MEK/ERK

GDNF signaling is known to promote proliferation and maintain the undifferentiated state of spermatogonial stem cells, suggesting local abundance of GDNF would be a critical determinant of cell fate. We revealed that cyclical activation of MEK/ERK signaling in Sertoli cells contributes to the stage-dependent expression of *GDNF*. As *GDNF* heterozygous mice show gradual loss of spermatogonial stem cells [10], activation of ERK1/2 signaling in Sertoli cells would be essential to maintain the stem cell pool. We also identified that RA signaling acts as a repressor of *GDNF*, and overexpression of dnRAR in Sertoli cells was sufficient for increased *GDNF* expression. Thus, RA signaling and upstream regulators of MEK/ERK in Sertoli cells may control stem cell activity through changes in stage-dependent GDNF expression. Although undifferentiated spermatogonia do not proliferate during stages IV-VI, when *GDNF* expression is still high, unknown factor(s) secreted from differentiating spermatogonia might suppress proliferation of undifferentiated spermatogonia in these stages as previously suggested [7].

It is known that germ cells play a role in regulation of the spermatogenic cycle [29, 32]. Because ERK1/2 in Sertoli cells were stochastically activated in germ cell-free testes, germ cells may coordinate stage-dependent activation of MEK/ERK signaling, similarly to RA signaling. We also found that modification of RA signaling did not affect the activation pattern of ERK1/2 and vice versa, suggesting that the RA and MEK/ERK signaling pathways are regulated independently. To understand the molecular basis of cyclicity in spermatogenesis, clarification of the type of external stimuli that regulate the stage-dependent activation of ERK1/2 signaling in Sertoli cells is needed. FGF signaling is a candidate upstream regulator for ERK1/2 signaling in Sertoli cells, although the significance of FGF signaling in testes is largely unknown. Because other factors also activate GDNF expression in cultured Sertoli cells [14], these might also be involved in cycle-related regulation of ERK1/2 *in vivo*.

Intrinsic factors in spermatogonial stem cells regulated by GDNF signaling

Our current study revealed that ERK1/2 is activated in the majority of GFR1-positive cells under the control of GDNF signaling and its suppression disturbs gene expression patterns characteristic of As and Apr cells. Intriguingly, this function of MEK/ERK signaling might explain different features between GFR1/Nanos2- and NANOS3/NGN3-positive cells [1, 2]. We found that MEK/ERK signaling maintained *GFR1* and *RET* gene expression and suppressed *NANOS3* and *NGN3* expression. In addition, suppression of MEK/ERK signaling promoted the expression of differentiating spermatogonia markers, such as *STRA8* and *c-KIT*, upon activation of RA signaling in GS cells. Therefore, activation of MEK/ERK signaling may prevent differentiation of GFR1-positive cells and promote cells with a capacity to self-renew more frequently than NANOS3-positive cells. This idea is consistent with previous reports that either overexpression of GDNF or hyperactivation of ERK1/2 signaling in spermatogonial stem cells interferes with their differentiation [10, 19]. Although it remains unclear how MEK/ERK signaling represses differentiation, MEK/ERK signaling plays an essential role in the maintenance of undifferentiated spermatogonial stem cells.

PI3K/AKT signaling is known to be important for self-renewal of GS cells by promoting their proliferation. However, our results suggest PI3K/AKT signaling also induces differentiation of GS cells in contrast to MEK/ERK signaling. We found that inhibition of PI3K/AKT signaling increased *GFRa1* and *RET* expression and largely repressed *STRA8* and *c-KIT* expression, especially in the presence of RA. Consistent with this, it has been reported that hyperactivation of AKT in GS cells stimulates c-KIT expression as well as proliferation [17]. Moreover, 3-phosphoinositide-dependent protein kinase 1 (PDK1)-

conditional KO mice, in which PI3K/AKT signaling is hypoactivated, exhibit differentiation block and accumulation of RET-positive cells [38]. Thus, PI3K/AKT signaling coincidently promotes proliferation and differentiation, and MEK/ERK signaling may compensate for the PI3K/AKT-dependent induction of differentiation. Taken together, the MEK/ERK and PI3K/AKT signaling pathways under the control of GDNF cooperatively promote self-renewal of spermatogonial stem cells by maintaining their undifferentiated state and inducing proliferation, respectively.

Conclusion

This study demonstrates that MEK/ERK signaling plays an important role in the maintenance of spermatogonial stem cells. The temporal activation of RA signaling and a signal mediated by MEK/ERK coordinate gene expression in Sertoli cells, which regulates the level of GDNF required for the maintenance of spermatogonial stem cells. Our results provide a novel insight into how cyclical self-renewal/proliferation and differentiation of spermatogonial stem cells are achieved.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

References

1. Sada A, Suzuki A, Suzuki H, et al. The RNA-binding protein NANOS2 is required to maintain murine spermatogonial stem cells. SCIENCE. 2009; 325:1394–1398. [PubMed: 19745153]

2. Nakagawa T, Sharma M, Nabeshima Y, et al. Functional hierarchy and reversibility within the murine spermatogenic stem cell compartment. SCIENCE. 2010; 328:62–67. [PubMed: 20299552]

3. Suzuki H, Sada A, Yoshida S, et al. The heterogeneity of spermatogonia is revealed by their topology and expression of marker proteins including the germ cell-specific proteins Nanos2 and Nanos3. DEVELOPMENTAL BIOLOGY. 2009; 336:222–231. [PubMed: 19818747]

4. Yoshida S. Elucidating the identity and behavior of spermatogenic stem cells in the mouse testis. REPRODUCTION. 2012

5. Hogarth CA, Griswold MD. The key role of vitamin A in spermatogenesis. THE JOURNAL OF CLINICAL INVESTIGATION. 2010; 120:956–962. [PubMed: 20364093]

6. Hess RA, Renato de Franca L. Spermatogenesis and cycle of the seminiferous epithelium. ADVANCES IN EXPERIMENTAL MEDICINE AND BIOLOGY. 2008; 636:1–15. [PubMed: 19856159]

7. de Rooij DG. Stem cells in the testis. INTERNATIONAL JOURNAL OF EXPERIMENTAL PATHOLOGY. 1998; 79:67–80. [PubMed: 9709376]

8. Hasegawa K, Saga Y. Retinoic acid signaling in Sertoli cells regulates organization of the blood-testis barrier through cyclical changes in gene expression. DEVELOPMENT. 2012; 139:4347–4355. [PubMed: 23095883]

9. de Rooij DG. The spermatogonial stem cell niche. MICROSCOPY RESEARCH AND TECHNIEQUE. 2009; 72:580–585.

10. Meng X, Lindahl M, Hyvonen ME, et al. Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. SCIENCE. 2000; 287:1489–1493. [PubMed: 10688798]

11. Johnston DS, Olivas E, DiCandeloro P, et al. Stage-specific changes in GDNF expression by rat Sertoli cells: a possible regulator of the replication and differentiation of stem spermatogonia. BIOLOGY OF REPRODUCTION. 2011; 85:763–769. [PubMed: 21653894]

12. Sato T, Aiyama Y, Ishii-Inagaki M, et al. Cyclical and patch-like GDNF distribution along the basal surface of Sertoli cells in mouse and hamster testes. PLOS ONE. 2011; 6:e28367. [PubMed: 22174794]

13. Grasso M, Fuso A, Dovere L, et al. Distribution of GFRA1-expressing spermatogonia in adult mouse testis. REPRODUCTION. 2012; 143:325–332. [PubMed: 22143971]

14. Simon L, Ekman GC, Tyagi G, et al. Common and distinct factors regulate expression of mRNA for ETV5 and GDNF, Sertoli cell proteins essential for spermatogonial stem cell maintenance. EXPERIMENTAL CELL RESEARCH. 2007; 313:3090–3099. [PubMed: 17574550]

15. He Z, Jiang J, Kokkinaki M, et al. Gdnf upregulates c-Fos transcription via the Ras/Erk1/2 pathway to promote mouse spermatogonial stem cell proliferation. STEM CELLS. 2008; 26:266–278. [PubMed: 17962702]

16. Oatley JM, Brinster RL. Regulation of spermatogonial stem cell self-renewal in mammals. ANNUAL REVIEW OF CELL AND DEVELOPMENTAL BIOLOGY. 2008; 24:263–286.

17. Lee J, Kanatsu-Shinohara M, Inoue K, et al. Akt mediates self-renewal division of mouse spermatogonial stem cells. DEVELOPMENT. 2007; 134:1853–1859. [PubMed: 17428826]

18. Sada A, Hasegawa K, Pin PH, et al. NANOS2 acts downstream of glial cell line-derived neurotrophic factor signaling to suppress differentiation of spermatogonial stem cells. STEM CELLS. 2012; 30:280–291. [PubMed: 22102605]

19. Ishii K, Kanatsu-Shinohara M, Toyokuni S, et al. FGF2 mediates mouse spermatogonial stem cell self-renewal via upregulation of Etv5 and Bcl6b through MAP2K1 activation. DEVELOPMENT. 2012; 139:1734–1743. [PubMed: 22491947]

20. Lucas BE, Fields C, Joshi N, et al. Mono-(2-ethylhexyl)-phthalate (MEHP) affects ERK-dependent GDNF signalling in mouse stem-progenitor spermatogonia. TOXICOLOGY. 2012; 299:10–19. [PubMed: 22564763]

21. Suzuki H, Tsuda M, Kiso M, et al. Nanos3 maintains the germ cell lineage in the mouse by suppressing both Bax-dependent and -independent apoptotic pathways. DEVELOPMENTAL BIOLOGY. 2008; 318:133–142. [PubMed: 18436203]

22. Pages G, Guerin S, Grall D, et al. Defective thymocyte maturation in p44 MAP kinase (Erk 1) knockout mice. SCIENCE. 1999; 286:1374–1377. [PubMed: 10558995]

23. Voisin L, Saba-El-Leil MK, Julien C, et al. Genetic demonstration of a redundant role of extracellular signal-regulated kinase 1 (ERK1) and ERK2 mitogen-activated protein kinases in promoting fibroblast proliferation. MOLECULAR AND CELLULAR BIOLOGY. 2010; 30:2918–2932. [PubMed: 20368360]

24. Kotaja N, Kimmins S, Brancorsini S, et al. Preparation, isolation and characterization of stage-specific spermatogenic cells for cellular and molecular analysis. NATURE METHODS. 2004; 1:249–254. [PubMed: 16144087]

25. Hasegawa K, Okamura Y, Saga Y. Notch signaling in Sertoli cells regulates cyclical gene expression of Hes1 but is dispensable for mouse spermatogenesis. MOLECULAR AND CELLULAR BIOLOGY. 2012; 32:206–215. [PubMed: 22037762]

26. Kanatsu-Shinohara M, Ogonuki N, Inoue K, et al. Long-term proliferation in culture and germline transmission of mouse male germline stem cells. BIOLOGY OF REPRODUCTION. 2003; 69:612–616. [PubMed: 12700182]

27. Suzuki A, Saba R, Miyoshi K, et al. Interaction between NANOS2 and the CCR4-NOT deadenylation complex is essential for male germ cell development in mouse. PLOS ONE. 2012; 7:e33558. [PubMed: 22448252]

28. Shaul YD, Seger R. The MEK/ERK cascade: from signaling specificity to diverse functions. BIOCHIMICA ET BIOPHYSICA ACTA. 2007; 1773:1213–1226. [PubMed: 17112607]

29. van Pelt AM, de Rooij DG. Synchronization of the seminiferous epithelium after vitamin A replacement in vitamin A-deficient mice. BIOLOGY OF REPRODUCTION. 1990; 43:363–367. [PubMed: 2271719]

30. Franca LR, Ogawa T, Avarbock MR, et al. Germ cell genotype controls cell cycle during spermatogenesis in the rat. BIOLOGY OF REPRODUCTION. 1998; 59:1371–1377. [PubMed: 9828180]

31. Timmons PM, Rigby PW, Poirier F. The murine seminiferous epithelial cycle is pre-figured in the Sertoli cells of the embryonic testis. DEVELOPMENT. 2002; 129:635–647. [PubMed: 11830565]

32. Sugimoto R, Nabeshima Y, Yoshida S. Retinoic acid metabolism links the periodical differentiation of germ cells with the cycle of Sertoli cells in mouse seminiferous epithelium. MECHANISMS OF DEVELOPMENT. 2012; 128:610–624. [PubMed: 22200512]

33. Klein AM, Nakagawa T, Ichikawa R, et al. Mouse germ line stem cells undergo rapid and stochastic turnover. CELL STEM CELL. 2010; 7:214–224. [PubMed: 20682447]

34. Nakagawa T, Nabeshima Y, Yoshida S. Functional identification of the actual and potential stem cell compartments in mouse spermatogenesis. DEVELOPMENTAL CELL. 2007; 12:195–206. [PubMed: 17276338]

35. Doupe DP, Alcolea MP, Roshan A, et al. A single progenitor population switches behavior to maintain and repair esophageal epithelium. SCIENCE. 2012; 337:1091–1093. [PubMed: 22821983]

36. Simons BD, Clevers H. Strategies for homeostatic stem cell self-renewal in adult tissues. CELL. 2011; 145:851–862. [PubMed: 21663791]

37. Doupe DP, Klein AM, Simons BD, et al. The ordered architecture of murine ear epidermis is maintained by progenitor cells with random fate. DEVELOPMENTAL CELL. 2010; 18:317–323. [PubMed: 20159601]

38. Goertz MJ, Wu Z, Gallardo TD, et al. Foxo1 is required in mouse spermatogonial stem cells for their maintenance and the initiation of spermatogenesis. THE JOURNAL OF CLINICAL INVESTIGATION. 2011; 121:3456–3466. [PubMed: 21865646]
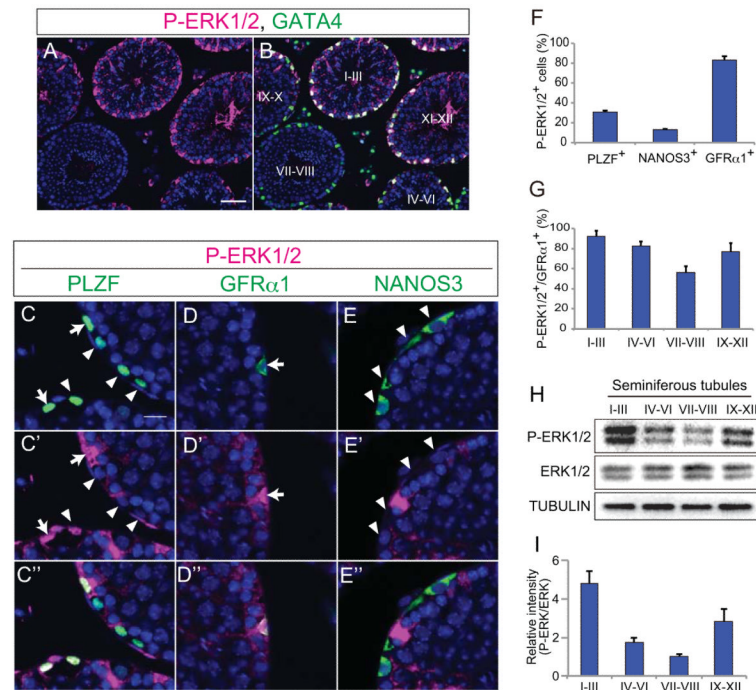
Figure 1. Stage-dependent activation of ERK1/2 in adult testes
(A,B) Immunostaining of an adult testis for phospho-ERK1/2 (magenta) and GATA4
(green), a marker for Sertoli and Leydig cells. (C-E) Immunostaining of phospho-ERK1/2
and PLZF (C-E), GFR 1 (C'-E') and NANOS3 (C''-E''). Arrows and arrowheads indicate
phospho-ERK1/2-positive and negative cells, respectively. Quantitative data are shown in
(F) (n=4). (G) Stage-dependent quantification of phospho-ERK1/2/GFR 1-double positive
cells (n=4). (H,I) Western blotting of phospho-ERK1/2 using stage-specific tubules (H), and
quantitative data of two independent experiments (I). Bars, 40 µm (A) and 15 µm (C). Error
bars, s.d.
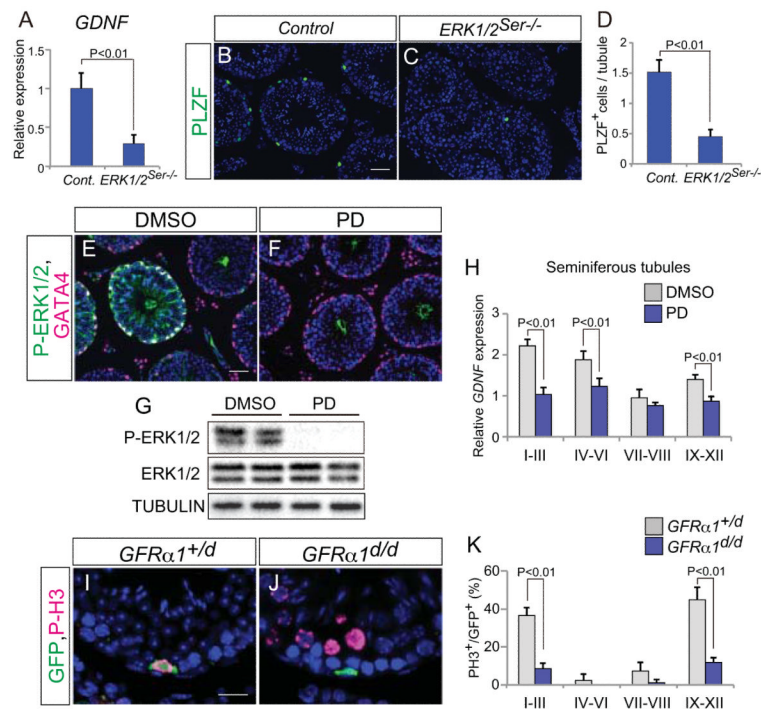
Figure 2. Involvement of ERK1/2 signaling in the stage-dependent GDNF expression
(A) *GDNF* expression in 6-week-old testes from control or *ERK1/2$^{ser-/-}$* mice (n=3). (B,C) Immunostaining of PLZF (green) in 6-week-old control or *ERK1/2$^{ser-/-}$* testes; quantitative data are shown in (D) (n=3). (E-G) PD0325901 (100 μM) was injected into adult testes and ERK1/2 phosphorylation was examined by immunostaining (E,F) and western blotting (G) 6 h after the injection. We tested eight independent testes for these experiments and confirmed reproducibility. (H) PD0325901 was injected into adult testes and stage-specific tubules were isolated 6 h after injection. *GDNF* expression in the tubules was evaluated with qRT-PCR (n=4). (I,J) Immunostaining of GFP (green) and phospho-Histone H3 (magenta) in *GFRα1$^{+/d}$* or *GFRα1$^{d/d}$* mice. Quantitative data (+/d, n=4; d/d, n=5) are shown in (K). Bars, 40 μm (A,E), 10 μm (I). Error bars, s.d.
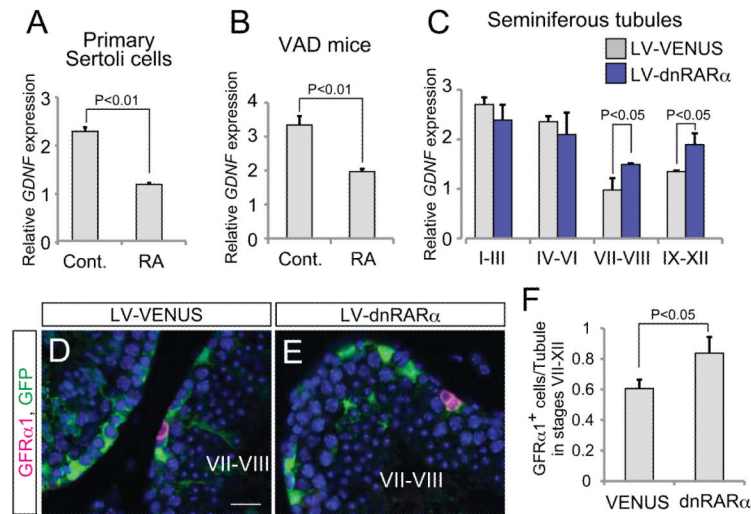
Figure 3. Effects of RA signaling on GDNF expression in Sertoli cells
(A) *GDNF* expression changes in primary Sertoli cells cultured in the presence of 1 μM RA for 24 h (n=3). (B) At 24 h after retinol injection into VAD mice, *GDNF* expression in whole testes was measured by qRT-PCR (n=3). (C) LV-dnRAR or LV-VENUS was injected into adult testes and stage-specific tubules were isolated at day 5. *GDNF* expression in the tubules was quantified by qRT-PCR (n=3). (D,E) Immunostaining of GFRα1 (magenta) and GFP (green) at day 5 after injection of LV-dnRAR or LV-VENUS. (F) Quantification of GFRα1-positive cells in tubules in stages VII-XII after injection of LV-dnRAR or LV-VENUS (LV-VENUS n=5; LV-dnRAR, n=4). Bars, 20 μm. Error bars, s.d.
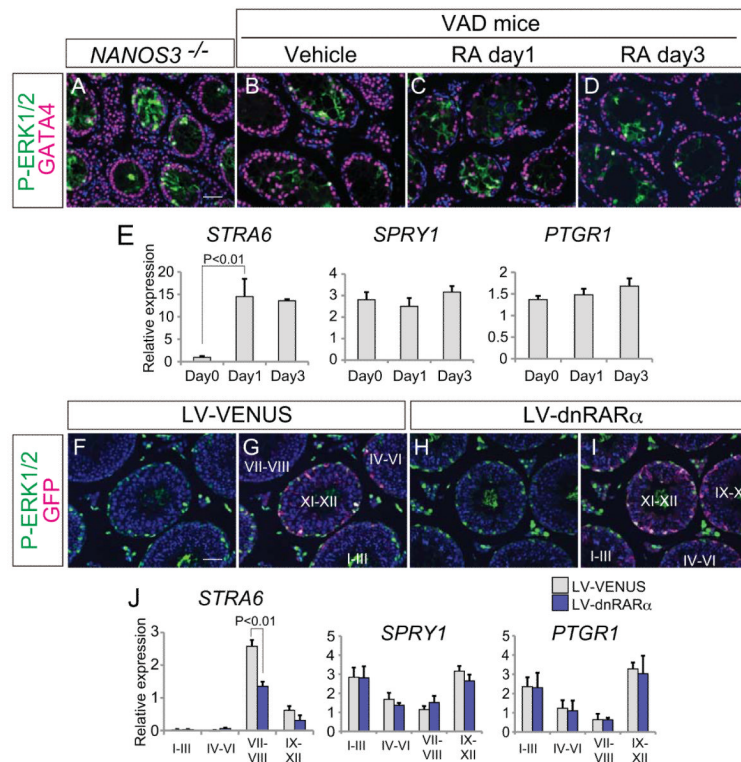
Figure 4. Activity of MEK/ERK signaling in Sertoli cells is independent of RA signaling
(A) Immunostaining of phospho-ERK1/2 (green) and GATA4 (magenta) in *NANOS3*^{−/−} testis. (B-D) Immunostaining of phospho-ERK1/2 (green) and GATA4 (magenta) in VAD mice after injection of retinol or vehicle. (E) Expression changes of stage-dependent genes regulated by RA signaling (*STRA6*) or MEK/ERK signaling (*SPRY1, PTGR1*) in VAD testes after injection of retinol or vehicle (vehicle, n=4; Day1, n=4; Day3, n=3). (F-I) Immunostaining of phospho-ERK1/2 (green) and GFP (magenta) in testes at day 5 after injection of LV-dnRAR or LV-VENUS. We checked four independent testes for this experiment. (J) Expression changes of stage-dependent genes regulated by RA signaling (*STRA6*) or ERK1/2 signaling (*SPRY1, PTGR1*) in testes at day 5 after injection of LV-dnRAR or LV-VENUS (LV-VENUS n=3; LV-dnRAR , n=3). Bars, 40 μm. Error bars, s.d.
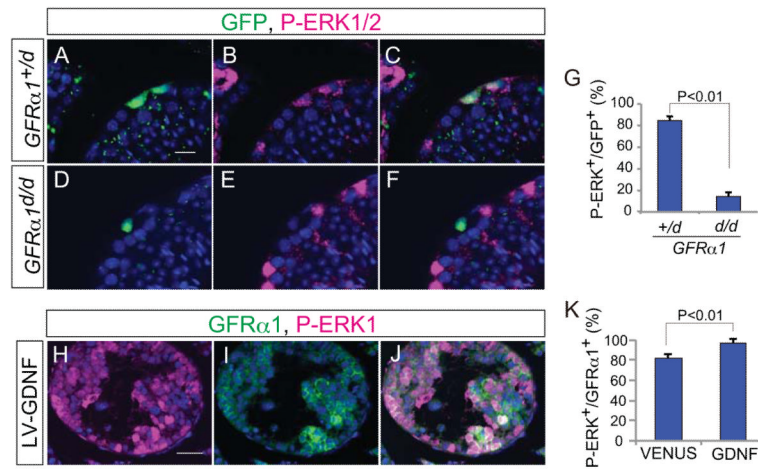
Figure 5. ERK1/2 are activated by GDNF signaling in GFRα1-positive cells
(A-F) Immunostaining of phospho-ERK1/2 (magenta) and GFP (green) in *GFRα1*$^{+/d}$ or *GFRα1*$^{d/d}$ mice at day 5 after injection of tamoxifen. (G) Quantification of phospho-ERK1/2/GFP-double positive cells in *GFRα1*$^{+/d}$ or *GFRα1*$^{d/d}$ mice (+/*d*, n=4; *d/d*, n=5). (H-J) Immunostaining of phospho-ERK1/2 (magenta) and GFRα1 (green) in testes 4 weeks after injection of LV-GDNF. (H) Quantification of phospho-ERK1/2/GFRα1-double positive cells in LV-GDNF- or LV-VENUS-injected testes (LV-VENUS, n=4; LV-GDNF, n=4). Bars, 10 μm (A), 40 μm (H). Error bars, s.d.
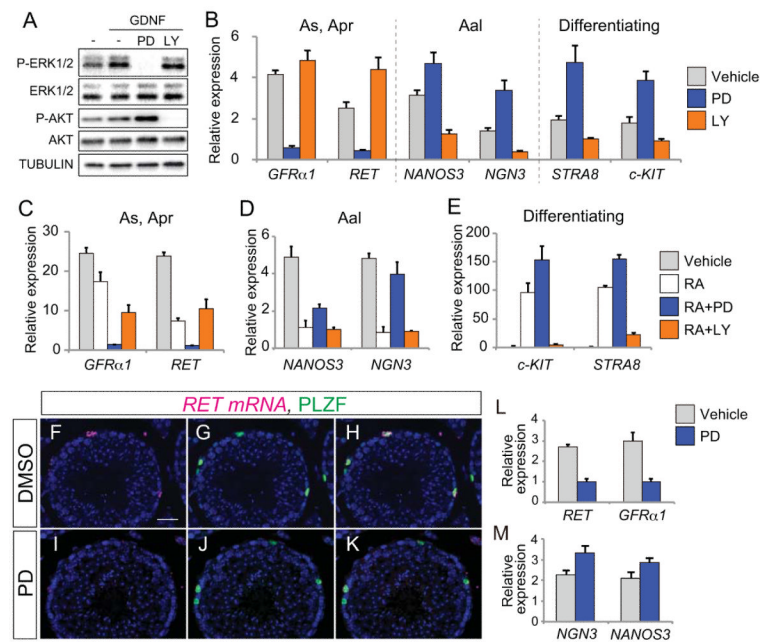
Figure 6. Undifferentiated state is maintained by ERK1/2 signaling in spermatogonial stem cells
(A) Western blotting of phospho-ERK1/2 and AKT in GS cells treated with 40 ng GDNF, 10 μM PD0325901 or 10 μM LY294002 for 20 min. (B) GS cells were cultured in the presence of PD0325901 or LY294002 for 24 h and gene expression was evaluated by qRT-PCR (n=3). (C-E) GS cells were cultured in the presence of RA combined with PD0325901 or LY294002 for 12 h, and gene expression associated with As, Apr (C), Aal (D) and differentiating spermatogonia (E) was measured by qRT-PCR (n=3). (F-K) At 6 h after PD0325901 injection, *RET* (magenta) and PLZF (green) were detected by *in situ* hybridization and immunostaining, respectively. We tested four independent testes for this experiment and confirmed reproducibility. (L,M) *RET* and *GFR 1* (L), and *NGN3* and *NANOS3* (M) mRNA expression in whole testes at 6 h after injection of PD0325901 (n=4). Bars, 40 μm. Error bars, s.d.
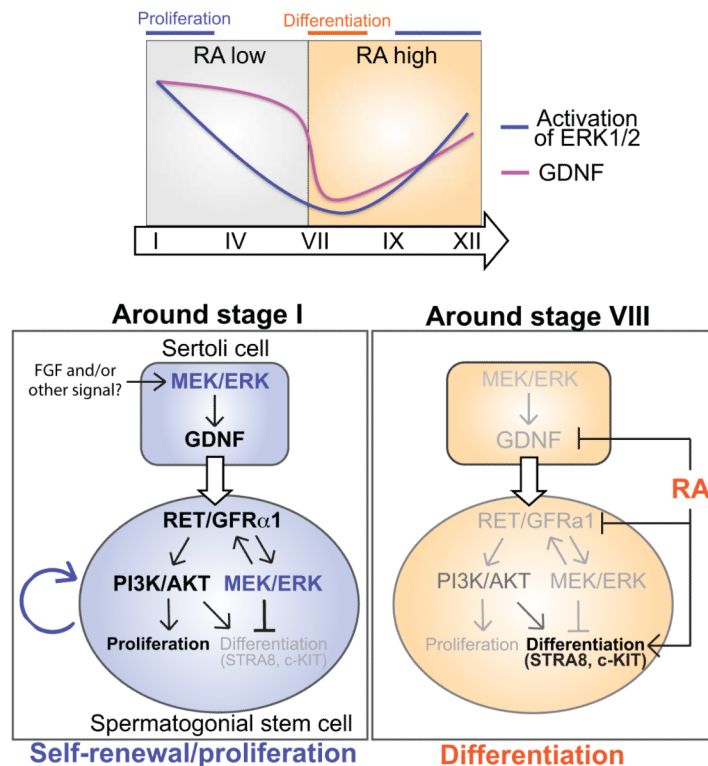
Figure 7. Model for stage-dependent regulation of spermatogonial stem cells
Around stage I in the seminiferous cycle (shown in the upper panel), the MEK/ERK pathway, regulated by an unknown signal, is activated and promotes GDNF expression in Sertoli cells, which leads to activation of MEK/ERK and PI3K/AKT signaling in spermatogonial stem cells via RET/GFR 1 co-receptors. Intrinsic MEK/ERK signaling maintains the undifferentiated state of spermatogonial stem cells by inducing GFR 1/RET expression in addition to suppression of differentiation-associated gene expression. Concomitantly, PI3K/AKT signaling stimulates proliferation of spermatogonial stem cells as well as their differentiation. This weak induction of differentiation may be compensated by ERK1/2 signaling. Around stage VIII, RA restricts local availability of GDNF by decreasing *GDNF* expression in Sertoli cells. RA also directly promotes differentiation of spermatogonial stem cells by suppressing intrinsic factors essential for maintaining their undifferentiated state and activating differentiation-associated genes such as *STRA8* and *c-KIT*. At this time, weak activation of PI3K/AKT signaling may also be required to induce differentiation.