Absence of Inhibin Alpha and Retinoblastoma Protein Leads to Early Sertoli Cell Dysfunction

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Abstract

Sertoli cells, the support cells of mammalian spermatogenesis, are regulated by a number of nuclear factors and express retinoblastoma (RB) tumor suppressor protein. We hypothesized that RB is an important mediator of Sertoli cell tumorigenesis in inhibin α knockout (Inha KO) mice. In our previous mouse studies, we found that conditional knockout (cKO) of Rb in Sertoli cells caused progressive Sertoli cell dysfunction. Initially, loss of RB had no gross effect on Sertoli cell function as the mice were fertile with normal testis weights at 6 weeks of age, but by 10–14 weeks of age, mutant mice demonstrated severe Sertoli cell dysfunction and infertility. Although double knockout (dKO) of Rb and Inha did not result in exacerbation of the tumorigenic phenotype of Inha-null mice, we found that the dKO mice demonstrate an acceleration of Sertoli cell dysfunction compared to Rb cKO mice. Specifically, in contrast to Rb cKO mice, Inha/Rb dKO mice showed signs of Sertoli cell dysfunction as early as 4 weeks of age. These results demonstrate that RB is not essential for Sertoli cell tumorigenesis in Inha KO mice but that loss of Inha accelerates the infertility phenotype of Rb cKO mice.

Introduction

Mammalian spermatogenesis is dependent on the proper functioning of Sertoli cells, the somatic support cells of germ cell maturation. During embryogenesis, immature Sertoli cells emerge at E10.5 and form the seminiferous tubules [1]. They continually replicate prenatally and postnatally and finally enter replicative senescence at P12–17 when they differentiate into their mature form [2]. During puberty, Sertoli cells must form extensive intercellular junctions to support an expanding population of differentiating germ cells undergoing the process of spermatogenesis, which involves extensive morphological differentiation of round spermatids into mature spermatozoa [1]. At 5–6 weeks of age, mice produce spermatozoa from the first wave of spermatogenesis, and previous studies suggest that this first wave of spermatogenesis is significantly different from later waves [3,4,5]. At this point, Sertoli cells must once again adapt because, unlike the first wave of spermatogenesis, subsequent waves of spermatogenesis not only depend on forming new cellular junctions but also depend on removing old ones [1]. Our previous work in this field indicated that retinoblastoma protein (RB) is essential to maintain cell cycle quiescence and junctional remodeling in mature adult Sertoli cells [6].

RB is an important intracellular regulator of the cell cycle. RB is a repressor of E2F transcription factors, which function to promote progression from G1 to the S phase of the cell cycle [7]. For the cell cycle to progress, RB is inactivated via phosphorylation by cyclin dependant kinases (CDKs). RB and RB pathway components are frequently altered in many human cancers [8], but little is known about the role of RB in Sertoli cell tumorigenesis.

Inhibins are αβ heterodimeric members of the transforming growth factor β (TGFβ) superfamily and act as competitive antagonists of activins, which are ββ homodimeric TGFβ ligands [9]. Targeted deletion of the inhibin α subunit (Inha) in mice revealed that loss of inhibin leads to the development of gonadal sex cord-stromal tumors, which originate from Sertoli cells in the male [10]. Further studies using Inha-null mice deficient in either follicle stimulating hormone (FSH) and/or luteinizing hormone (LH) revealed that these tumors were gonadotropin-dependent [11,12,13], but the intracellular regulators of Sertoli cell tumorigenesis remained unknown [14]. Subsequent studies showed that RB pathway components, cyclin D2 (Cdk4) and cyclin dependant kinase 4 (Cdk4), are increased in Inha knockout (KO) tumors while the CDK inhibitor, p27 (Cdkn1b), is decreased [15]. Double knockout (dKO) studies in our laboratory revealed that p27 [15] and cyclin D2 [16] are important modifiers of the Inha KO phenotype as Inha/Cdkn1b dKOs had accelerated cancer development, whereas Inha/Cdk2 dKOs had attenuated tumor formation.

Based on these findings, we hypothesized that RB is essential for the progression of Inha KO tumorigenesis and, similar to Inha/Cdkn1b dKO, that Inha/Rb dKO mice would demonstrate rapid tumor formation. We endeavored to carefully characterize this double knockout of Rb and Inha in the male to better understand the role of RB in Sertoli cell cycle control and tumorigenesis.
Unexpectedly, the loss of RB on an Inha deficient background does not worsen disease progression. However, our findings show that loss of inhibin accelerates Sertoli dysfunction in the Sertoli cell-specific RB knockout mice [6].

Materials and Methods

Mouse Lines and Genotyping

Generation of mice containing a null mutation in the Inha gene [10], a null [17] or floxed [18] mutation in the Rb gene, and a transgene of Cre recombinase driven by the anti-Mullerian hormone promoter (Amh-Cre) [19] have been described previously. Tail DNA was utilized for PCR genotyping that was performed for all alleles according to the manufacturer’s protocol (New England Biolabs, Ipswich, MA). Primers for the Inha alleles have been described [20] (E2-2/Ex2: 5’-GGGTCTTCTCGGCTTTG-GC-3’; INTRON: 5’-CCTGGGGTGAGAAGTATG-3’; Hprt3: 5’-GGATATGCGCCCTGACTATA-3’) and produce wild-type (550-bp) and null (850-bp) products. Primers for Rb exon 3 alleles have been described [21] (RX3: 5’-GGAATGACATCTT- TATCCGAG-3’; RI3: 5’-CAACCTTCTCGGCCCCGTG-3’; PGK: 5’-GAGGAAACAGATCTGACGGCC-3’) and produce wild-type (724-bp) and null (400-bp) products. Primers for Rb exon 19 alleles have been described [21] (Rb212: 5’-GAAGAAAGATG- CAGGACATTTG-3’; Rb18: 5’-GGCGTTGTGCGCATAT- G-3’) yielding a 748-bp product for the Rb-floxed allele, a 699-bp product for the Rb wild-type allele, and a 260-bp product for the recombined allele. PCR for Amh-Cre has been described [6] (McreAMH: 5’-AGTCGAGGCTCTGGCATTA-3’; McreGene: 5’-AATCGGGAACATCTCCGAGT-3’) and produces a 443-bp product.

Animal Care and Treatment

Mice were maintained on a 129SvEv/C57BL/6 background and housed with unlimited access to food and water and exposure to 12 h:12 h light:dark cycles in accordance with the standards of the Association for Assessment and Accreditation of Laboratory Animal Care. The Institutional Animal Care and Use Committee at the Baylor College of Medicine approved the study under approval number AN-716. Survival curves were generated as described [13]. Mice were weighed weekly from 4 weeks to a maximum of 26 weeks to monitor for symptoms of cancer cachexia and sacrificed when body weights decreased to less than 16.0 g. For serum collection, mice were anesthetized, and blood was collected by cardiac puncture. Micropipette tubes (BD) were utilized for serum isolation and sent to the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core (http://www.healthsystem.virginia.edu/internet/crr) for detection of FSH, LH, testosterone (T), and estradiol (E2).

Morphological and Histological Analysis

Immediately after cardiac puncture, mice were euthanized, and the desired tissues were harvested and weighed. In general, one testis/tumor was placed into fixative and the other was frozen for sectioning, and staining for periodic acid Schiff and hematoxylin were performed by the Histology Core of the Department of Pathology of Baylor College of Medicine.

RNA Extraction and Quantitative PCR

RNA was extracted from 4 week-old testes using the RNeasy Mini Kit (Qiagen, Valencia, CA). For quantitative PCR, the isolated RNA (n = 3 for each genotype) was converted to cDNA using SuperScriptII (Invitrogen). Primers (E2f1, Cdkn2a, Timp1) used in a SYBR green based qPCR assay were previously reported [6,22]. Bcl3 was assayed with a Taqman probe (Mm00519260_m1, Applied Biosystems). Quantitative PCR reactions were performed using Gapdh as an endogenous control for relative quantification [22].

Statistical Analysis

Statistical analysis utilized JMP 8.0.1 software (SAS Institute). Statistical significance was determined by one-tailed t test assuming unequal variance for two sample comparison and by one-way analysis of variance (ANOVA) followed by Tukey’s honestly significant difference (HSD) test for multiple sample comparisons. Survival curves were compared using the log-rank test. Groups were considered not significantly different from one another if p>0.05.

Results

Loss of Sertoli cell-expressed RB does not adversely affect disease progression in Inha-null mice

Inha KO mouse tumors arise from Sertoli cells or their precursors [10]. Since we wanted to delete RB from these tumors and Rb-null mice are embryonic lethal [17], we employed a conditional knockout (cKO) system that we had previously used to delete Rb in Sertoli cells [6] by utilizing a floxed Rb allele [18] and a Cre recombinase driven by the anti-Mullerian hormone (Amh) promoter [19]. Genotypes examined include: Inha+/- Rbflox/+ (control, Inha+/- Rb+/+), Inha+/- Rbflox/+ Amh-Cre (Rb cKO), Inha+/- Rbflx/Amh-Cre (Inha+/ ), and Inha+/- Rbflx/Amh-Cre (Inha+/Rb

Figure 1. Proof of Rb recombination in testes of 6 week-old mice. PCR amplification of the region surrounding exon 19 was performed on DNA extracted from whole testes of Inha+/+ and Inha/Rb dKO mice. As shown schematically in (A), primers flanking the loxP sites amplify the recombined Rb conditional allele to produce a 260-bp product, which is only seen in the Cre-positive mice (B). Amplifications of a 748-bp product for the Rb-floxed allele and a 699-bp product for the Rb wild-type/null allele were used as loading controls (B). doi:10.1371/journal.pone.0011797.g001
As shown in Figure 1, recombination was confirmed to occur in the dKO mice using 6 week-old whole testes. After we had confirmed that the \( \text{Rb} \) allele was recombined in our dKO mice, all groups of mice were weighed weekly starting at 4 weeks of age to monitor disease progression since \( \text{Inha} \) KO mice experience death secondary to cancer cachexia [23]. We monitored these groups until they reached 26 weeks of age and found that while 100% of control and \( \text{Rb} \) cKO mice survived until the end of the observation period, only 8% of \( \text{Inha}^{−/−} \) and 10% of \( \text{Inha/Rb} \) dKO mice survived to that age (Figure 2A). The difference in mortality between \( \text{Inha}^{−/−} \) and \( \text{Inha/Rb} \) dKO mice was not significant (\( p > 0.05 \) by log-rank test), and both groups had 50% survival at 14 weeks of age (Figure 2A).

Disease progression was also monitored by examining other parameters. Tumor burden of the combined gonads was determined at 6 weeks, 10 weeks, and end of life. \( \text{Inha/Rb} \) dKO mice did not differ significantly from \( \text{Inha}^{−/−} \) mice at every age examined (Figure 2B, \( p > 0.05 \)). Gonadal tumors in \( \text{Inha}^{−/−} \) mice secrete an excess of activins, which signal through type 2A activin receptors (ACVR2A) to cause a cachexia wasting syndrome characterized by decreased body weights and liver weights [23,24]. An examination of body weights from 4–10 weeks of age showed no significant differences between \( \text{Inha}^{−/−} \) and \( \text{Inha/Rb} \) dKO (Figure 3A, \( p > 0.05 \)). Similarly, liver weights from 6 weeks, 10 weeks, and end of life were also not significantly different between these groups (Figure 3B, \( p > 0.05 \)).

Figure 2. Double knockout of \( \text{Inha} \) and \( \text{Rb} \) in Sertoli cells does not significantly affect disease progression. Control mice have 100% survival until 26 weeks of age (A). During the same period, \( \text{Inha}^{−/−} \) (\( n = 25 \)) and \( \text{Inha/Rb} \) dKO (\( n = 20 \)) mice reach 50% survival at 14 weeks of age and are not significantly different by log-rank test (A, \( p > 0.05 \)). Tumor burden is also not significantly different between these two groups at 6 weeks, 10 weeks, and end of life (B, \( p > 0.05 \)).

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Figure 3. Double knockout of \( \text{Inha} \) and \( \text{Rb} \) in Sertoli cells does not significantly affect the activin-induced wasting syndrome. Body weights (A) and liver weights (B) are not significantly different between age-matched \( \text{Inha}^{−/−} \) and \( \text{Inha/Rb} \) dKO mice (\( p > 0.05 \)). Control liver weights are shown for comparison. Different letters represent statistically different groups.

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Inha/Rb Double Knockout

Table 1. Serum Hormone Levels for 10 wk-old Males.

| Genotype       | FSH (ng/ml) | LH (ng/ml) | T (ng/dl) | E2 (pg/ml) |
|----------------|-------------|------------|-----------|------------|
| Inha<sup>−/−</sup> Rb<sup>−/−</sup> (n = 5) | 15.7 ± 1.7  | 0.08 ± 0.03 | 46.7 ± 8.2 | 5.2 ± 0.2* |
| Inha<sup>−/−</sup> (n = 9) | 26.8 ± 3.6  | 0.14 ± 0.04 | 60.1 ± 9.8 | 83.7 ± 15.0* |
| Inha/Rb dKO (n = 9) | 23.0 ± 2.6  | 0.16 ± 0.03 | 90.4 ± 22.1 | 25.2 ± 14.2* |

Values are means ± standard errors. Statistically different values by Tukey-Kramer HSD are represented by different letters in superscript (a vs. b, p < 0.05). N.S., Not significant by one-way ANOVA (p > 0.05). *FISH values of Inha<sup>−/−</sup> Rb<sup>−/−</sup> vs. Inha<sup>−/−</sup> and Inha<sup>−/−</sup> Rb<sup>−/−</sup> vs. Inha/Rb dKO are significantly different by one-tailed t test (p < 0.05).

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Serum hormone levels were also measured in 10 week-old mice, due to their implications in disease progression [12,13,25]. No significant differences were detected between Inha<sup>−/−</sup> and Inha/Rb dKO levels of FSH, LH, or testosterone (T) (Table 1). The only significant difference detected was in estradiol (E2) levels, which were significantly decreased in Inha/Rb dKO mice as compared to Inha<sup>−/−</sup> mice (Table 1). Although prior studies have indicated that loss of estrogen receptors in Inha-null tumors prevented early tumorigenesis and death [25], we saw no functional consequence of this decreased estradiol level as disease progression in Inha/Rb dKO mice did not differ significantly from Inha<sup>−/−</sup> mice, as outlined previously.

Loss of Inha accelerates the progressive Sertoli cell dysfunction observed in Sertoli cell-specific RB deficient mice

Since we could not discern any striking differences between Inha<sup>−/−</sup> and Inha/Rb dKO mice using the criteria described above, we sought to examine mice from younger stages of disease progression. Histology of 4 week-old and 6 week-old Inha/Rb dKO testes did not show increased tumorigenesis when compared to age-matched Inha<sup>−/−</sup> testes (data not shown). However, when we examined the non-tumorigenic areas that contained intact seminiferous tubules, we observed an acceleration of the progressive infertility phenotype of the Rb cKO mice [6]. In Rb cKO mice, histological signs of Sertoli cell dysfunction, such as vacuolization, are not observed until roughly 8 weeks of age (Figure 4, top row middle inset) [6]. Loss of the tubular lumen, severely decreased tubular widths, and severe loss of germ cells generally occurs at 10 weeks of age (Figure 4, bottom row middle inset) [6]. In Inha/Rb dKO testes, there were prominent signs of vacuolization (Figure 4, top row right panel, arrows) in 4 week-old tubules that were not observed in either Inha<sup>−/−</sup> or Rb cKO testes (Figure 4, top row). However, 4 week-old Inha<sup>−/−</sup>, Rb cKO, and
Inha/Rb dKO testes showed similar germ cell compositions (Figure 4, top row). In 6 week-old Inha/Rb dKO testes, there were signs of severe Sertoli cell dysfunction, such as loss of tubular lumens, decreased tubular widths, and loss of advanced germ cells, that resembled much older Rb cKO mice (Figure 4, bottom row). Examination of 6 week-old Inha−/− and Rb cKO testes revealed multiple tubules with full complements of germ cells, including elongating spermatids, and intact tubular widths and lumens (Figure 4, bottom row). Some Inha−/− tubules showed loss of elongating spermatids (Figure 4, asterisk), but overall, the seminiferous tubules of 6 week-old Inha−/− and Rb cKO mice showed far less dysfunction than 6 week-old Inha/Rb dKO mice.

To investigate the cause of the accelerated Sertoli cell dysfunction in Inha/Rb dKO mice, we explored changes in mRNA levels of genes that were implicated in the pathogenesis of the Rb cKO phenotype [6]. We used RNA from whole testes of 4 week-old mice that exhibited only minor histological differences from control mice to examine select gene changes. Genes related to Rb cKO apoptotic defects (E2f1, Cdkn2a, and Bbc3/PUMA) and differentiation defects (Timp1) were all significantly increased in Inha/Rb dKO testes as compared to control (Figure 5).

**Discussion**

Whereas absence of p27 in Inha KO mice accelerates the formation of Sertoli cell tumors [15], our present studies indicate that RB plays a minor or redundant role in Inha KO tumorigenesis. By following the disease progression of Inha/Rb dKO mice as compared to Inha−/− mice, we determined that loss of RB does not significantly affect a number of parameters associated with Inha KO tumorigenesis, including survival, tumor burden, body weight, or liver weight. However, our findings do show that the progressive Sertoli dysfunction exhibited in Rb cKO mice [6] is accelerated by the additional loss of inhibin α.

Activin levels are pathologically increased in Inha-null mice [23] and are the major cause of tumorigenesis in these mice. This is suggested by studies in which decreased levels of free activins decreased tumor progression and disease severity [26]. Also, double knockout of Inha and Smad3, the activin-responsive transcription factor, resulted in attenuation of Sertoli cell tumorigenesis [20,27]. These studies suggest that pathologically increased activins are in large part responsible for Sertoli cell tumorigenesis in Inha-null mice. Why is it that Sertoli cells react by becoming tumorigenic since the entire organism is exposed to increased activins in the bloodstream? Previous studies conducted in our lab indicated that gonadotropins, especially FSH, are essential for the progression of Inha KO tumorigenesis in male mice [11,12]. Perhaps, it is the gonadotropin-responsiveness of Sertoli cells that makes them uniquely susceptible to pathological activin signaling. FSH signaling in Sertoli cells can activate the Ras-related mitogen activated signaling pathway involving ERK1 (mitogen-activated protein kinase 3) and ERK2 (mitogen-activated protein kinase 1), which subsequently upregulate cyclin D1 [28]. CDK4/cyclin D complexes and other Ras-related kinases modulate the phosphorylation of SMAD3 [29,30,31], and this
modulation of SMAD3 causes its downstream signaling to change from tumor suppressive to oncogenic [29,30]. We were very surprised at the minor effect of loss of RB on the disease progression in Inha-null mice. However, since our hypothesis was based on double knockout models that suggested that RB pathway components, specifically p27 and cyclin D2, modulate Inha KO tumorigenesis, we speculate that crosstalk between the RB and activin/FSH pathways were responsible for our previous results. In our model (Figure 6), the pathway of FSH signaling is crucial for establishing crosstalk between signaling of the components of the RB pathway of cell cycle control (cyclin D1/2) and activin signaling to result in tumorigenesis; however, retinoblastoma protein itself is not crucial for oncogenesis in this model. It will be important in the future to study the expression levels of activin pathway components and RB pathway components (e.g., cyclin D1) in our Rb/Inha dKO mouse to further clarify if our model of Inha KO tumorigenesis is correct.

Retinoblastoma protein is a major determinant of Sertoli cell maturation, and mice with Sertoli cell-specific depletion of RB have progressively dysfunctional testes after puberty [6]. In light of this knowledge, it is quite interesting that deletion of Rb on an Inha-null background causes acceleration of the phenotype of progressive Sertoli cell dysfunction. This could be due to increased FSH signaling in Inha-null mice as FSH levels are not increased in Rb cKO mice until older ages [6]. Additionally, activation of SMAD3 in Inha-null Sertoli cells may cause an elevation of tumorigenic gene expression that may subsequently increase E2F1- and p53-target genes (Figure 6). We propose that these pathways converge to affect Sertoli cell function in Inha/Rb dKO mice.

Previously, we proposed that RB was important for Sertoli cell differentiation because of its interactions with androgen receptor [6]. The current studies raise the possibility that the pathological expression of cell cycle genes on an RB-deficient background are
also directly related to Sertoli cell differentiation. Strikingly, we found that Temp1, a gene important to the inhibition of collagen-remodeling that is highly expressed in immature Sertoli cells, was upregulated in Inha/Rb dKO mice. Temp1 was also highly upregulated in Sertoli cell-specific Rb cKO mice. The early upregulation of Temp1 in Inha/Rb dKO testes as compared to Rb cKO testes suggests that differentiation genes like Temp1 may be related to cell cycle pathways downstream of Rb and SMAD3. Elucidation of our proposed model will be vital to better understanding the functions and regulation of the Sertoli cell.

References

1. Skinner MK, Griswold MD (2005) Sertoli cell biology. Amsterdam; Boston: Elsevier Academic Press. xv, 494 p., [12] p. of plates.
2. Vergouwen RP, Jacobs SG, Haikamp R, Davids JA, de Rooij DG (1991) Proliferative activity of gonocytes, Sertoli cells and interstitial cells during testicular development in mice. J Reprod Fertil 93: 233–243.
3. Falender AE, Freiman RN, Geles KG, Lo KG, Hwang K, et al. (2005) Maintenance of spermatogenesis requires TAF4b, a gonad-specific subunit of TFIIID. Genes Dev 19: 794–803.
4. Beamer WG, Cundiff-Beamer TL, Shultz KL, Langley SH, Roderick TH (1988) Juvenile spermatogonial depletion (jdu): a genetic defect of germ cell proliferation of male mice. Biol Reprod 38: 899–906.
5. Chen C, Ouyang W, Grippura V, Zhou Q, Carus K, et al. (2005) ERM is required for transcriptional control of the spermatogonial stem cell niche. Nature 436: 1030–1034.
6. Nalam KL, Andreu-Veyra C, Braun RE, Akiyama H, Matzuk MM (2009) Retinoblastoma protein plays multiple essential roles in the terminal differentiation of Sertoli cells. Mol Endocrinol 23: 1900–1913.
7. Nguyen DX, McCance ID (2005) Role of the retinoblastoma tumor suppressor protein in cellular differentiation. J Cell Biochem 94: 870–879.
8. Burkhardt DL, Sage J (2008) Cellular mechanisms of tumour suppression by the retinoblastoma gene. Nat Rev Cancer 8: 671–682.
9. Chang H, Brown CW, Matzuk MM (2002) Genetic analysis of the mammalian transforming growth factor-beta superfamily. Endocr Rev 23: 787–823.
10. Matzuk MM, Finegold MJ, Mather JP, Krummen L, Lu H, et al. (1994) Development of cancer, the basis for cancer therapy. Science 266: 1571–1575.
11. Kumar TR, Wang Y, Matzuk MM (1996) Gonadotropins are essential modifier of the gonadal tumor suppressor gene in mice. J Biol Chem 271: 30008–30013.
12. Kumar TR, Palapattu G, Wang P, Woodruff TK, Boime I, et al. (1999) Retinoblastoma tumour suppressor gene is required for transcriptional control of the spermatogonial stem cell niche. Nature 436: 1030–1034.
13. Nagaraja AK, Agno JE, Kumar TR, Matzuk MM (2008) Luteinizing hormone stimulates mammalian transforming growth factor-beta superfamily. Endocr Rev 29: 19–28.
14. Li Q, Graff JM, O’Connor AE, Loveland KL, Matzuk MM (2007) SMAD3 regulates gonadal tumorigenesis. Mol Endocrinol 21: 2472–2486.
15. Cipriano SC, Chen L, Burns KH, Koff A, Matzuk MM (2001) Inhibition of p27 interact to regulate gonadal tumorigenesis. Mol Endocrinol 15: 983–996.
16. Burns KH, Agno JE, Szczeklik P, Matzuk MM (2003) Cyclin D2 and p27 are tissue-specific regulators of tumorigenesis in inhibin alpha knockout mice. Mol Endocrinol 17: 2053–2069.
17. Jacke T, Faletz A, Schmitt EM, Bronson RT, Goodell MA, et al. (1992) Effects of an Rb mutation in the mouse. Nature 359: 295–300.
18. Marino S, Vooijs M, van Der Gelden H, Jonkers J, Berns A (2000) Induction of medulloblastomas in p53-null mutant mice by somatic inactivation of Rb in the external granular layer cells of the cerebellum. Genes Dev 14: 994–1004.
19. Holdcroft RW, Braun RE (2004) Androgen receptor function is required in Sertoli cells for the terminal differentiation of haploid spermatids. Development 131: 459–467.
20. Li Q, Graff JM, O’Connor AE, Loveland KL, Matzuk MM (2007) SMAD3 regulates gonadal tumorigenesis. Mol Endocrinol 21: 2472–2486.
21. Vooijs M, van der Valk M, te Riele H, Berns A (1998) Fip-mediated tissue-specific inactivation of the retinoblastoma tumor suppressor gene in the mouse. Oncogene 17: 1–12.
22. Andreu-Veyra C, Chen R, Matzuk MM (2007) Effects of granulosa cell-specific deletion of Rb in Inha-alpha null female mice. Endocrinology 148: 3837–3849.
23. Matzuk MM, Finegold MJ, Mather JP, Krummen L, Lu H, et al. (1994) Development of cancer, the basis for cancer therapy. Science 266: 1571–1575.
24. Coerver KA, Woodruff TK, Finegold MJ, Mather J, Bradley A, et al. (1996) Activin signaling through activin receptor type II causes the cachexia-like symptoms in inhibin-deficient mice. Mol Endocrinol 10: 534–543.
25. Burns KH, Agno JE, Chen L, Haupt B, Ojibonna SC, et al. (2003) Sexually dimorphic roles of steroid hormone receptor signaling in gonadal tumorigenesis. Mol Endocrinol 17: 2039–2052.
26. Li Q, Kumar R, Underwood K, O’Connor AE, Loveland KL, et al. (2007) Prevention of cachexia-like syndrome development and reduction of tumor progression in inhibin-deficient mice following administration of a chimeric activin receptor type II-murine Fc protein. Mol Hum Reprod 13: 673–683.
27. Looyenga BD, Hammer GD (2007) Genetic removal of Smad3 from inhibin-null mice attenuates tumor progression by uncoupling extracellular mitogenic signals from the cell cycle machinery. Mol Endocrinol 21: 2440–2457.
28. Crepieux P, Marion S, Martinat N, Fafur V, Vern YL, et al. (2001) The ERK-dependent signalling is stage-specifically modulated by FSH, during primary Sertoli cell maturation. Oncogene 20: 4696–4709.
29. Matsuzaki K, Kitano C, Murata M, Sekimoto G, Yoshida K, et al. (2009) Smad2 and Smad3 phosphorylated at both linker and COOH-terminal regions transmit malignant TGF-beta signal in later stages of human colorectal cancer. Cancer Res 69: 5332–5339.
30. Sekimoto G, Matsuzaki K, Yoshida K, Mori S, Murata M, et al. (2007) Reversible Smad-dependent signaling between tumor suppression and oncogenesis. Cancer Res 67: 5090–5096.
31. Matsuura I, Denissova NG, Wang G, He D, Long J, et al. (2004) Cyclin-dependent kinases regulate the antiproliferative function of Smads. Nature 430: 226–231.

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Author Contributions

Conceived and designed the experiments: RLN CVAV MMM. Performed the experiments: RLN. Analyzed the data: RLN. Wrote the paper: RLN.