Repulsive Guidance Molecule b (RGMb) Is Dispensable for Normal Gonadal Function in Mice

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Abstract

Bone morphogenetic protein (BMP) signaling plays an important role in spermatogenesis and follicle development. Our previous studies have shown that repulsive guidance molecule b (RGMb, also known as Dragon) is a coreceptor that enhances BMP2 and BMP4 signaling in several cell types and that RGMb is expressed in spermatocytes and spermatids in the testis and in oocytes of the secondary follicles in the ovary. Here, we demonstrated that specific deletion of Rgm in germ cells in testis and in oocytes of the secondary follicles in the ovary. Here, we demonstrated that specific deletion of RGMb-deficient mice die at early postnatal ages, homozygous RGMb-deficient mice die at early postnatal ages, but RGMb-deficient mice die at early postnatal ages, however, BMP2 or BMP4 signals are transduced through BMPRII but not through ActRIIA and ActRIIB. The utilization of both BMPRII and ActRIIA leads to an increased signal in response to BMP2 or BMP4 [18–20]. In addition, the RGM proteins can also function as ligands for the neogenin receptor [21–24].

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INTRODUCTION

Bone morphogenetic proteins (BMPs) make up the largest subfamily of the TGFβ superfamily. All members in this subfamily signal through type I and type II serine/threonine kinase receptors. Ligand binding to receptors induces phosphorylation of the type I receptor by the constitutively active type II receptor. Type I receptors then phosphorylate the downstream receptor-activated Smads (R-Smads). The BMP subfamily signals via Smad1, Smad5, and Smad8, while the TGF-β and activin subfamilies signal via Smad2 and Smad3. All R-Smads then form heteromeric complexes with Smad4 before migrating from the cytoplasm to the nucleus to control gene transcription [1].

BMPs play critical roles in a wide variety of developmental, physiologic, and pathologic processes, including spermatogenesis and follicle development. BMP4, BMP8a, and BMP8b have been shown to regulate primordial germ cell specification during embryonic development and are required for normal spermatogenesis in adult mice [2–5]. The significance of BMPs in ovarian function is underscored by Inverdale and Booroola sheep, which show dramatic changes in ovulation rates due to a natural point mutation in the BMP15 gene and in the BMPRIIB gene, respectively [6, 7]. Genetic deletion of BMP6, ALK3, ALK6, Smad1, or Smad5 results in impaired follicle development in mice [8–12]. Therefore, it is quite clear that BMP signaling plays important roles in regulating reproduction.

Repulsive guidance molecule b (RGMb) is a member of the RGM family, which consists of RGMa, RGMb (Dragon), and RGMc (hemojuvelin). RGM proteins share 50%–60% sequence homology and have similar structural features, including a signal sequence, conserved proteolytic cleavage site, partial von Willebrand factor type D domain, and a glycosphatidylinositol (GPI) anchor. RGM proteins are retained on the outer layer of the plasma membrane through the GPI anchor motif [13, 14]. Our biochemical studies have revealed that all three RGM members function as coreceptors that enhance BMP signaling [14–17]. RGM proteins bind directly to BMP2 and BMP4 but not BMP7, activin, TGFβ1, TGFβ2, and TGFβ3. RGM proteins also interact with BMP type II and type I receptors. Importantly, we found that RGM members facilitate the use of ActRIIA by BMP2 and BMP4 ligands. BMP2 or BMP4 signal is normally transduced through BMPRII but not through ActRIIA and ActRIIB.

Although RGMb was first cloned from neural tissues, it is also expressed outside the nervous system in such sites as the gonads and kidney [14, 20, 25]. The biological function of RGMb is only beginning to be elucidated. We have shown that homozygous RGMb-deficient mice die at early postnatal ages,
suggesting an essential role of RGMb for survival [26]. RGMb inhibits IL-6 expression in macrophages [26] and promotes neurite outgrowth and peripheral nerve regeneration through the BMP pathway [27]. RGMb binds to PD-L2 to promote respiratory tolerance [28]. RGMb also regulates renal epithelial cell apoptosis in injured kidneys [23]. In addition, RGMb expression increases with colon cancer development and promotes colon cancer cell growth [29].

RGMb is highly expressed in the testis and ovary [14, 25]. Using immunohistochemistry and in situ hybridization, we previously showed that RGMb is expressed in spermatocytes and round spermatids but not in spermatogonia and Sertoli cells in adult mouse testis. Within the adult ovary, RGMb is detected exclusively in oocytes. A stronger signal is seen in the oocytes of secondary follicles compared to the oocytes of antral follicles, and no RGMb expression was found in primordial and primary follicles [14]. To identify the specific role of RGMb in the testis and ovary, we generated germ cell-specific Rgmb knockout mice by crossing breeding floxed Rgmb mice with Stra8-icre mice. We found that gonad-specific RGMb ablation did not alter gonadal structure and functions.

MATERIALS AND METHODS

Generation of Gonad-Specific Rgmb Knockout Mice (Conditional Knockout)

The Rgmb targeting vector was generated by the Massachusetts General Hospital Gene Knockout Center. The targeting vector contains Loxp-sites flanking Exon I of the Rgmb gene and a neomycin gene flanked by FRT-sites (Fig. 1A). After homologous recombination, the expected targeted allele was confirmed by Southern blot analysis of ES DNA using probes inside and outside the targeted region. Germ line transmission of the floxed allele was achieved after breeding of chimeras derived from floxed ES cells (C57BL/6) with C57BL/6 mice. The FRT-flanked neomycin gene was removed by crossing breeding floxed mice with FLP mice. Germ line excision of the loxP-flanked region in spermatocytes and oocytes to establish conditional gonad knockout mice (cKO) was achieved by interbreeding with Stra8-ice mice, a transgenic line, in which cre expression is driven by a 1.4-Kb promoter region of the germ cell-specific stimulated by retinoic acid gene 8 (Stra8) [30]. Stra8 is first expressed in the mitotic germ cells of postnatal testes and subsequently in spermatogonia of adult testis. Stra8 is also expressed in ovarian germ cells from E12.5 to E16.5 in female embryos, but it is not expressed in adult ovaries [31, 32]. Stra8 plays a role in premeiotic DNA replication and the events of prophase I in germ cells of both sexes [33].

FPL transgenic mice and Stra8-ice transgenic mice were purchased from the Jackson Laboratory. Both of the mouse lines are on C57BL/6J background.

Immunohistochemistry

Immunohistochemistry for RGMb in the testis and ovary was performed as previously described [14]. Antigen retrieval was performed on paraffin sections in 0.1 M citrate buffer (pH 6.0). Tissue sections were incubated overnight with a previously validated rabbit anti-RGMb antibody [13–15, 20]. The signals were developed using Histostain Plus LAB-SA Detection System (Invitrogen). Sections were then counterstained with Harris hematoxylin.

Western Blotting

Western blot analyses were performed as previously described [14]. Briefly, the samples were lysed in TBS (Tris-HCl, 50 mM, NaCl 150 mM, 2% n-octylglucoside, pH 7.4) containing protease inhibitor mixture (Pierce Biotechnology) and phosphatase inhibitor mixture (Pierce Biotechnology) for 30 min on ice. After centrifugation for 10 min at 4°C, the supernatant was assayed for protein concentration by colorimetric assay (BCA kit, Pierce). The lysates were subjected to Western blotting analysis under reducing conditions using the previously validated 307.1H6 RGMb antibody [28], anti-phospho-Smad1/5/8 antibodies (Cell Signaling Technology), anti-Smad1 antibodies (Cell Signaling Technology), and anti-β-actin antibodies (Sigma-Aldrich) as indicated.

RESULTS

Fertility Test

To test the fertility of Rgmb cKO male mice, 6-wk-old male Rgmbf/f or Rgmbf/f-Stra8-icre males derived from the breeding strategy described in Figure 1B were mated to 6-wk-old WT C57BL/6 female mice. Similarly, to test the fertility of Rgmb cKO female mice, 6-wk-old female Rgmbf/f or Rgmbf/f-Stra8-icre females derived from the breeding strategy illustrated in Figure 5A were mated to 6-wk-old WT male mice. Rgmbf/f and Rgmbf/f-Stra8-ice females from the breeding strategy in Figure 1B showed fertilities similar to Rgmbf/f, thus, they were also included as control. Cages were monitored every 1 or 2 days for the presence and number of newborn pups. The pairing was terminated when female mice failed to produce a litter for 3 mo.

Histology

Tests were isolated from mice at 2 and 13 mo of age and ovaries from mice at 14 days and 4 mo of age. The tissues were fixed in Bouin solution (Sigma-Aldrich), and the ovaries were fixed in 4% paraformaldehyde overnight. The tissues were then processed for paraffin embedding. Sections (6 μm in thickness) were stained with hematoxylin and eosin (H&E) and photographed.
levels in the Rgmb cKO testes. Consistent with our previous observation [14], RGMb protein was highly expressed in spermatocytes and round spermatids but not in spermatogonia and elongating spermatids in control testis as determined by immunohistochemistry. RGMb expression was not detected in the Rgmb cKO testes (Fig. 2C).

Genotyping on progeny from the breeding between male Rgmbf/f-Stra8-icre (Rgmb cKO) mice and female WT mice showed recombined allele and WT allele but no floxed alleles, while the progeny from the control mating (male Rgmbf/wt and female WT mice) carried floxed allele at the expected ratio (Fig. 2D), indicating that all the floxed alleles had undergone cre-mediated recombination in the ovaries of some female Rgmbf/f mice, resulting in cre-mediated excision, Rgmbdel mice were also observed.

Our previous studies have shown that RGMb is a coreceptor that enhances BMP signaling. Therefore, we examined Smad1/5/8 phosphorylation levels in Rgmb cKO testis. As shown in Figure 2B, phospho-Smad1/5/8 levels in testis were similar between Rgmb cKO and control mice.

We then examined the testis weights of Rgmb cKO mice. The testes of Rgmb cKO mice were slightly but significantly heavier than those from control littermates at 2 mo of age (Fig. 3A). However, the testes weights became similar between the two genotypes in older mice at 13 mo of age (Fig. 3B).
Histologically, Rgmb cKO testes were not distinguishable from the control testes at 2 mo of age (Fig. 3C). In the testes from 13-mo-old Rgmb cKO mice, some seminiferous tubules lacked germ cells and developed vacuoles, but this type of abnormal tubules was also seen in the control testis (Fig. 3D).

To determine whether deletion of Rgmb in male germ cells affects fertility, we paired 6-wk-old male Rgmbf/f or Rgmbf/f-Stra-icre with 6-wk-old WT C57BL/6 female mice and monitored the number of pups born over the reproductive ages. The accumulative numbers of progeny were similar between the two genotypes (Fig. 4).

Global Rgmb Knockout (gKO) Mice Showed Normal Follicle Development

Our previous study showed that Rgmb gKO mice die 2–3 wk after birth [26]. We also previously demonstrated a complete ablation of RGMb protein in the oocytes of Rgmb gKO mice [25]. We collected ovaries from 14-day-old WT and Rgmb gKO mice for histological analysis. As shown in Figure 5, the structures of Rgmb gKO ovaries appeared to be normal. Both WT and Rgmb gKO ovaries contain small antral follicles and follicles at earlier stages.

Oocyte-Specific Rgmb Knockout (cKO) Mice Showed Normal Follicle Development and Fertility

To examine the role of RGMb in the ovarian functions in adult mice, we obtained oocyte-specific Rgmb knockout mice (cKO) using Stra8-icre mice. Stra8-icre was found to be not functional in oocytes [30]. However, endogenous Stra8 is expressed in ovarian germ cells from E12.5 to E16.5 in female embryos, although it is not expressed in adult ovaries [31, 32]. Therefore, we examined whether cre-mediated floxed fragment excision takes place in the ovaries of Rgmbf/del-Stra8-icre mice by examining the genotypes of progeny from the breeding of female Rgmbf/del-Stra8-icre with male WT mice (Fig. 6). We already showed a complete deletion of Rgmb in male germ cells in Rgmbf/del-Stra8-icre testis (Fig. 2C). In other words, all the sperm from Rgmbf/del-Stra8-icre males to mate with WT females, both floxed alleles and WT alleles but not recombined alleles were detected.

**FIG. 2.** Ablation of Rgmb in Rgmb cKO testis. A) Rgmb mRNA levels in the testes of control (Ctrl, Rgmbf/f) and Rgmb cKO (Rgmbf/f-Stra8-icre) mice. Testes collected from male WT and Rgmb cKO mice at 2 mo of age were analyzed for Rgmb mRNA levels by real-time PCR. B) RGMb protein and BMP signaling in the testes of control and Rgmb cKO mice. Testes collected from male WT and Rgmb cKO mice at 6 mo of age were analyzed for RGMb protein and Smad1/5/8 phosphorylation levels by Western blotting. C) Cellular localization of RGMb in the testes of control (Rgmbf/wt) and Rgmb cKO (Rgmbf/f-Stra8-icre) mice at 2 mo of age. All sections were stained with DAB (brown) and counterstained with hematoxylin (blue). RGMb is expressed in spermatocytes and round spermatids but not in spermatogonia. Bars = 60 μm. D) Representative genotypes of the offspring from an Rgmbf/wt-Stra8-icre male mated with a WT female. Genomic DNA isolated from tail tips were used to perform genotyping using primers 1 and 2 (P1/2) and primers 1 and 6 (P1/6), as illustrated in Figure 1. Only WT and recombined alleles were observed, and no floxed allele was transmitted to the offspring. In contrast, in the offspring from the breeding of an Rgmbf/del male with a WT female, both floxed alleles and WT alleles but no recombined alleles were detected. n = 3 for A. *P < 0.01.
suggests a complete cre-mediated recombination in all the oocytes of the five female Rgmb<sup>f/del</sup>-Stra8-icre parental mice. These five female mice were designated as Rgmb cKO. Other female Rgmb<sup>f/del</sup>-Stra8-icre mice were able to transmit the floxed allele to their progeny, indicating that cre-mediated excision did not occur in their ovaries. Western blotting demonstrated reduced RGMb protein expression in the ovaries of Rgmb cKO mice compared with control mice (Fig. 6C). Consistent with our previous results [14], RGMb was expressed in the oocytes of secondary follicles but not in the primordial and primary follicles in control mice (Fig. 6D). RGMb expression was not detected in the oocytes of Rgmb cKO mice (Fig. 6D).

We also examined BMP signaling in RGMb cKO ovaries. Western blotting showed that phospho-Smad1/5/8 levels in ovary were similar between Rgmb cKO and control mice (Fig. 6C).

We then examined the ovary structure of the Rgmb cKO mice at 4 mo of age (Fig. 6E). Follicle development in Rgmb cKO mice appeared to be similar to that in control mice.

We followed the breeding of three female Rgmb cKO mice with WT male mice to test the effect of Rgmb ablation on their fertility. Rgmb<sup>f/del</sup>, Rgmb<sup>f/wt</sup>, and Rgmb<sup>f/wt-Stra8-icre</sup> females showed similar fertilities, and thus they were all used as control females. The accumulative number of progeny was similar between female Rgmb cKO and control mice (Fig. 7).

FIG. 3. Testis weight and structure in Rgmb cKO mice. Testes from control (Ctrl, Rgmb<sup>f/f</sup>) and Rgmb cKO (Rgmb<sup>f/f-Stra8-icre</sup>) mice at 2 mo (A) and 13 mo (B) of age were collected and weighed. Testes were also fixed and processed for H&E staining to visualize seminiferous tubules (C and D). Seminiferous tubules that lacked germ cells and developed vacuoles are labeled with red asterisks. n = 3 for A and B. *P < 0.05.
Deletion of Rgmb Did Not Alter Rgma and Rgmc Expression in Gonads

RGMb is a member of the RGM family, which also contains RGMa and RGMc. We therefore examined whether RGMa and RGMc are expressed in gonads and whether RGMb deletion altered RGMa and RGMc expression, thus offsetting the loss of RGMb. As determined by RT-PCR, Rgma and Rgmc mRNAs were expressed in the testis and ovary (Fig. 8A). Rgma and Rgmc mRNA levels in testis were similar between control and Rgmb cKO mice at 2 mo of age (Fig. 8, B and C). Rgma and Rgmc mRNA expression was not altered either in the ovaries of global Rgmb knockout mice at 14 days of age as compared with the ovaries of their WT littermates (Fig. 8, D and E). These results suggest that ablation of Rgmb did not have any effects on Rgma and Rgmc expression in the testis and ovary.

DISCUSSION

Our previous studies have revealed that RGMb is expressed in spermatocytes and spermatids in the testis and in oocytes in the ovary [14, 25]. In the present study, we examined the role
FIG. 6. Ablation of Rgmb in Rgmb cKO ovary. A) Outline of the breeding strategy for Rgmb conditional knockout female mice. Rgmb<sup>f/f</sup>-Stra8-icre males were crossed with Rgmb<sup>f/f</sup> females to produce Rgmb<sup>f/del</sup>-Stra8-icre and Rgmb<sup>f/del</sup> female mice. B) Representative genotypes of the offspring from an Rgmb<sup>f/del</sup>-Stra8-icre female mated with a WT male. Genomic DNA isolated from tail tips were used to perform genotyping using primers 1 and 2 (P1/2) and primers 1 and 6 (P1/6), as illustrated in Figure 1. Only WT and recombined alleles were observed, and no floxed allele was transmitted to the offspring. C) RGMb protein levels and BMP signaling in the ovaries of control (Ctrl, Rgmb<sup>f/del</sup>) and Rgmb cKO (Rgmb<sup>f/del-Stra8-icre</sup>) mice. Ovaries collected from female Ctrl and Rgmb cKO mice at 4 mo of age were analyzed for RGMb protein and Smad1/5/8 phosphorylation levels by Western blotting. Control and Rgmb cKO testes were used as controls. D) Cellular localization of RGMb in the ovary of control (Rgmb<sup>f/del</sup>) and Rgmb cKO (Rgmb<sup>f/del-Stra8-icre</sup>) mice. All sections were stained with DAB (brown) and counterstained with hematoxylin (blue). RGMb is expressed in oocytes of the secondary follicles but not in the primordial and primary follicles. Bars = 60 μm. E) Histology of ovaries from conditional Rgmb knockout (cKO) mice. Ovaries from control (Ctrl, Rgmb<sup>f/del</sup>) and Rgmb cKO (Rgmb<sup>f/del-Stra8-icre</sup>) mice at 4 mo of age were collected for H&E staining.
of RGMb in spermatogenesis and follicle development by specific deletion of RGMb expression in germ cells. To our surprise, deletion of Rgmb did not affect gonadal structures and fertility. Moreover, global knockout of RGMb did not alter ovarian structure in postnatal mice. These results led us to conclude that RGMb is not essential for normal spermatogenesis and follicle development.

We have also previously shown that RGMb is a BMP coreceptor that stimulates the BMP2/BMP4-Smad1/5/8 pathway in many cell types. However, Smad1/5/8 phosphorylation levels did not change by deletion of RGMb in the testis or ovary. These unexpected results do not appear to support a significant role of RGMb's BMP signaling function in the gonad. The failure of RGMb deletion in lowering BMP signaling may at least partially explain the unaltered phenotypes in the testis and ovary in Rgmb cKO mice.

RGMb also functions as a ligand for the neogenin receptor [22, 23], and this receptor has been found to be expressed in spermatocytes within the testis and in granulosa cells within the ovary [35]. Our previous study revealed that RGMb signals through neogenin to inhibit E-cadherin expression and induce cell apoptosis in renal epithelial cells both in cell culture and in injured kidneys [23]. However, the RGMb and neogenin interaction does not appear to be biologically significant in gonads, as deletion of Rgmb did not alter gonadal structures and functions.

The other two members of the RGM family, Rgma and Rgmc, also function as coreceptors for the BMP signaling and as ligands for the neogenin receptor [21, 24]. Loss of Rgmb may have been compensated by Rgma, Rgmc, or other components in their pathways. Indeed, our present study demonstrated that Rgma and Rgmc were expressed in the testis and ovary. Moreover, Rgma and Rgmc appeared to be even more highly expressed than Rgmb in the testis because we observed that the threshold cycles for Rgma and Rgmc were 22 and 21, respectively, while the threshold cycles for Rgmb were 24 in our real-time PCR assays. In the ovary, Rgma, Rgmb, and Rgmc showed threshold cycles of 22, 24, and 29, respectively; thus, Rgma appeared to be the most highly expressed RGM family member in the ovary. Although the cellular localization of Rgma and Rgmc in gonads remains to be determined, high expression of the three members suggests a potential functional redundancy. This hypothesis can be tested by compound knockout of the three genes in the testis and ovary. Nevertheless, our results support the notion that germ cell development does not depend on the expression of a single gene but that crucial pathways are highly safeguarded.

Rgmb protein expression was dramatically reduced but not completely lost in the testis and ovary from Rgmb cKO mice compared to control mice. However, breeding of Rgmb<sup>f/f</sup>-Stra8-icre or Rgmb<sup>f/del</sup>-Stra8-icre females with WT mice showed a loss of the floxed allele and an occurrence of excision of the floxed fragment in all the progeny. These results indicate a complete cre-mediated recombination in the gonads of Rgmb<sup>f/del</sup>-Stra8-icre mice with WT mice showed a loss of the floxed allele and an occurrence of excision of the floxed fragment in all the progeny. This observation is in contrast with a previous study that did not see Stra8-cre expression in the ovary at embryonic stages when endogenous Stra8 is expressed in ovarian germ cells and that did not observe cre-mediated recombination [30]. The discrepancy between the two studies remains unknown, but it may reflect different activities of the Stra8 promoter of the transgene among individual embryonic ovaries.
RGMb IS NOT REQUIRED FOR NORMAL GONADAL FUNCTION


