Steroidogenic enzyme gene expression and testosterone production are developmentally modulated by Bone morphogenetic protein receptor-1B in mouse testis.

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Short title: BMPR-1B regulates steroidogenic enzymes and testosterone

Summary

Bone morphogenetic proteins (BMPs) and receptors (BMPR-1A, BMPR-1B, BMPR-2) have been shown to be vital for female reproduction, while their roles in males are poorly described. Our study was undertaken to specify the function of BMPR-1B in steroidogenic enzyme gene expression, testosterone production and reproductive development in male mice, given that *Bmpr1b* mRNA is expressed in mouse testis and *Bmpr1b* knockout results in compromised fertility.

Male mice were passively immunized for 6 days with anti-BMPR-1B in the presence or absence of exogenous gonadotrophins. We then measured the effects of anti-BMPR-1B on testicular hydroxysteroid dehydrogenase isoforms (*Hsd3b1, Hsd3b6,* and *Hsd17b3*) and aromatase (*Cyp19*) mRNA expression, testicular and serum testosterone levels, and testis and seminal vesicle weight. *In vitro* testosterone production in response to anti-BMPR-1B was determined using testicular culture, and Leydig cell culture in the presence or absence of gonadotrophins. In Leydig cell culture the contribution of seminiferous tubules and Leydig cells were examined by preconditioning the media with these testicular constituents.

In adult mice, anti-BMPR-1B increased testosterone and *Hsd3b1* but decreased *Hsd3b6* and *Cyp19* mRNA. In adult testicular culture and seminiferous tubule conditioned Leydig cell culture, anti-BMPR-1B reduced testosterone, while in normal and Leydig cell conditioned Leydig cell culture it increased testosterone levels. In pubertal mice, anti-BMPR-1B reduced gonadotrophin stimulated seminal vesicle growth.

In conclusion, BMPR-1B has specific developmental functions in the autocrine and paracrine regulation of testicular steroidogenic enzyme gene expression and testosterone production in adults and in the development of seminal vesicles during puberty.

Key words

Steroidogenic enzyme genes, transforming growth factors, male reproduction, steroidogenesis, bone morphogenetic protein receptor

Introduction

In mammalian males, steroidogenesis, spermatogenesis and fertility are regulated by the hypothalamic-pituitary-gonadal axis. The secretion of hypothalamic gonadotropin-releasing hormone stimulates the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary, which are essential for reproduction [1]. In males, LH stimulates Leydig cell testosterone production, while FSH in combination with testosterone induces Sertoli cell differentiation and male germ cell development [1]. Furthermore, increased androgen production facilitates the vascularization, growth, and secretory activity of the prostate and seminal vesicles [2]. In addition to LH and FSH, there is growing evidence that bone morphogenetic proteins (BMPs) also play key roles in male reproduction [3,4,5]. BMPs form the largest subgroup of growth factors in the TGF-β superfamily which signal by heterodimerization of type 1 and type 2 BMP receptors (BMPR-1A, BMPR-1B and BMPR-2) via modulation of downstream SMAD signal transducers [6].

BMPR-1B shares a high homology with BMPR-1A while BMPR-IA is expressed at higher levels than BMPR-IB on the cell membrane [7], and higher mRNA expression of *Bmpr1a* than *Bmpr1b* has also been observed in the testis [8]. BMPs and their putative receptors modulate testosterone synthesis, germ cell maturation, reproductive tissue integrity and epithelial secretory function [9,10,11]. Heterozygous mutations of *Bmp4* or *Bmp7* [9,12] and homozygous mutations of *Bmp8a* or *Bmp8b* [5,11] result in either compromised fertility or infertility in male mice. In mice, homozygous *Bmpr1a* and *Bmpr2* knockouts are lethal [13,14], while homozygous *Bmpr1b* mutation leads to infertility attributed to defective development of the seminal vesicles [15]. Seminal vesicles express *Bmpr1a* and *Bmpr1b* mRNA in immature mice [10], however, whether *Bmpr1b* mutants have altered testicular function has not been reported. In SMAT-1 cells, derived from immature Sertoli cells, BMPR-1B antagonized the ability of anti-mullerian hormone to stimulate BMPR-1A and down-regulate transcription of *P450scc* [16], essential for steroid synthesis [1]. TGF-β superfamily members have been shown to alter steroidogenic enzyme expression in male rats and fish [17,18]. This suggests that BMP receptor signaling could modulate androgen production via their effect on steroidogenic enzymes, such as hydroxysteroid dehydrogenase (HSD) and aromatase (CYP19).

3β-HSD is a major enzyme in the steroidogenic pathway that converts pregnenolone into progesterone, a testosterone precursor [1]. There are several isoforms, of which *in vivo Hsd3b6* is gonadotrophin dependent while *Hsd3b1* is gonadotrophin independent in mice [19]. 17β-HSD3 (17-ketoreductase) is another key enzyme involved in the conversion of testosterone precursors into testosterone [1], which is gonadotrophin dependent *in vivo* [20]. Aromatase converts androgens including testosterone into estrogens [1,21]. When stimulated by gonadotrophin, aromatase can acutely decrease testosterone *in vitro* via its ability to convert testosterone into estradiol [22]. Testosterone is present in highest concentrations in the testis while levels in serum are lower under normal circumstances [23], and this is due to the high need of testosterone for spermatogenesis and the adequacy of lower levels in serum for the development of secondary sexual characteristics [1].

Bmpr1b mRNA was detected in rabbit testis throughout postnatal development, in adult mouse testis and in testicular cell lines SMAT-1 and MA-10 derived from Leydig cell tumors [24]. *Bmpr1a* has been detected in embryonic mouse testis [25]. In immature mice *Bmpr1a* was expressed in Sertoli cells and spermatogonia [3,4]. BMPR-1A protein was also found in spermatogonia [3], while *Bmpr2* was detected in Sertoli cells [4]. In goats *BMPR1B* is expressed in higher amounts in undifferentiated spermatogonia then differentiated spermatogonia [26], highlighting a role in differentiation.

In humans, homozygous mutation of *BMPR1B* and *PDHA2* resulted in infertility in men

due to sperm defects, and in heterozygous mutation mild necrospermia [27]. Defects in fertility were attributed to germ cell specific *PDHA2* [27], while the role of *BMPR1B* may have been overlooked. *BMPR1B*, is expressed in human testicular cancer with germ cell origins [28], and in zebrafish, *bmpr1b* mutation has been directly linked to germ cell tumors of the testis [29]. In mice Smad1 and Smad5 were severely down-regulated in multiple endocrine neoplasia type 1 Leydig cell tumors [30]. In adult humans, BMPR-1A, BMPR-1B and BMPR-2 protein and mRNA were detected in the prostate [31], and significant down-regulation of *Smad1* occurred in prostate cancer [32,33]. BMPR-1B signaling is implicated in testicular cancer and the reduced proliferation of prostate cancer (PC3 cells), breast cancer, and brain cancer [28,34-36]. Collectively, this suggests that ligand signaling via BMPR-1B may have a role in the inhibition of cell proliferation.

Based on the localization of BMPR-1B protein and mRNA in testicular cells, the reproductive profile of BMPR-1B mutant male humans and mice, and the altered expression patterns of *BMPR1B* in testicular cancer, we suspect the receptor to have a role in steroidogenesis and growth of reproductive tissues.

In this study, we performed BMPR-1B immunization *in vivo* to investigate the functions of BMPR-1B in the regulation of key isoforms of HSD (*Hsd3b1, Hsd3b6, Hsd17b3*) and aromatase (*Cyp19*), testosterone production, and reproductive development using a mouse model. *In vitro*, we investigated the autocrine and/or paracrine roles of BMPR-1B in testosterone production using anti-BMPR-1B in testicular culture and Leydig cell culture, as well as examining the contribution of Leydig cells and seminiferous tubules to anti-BMPR-1B reactivity.

Methods

Antibodies and Reagents

The anti-BMPR-1B was raised in chickens against a synthetic peptide equivalent to amino acids 103-117 as previously described [37-39]. Using the NCBI Basic Local Alignment Search Tool (BLAST), we confirmed that the peptide sequence had 100% homology to mouse BMPR-1B and no similarity was found for BMPR-1A the closest family member receptor to BMPR-1B [40]. Non-immune Ig was derived from non-immunized chickens. Equine CG (Bioniche Animal Health, Armidale, NSW) having properties of both LH and FSH to target both Leydig and Sertoli cells [41], hCG (Sigma) to target Leydig cells, and FSH (Vetrepharm, A/Asia Pty. Ltd., Camperdown, NSW) to target Sertoli cells, were used to stimulate steroidogenesis [1].

Animal Care and Experiments

The University of New England Animal Ethics Committee approved the use of animals needed to conduct this research, which was in accordance with the National Health and Medical Research Council: Australian code of practice for the care and use of animals for scientific purposes. Male Swiss Quackenbush mice (Physiology Animal House, University of New England, NSW, Australia) were housed in sanitary conditions in a light-controlled room (12:12) at a constant temperature of 21 °C and had access to a constant supply of standard rodent chow and water. Mice aged 3 and 8 weeks were placed into 4 groups (A, B, C, D) containing 5 animals in each with no significant difference in body weight. Mice were treated subcutaneously using 100 µl of PBS as the carrier. As controls, group A received 50 µg of non-immune Ig, group B received 50 µg anti-BMPR-1B, group C received 2 IU eCG and group D received a combination of 2 IU eCG and 50 µg anti-BMPR-1B. The animals were treated for

6 days and sacrificed on day 7 by CO_2 asphyxiation. Blood samples were collected via cardiac puncture and the serum harvested to measure testosterone. The testes and seminal vesicles were weighed, and 100 mg of testis was homogenized to measure testicular testosterone and 100 mg testis was stored in RNALater (Ambion, Austin, TX) for RNA isolation. The experiments were repeated twice. n=15.

RNA Extraction, RT PCR and qPCR

RNA was extracted using TRI Reagent (Sigma-Aldrich Co, St Louis, MO) according to the manufacturer's instructions. RNA integrity was checked on a 1 % RNA agarose gel and quantified using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc., Wilmington, DE). Reverse transcription was performed by annealing 2 µg of total RNA with 20 ng of Oligo$(dT)_{15}$Primer (Fisher Biotec, Subiaco, WA) at 70 °C for 5 min, then placed on ice. This was followed by extension carried out at 40 °C for 60 min using 10 mM dNTP Mix (Fisher Biotec), 1 x RT Buffer, 400 U M-MLV Reverse Transcriptase (Promega, Alexandria, NSW) and 1 U of rRNasin(R)RNase Inhibitor (Promega). Gene specific primers (Table 1) (GeneWorks Pty Ltd, Hindmarsh SA) were derived from other authors or designed using NCBI Primer-BLAST [40]. A routine PCR using 80 ng of cDNA was carried out to confirm the specificity of primers and their expected product lengths were checked using 2 % agarose gel electrophoresis. The PCR products were gel eluted and sequenced at the Ramachiotti Centre for Genomics, UNSW, Sydney for confirmation. Quantitative PCR (qPCR) reactions were set up in duplicate using 16 ng of cDNA, in Fast EvaGreen qPCR Mix (Biotium, Hayward, CA) using a CAS-1200 automated PCR Setup robot (Corbett Robotics, Eight Mile Plains, QLD) and qPCR was performed using a Rotor-Gene R6 6000 Real-time Analyzer (Corbett Life Science, Concorde, NSW). n=5.

Tissue Culture

Testes were dissected from mice aged 3 and 8 weeks for two separate tissue culture experiments. 20 mg of dissected tissue was placed in each well of a 24-well polypropylene tissue culture plate (Sarstedt, Australia Pty Ltd) in one of 4 groups consisting of the control, anti-BMPR-1B, eCG, and anti-BMPR-1B plus eCG in 1 ml of Dulbeco Modified Eagle Medium/F-12 (DMEM:F12) (Gibco Invitrogen, Australia Pty Ltd, Mount Waverly, VIC) enriched with 0.1 % BSA (Sigma Aldrich Pty Ltd, Castle Hill, NSW). Anti-BMPR-1B was used at 50 µg/ml, eCG at 0.1 IU/ml and PBS was used for the control. Tissues were incubated for 3 hours at 32 °C in humidified air with 5.0 % CO_2. After incubation, the culture media were collected and assayed for testosterone. The experiment was repeated 4 times. n=20.

Cell Culture

Testes from 8-week-old mice were extracted, decapsulated, and mechanically dispersed as described by Kerr et al. [42] and Leydig cells were purified by discontinuous Percoll density gradients similar to Klinefelter et al. [43]. Briefly, freshly dispersed cells were filtered through 10 µm nylon gauze to collect interstitial cells, which were washed and loaded onto Percol gradients prepared at 0 %, 20 %, 35 %, 43 %, 68 % and 90 % in Hanks Balanced Salt Solution (8.0 % NaCl, 1.0 % D-glucose, 0.4 % KCl, 0.06 % KH_2PO_4, 0.1 % $MgCl_2$, 0.1 % $MgSO_47H_2O$, 0.09 % $NaHPO_47H_2O$, 0.14 % $CaCl_2$, 0.01 % Phenol Red, pH 7.4) enriched with 0.1 % BSA. The gradients were centrifuged at 1230 g for 30 min at 4 °C. After centrifugation, 5 bands/zones of visible cells were present. Fraction 4 (43-68 % interface) was harvested for cell culture. Cells were counted with a hematocytometer and cell viability was determined using trypan blue exclusion [44]. Fraction 4 contained enriched Leydig cells showing > 90 % steroidogenic cells as determined by Δ^5-3βHSD staining. A cell stock, prepared in DMEM:F12, was enriched with

0.1 % BSA at a concentration of 1,000 cells/50 µl. Three separate experiments in (1) unconditioned, (2) Leydig cell conditioned and (3) seminiferous tubule conditioned media were undertaken. All Leydig cells were cultured in 96-well polypropylene cell culture plates for 3 hours in DMEM:F12 enriched with 0.1 % BSA (Sarstedt Pty Ltd) at 32 °C and 5.0 % CO_2 in humidified air. Anti-BMPR-1B was used at a concentration of 50 µg/ml, hCG was used at 1 IU/ml, FSH was used at 0.1 µg/ml and PBS was used for the control. After incubation, the culture media were harvested and assayed for testosterone. Each experiment was repeated 4 times. n=20. *Experiment 1*: In each well, 50 µl cell stock was cultured with 100 µl of DMEM:F12 in groups of control, anti-BMPR-1B, hCG, and anti-BMPR-1B plus hCG. *Experiment 2:* Leydig cell conditioned media was prepared and pooled from Leydig cell culture from four respective groups (control, anti-BMPR-1B, hCG, and anti-BMPR-1B plus hCG). In each well, 50 µl of cell stock was cultured with 100 µl Leydig cell conditioned media in their respective groups. *Experiment 3:* Seminiferous tubule conditioned media was created by dissecting 3 x 1 cm random segments of seminiferous tubules, which were cultured for 6 hours in glass Petri dishes containing 5 ml of DMEM:F12 in control, anti-BMPR-1B, FSH, and anti-BMPR-1B plus FSH treatment groups. In each well, 50 µl of cell stock and 100 µl of seminiferous tubule conditioned media in groups of control, anti-BMPR-1B, FSH, and anti-BMPR-1B plus FSH were used. Pooled conditioned media samples used in experiments 2 and 3 were assayed for testosterone/residual testosterone for subtraction during analysis.

Steroid extraction and testosterone radioimmunoassay

Steroid extraction was carried out by vortexing 50 µl serum samples in glass tubes for 10 minutes with 0.7 ml of diethyl ether. The tubes were then placed on dry ice until the aqueous phase was frozen. The ether was decanted into polypropylene tubes (Sarstedt, Australia Pty Ltd) and evaporated under vacuum at 25 °C. The samples were re-hydrated in 100 µl of gel buffer (1 % PBS, 0.1 % gelatin, 0.02 % NaN_3 in H_2O), incubated for 12 hours at 4 °C and assayed for testosterone using a radioimmunoassay described by McFarlane et al. [45].

Analysis

Leydig cell testosterone was normalized to ng per 10 K cells. Data analysis of qPCR Ct values was performed by the $2^{-\Delta\Delta Ct}$ method using *β-actin* as the reference gene. Statistical analysis was performed using a general linear model (GLM) procedure (multiway ANOVA) in SAS statistical software (SAS Institute Inc. Cary, NC, USA), followed by the Student-Newman Keuls post hoc test. Values were considered to be significantly different at $p<0.05$ and presented as mean ± standard error (SEM).

Results

Effects of anti-BMPR-1B on expression of Cyp19, Hsd3b1, Hsd3b6 and Hsd17b3 mRNA in pubertal and adult mouse testis

Fig. 1 shows expression of *Cyp19*, *Hsd3b1*, *Hsd3b6* and *Hsd17b3* relative to *β-actin* in the testes of control, anti-BMPR-1B, eCG, and anti-BMPR-1B plus eCG treated groups of pubertal and adult mice. In pubertal testes relative expression of *Cyp19*, *Hsd3b1*, *Hsd3b6* and *Hsd17b3* in the control was 1.69±0.3, 55.0±5.0, 22.6±3.4 and 7.33±1.5 respectively. In pubertal testis, anti-BMPR-IB had no effect on *Cyp19* (2.67±0.6), *Hsd3b1* (58.4±7.0), *Hsd3b6* (18.3±3.1) or *Hsd17b3* (10.2±3.4) while eCG increased *Hsd3b1* 1.9 fold (105±9.7), ($p\leq0.0004$) and decreased *Hsd3b6* 1.8 fold (12.6±1.3) ($p\leq0.02$), while having no effect on *Hsd17b3* (7.76±1.3) or *Cyp19* (1.51±0.2). Anti-BMPR-1B mildly increased eCG-stimulated expression of *Hsd3b6* (17.6±2.7) but did not affect *Cyp19* (2.26±0.9), *Hsd3b1* (88.2±11) or *Hsd17b3*

(11.7±2.8). In adult testes relative expression of *Cyp19*, *Hsd3b1*, *Hsd3b6* and *Hsd17b3* was 1.46±0.1, 42.8±4.6, 6.57±1.5, and 15.6±1.5 respectively. Anti-BMPR-1B reduced *Cyp19* (1.03±0.05) by 1.4 fold ($p \leq 0.005$), increased *Hsd3b1* (64.8±5.4) by 1.5 fold ($p \leq 0.006$) and decreased *Hsd3b6* (3.69±0.3) by 1.8 fold ($p \leq 0.01$), while having no effect on *Hsd17b3* expression (13.6±1.4) in adults. eCG decreased the expression of *Cyp19* (0.91±0.2) by 1.6 fold ($p \leq 0.02$), *Hsd3b1* (27.9±5.9) by 2 fold ($p \leq 0.01$), increased *Hsd3b6* (16.7±1.9) by 2.5 fold ($p \leq 0.0009$) and increased *Hsd17b3* (29.5±6.2) by 1.9 fold ($p \leq 0.02$). Anti-BMPR-1B had no effects on *Cyp19* (1.17±0.2), *Hsd3b1*(34.9±7.1), *Hsd3b6* (13.9±2.4) and *Hsd17b3* (28.1±3.2) expression in eCG-stimulated testis. The mRNA expression of the HSD isoforms was up to ~ 100 times greater than that of *Cyp19*.

Effect of anti-BMPR-1B on serum and intra-testicular testosterone in pubertal and adult male mice

Fig. 2 shows serum testosterone and intra-testicular testosterone in control, anti-BMPR-1B, eCG, and anti-BMPR-1B plus eCG treatment groups of pubertal and adult male mice. In immature mice, eCG increased serum testosterone (1.42±0.1 ng/ml) 1.5 fold compared to the control (0.97±0.1 ng/ml) ($p \leq 0.004$). Anti-BMPR-1B marginally inhibited basal (0.83±0.1 ng/ml) and eCG stimulated (1.23± 0.1 ng/ml) serum testosterone production. In adults, serum testosterone in the anti-BMPR-1B group was increased 4.3 fold (8.16±2.7 ng/ml) compared to the control (1.92±0.4 ng/ml) ($p \leq 0.04$). Serum testosterone was also increased in the eCG treated group 5.5 fold (10.5±1.5 ng/ml) ($p \leq 0.0001$), while anti-BMPR-1B did not alter eCG-stimulated testosterone (10.9±1.7 ng/ml). In pubertal mice, eCG increased testicular testosterone (27.0±2.2 ng/ml) 3.8 fold compared to the control (7.09±1.6 ng/ml) ($p \leq 0.0001$), while anti-BMPR-1B had no effect on either basal (5.54±1.1 ng/ml) or eCG-mediated testosterone production (21.0±2.3 ng/ml). In adults, anti-BMPR-1B increased testicular testosterone 3.1 fold (110±32 ng/ml) ($p \leq 0.04$) compared to the control (35.0±6.6 ng/ml), and eCG increased testosterone 5.2 fold (184±26 ng/ml) ($p \leq 0.0001$), while anti-BMPR-1B had no effect on eCG-stimulated testosterone (197±26 ng/ml).

Effect of anti-BMPR-1B on testosterone levels in testicular culture of pubertal and adult mice

Fig. 3 shows testosterone levels relative to the control in pubertal and adult testicular culture in response to treatment with anti-BMPR-1B, eCG, and anti-BMPR-1B plus eCG. In pubertal testis, eCG resulted in a 2.7 fold increase in testosterone (2.68±0.2) ($p \leq 0.0001$), while anti-BMPR-1B had no effect on either basal (1.06±0.2) or eCG-mediated testosterone (1.93±0.3) relative to the control (0.63±0.2). In adult testicular culture anti-BMPR-1B decreased testosterone production by 1.5 fold (0.66±0.1) compared to the control (1.0±0.1) ($p \leq 0.05$) and eCG increased testosterone (1.92±0.2) 1.9 fold ($p \leq 0.0003$), while anti-BMPR-1B reduced the eCG-mediated increase in testosterone 1.5 fold (1.32±0.1) ($p \leq 0.02$).

Effect of anti-BMPR-1B on testosterone in purified Leydig cell culture in unconditioned media, Leydig cell conditioned media and seminiferous tubule conditioned media

Fig. 4 shows Leydig cell testosterone in response to anti-BMPR-1B, gonadotrophins, and anti-BMPR-1B plus gonadotrophin in unconditioned (normal media), Leydig cell conditioned media and seminiferous tubule conditioned media. Anti-BMPR-1B increased testosterone by 1.4 fold (20.2±1.0 ng) compared to the control (14.0±0.8 ng) ($p \leq 0.0001$), and hCG increased testosterone by 45 fold (634±74 ng) ($p \leq 0.0001$), while anti-BMPR-1B had no effect on hCG-induced testosterone (521±87 ng). In anti-BMPR-1B treated, Leydig cell conditioned media, testosterone increased by 1.6 fold (20.2±1.7 ng) ($p \leq 0.001$) compared to the control (12.6±1.1 ng). Leydig cell conditioned media previously treated with hCG had a powerful

inhibitory effect on Leydig cell testosterone production to below detection levels, while anti-BMPR-1B strongly inhibited the decrease seen in the hCG treated group (722±205 ng) ($p \leq 0.0005$). Testosterone in the seminiferous tubule conditioned media control (33.2±2.5 ng) was 2.4 fold increased from the normal media control ($p \leq 0.001$). In anti-BMPR-1B treated seminiferous tubule conditioned media, testosterone decreased by 1.3 fold (25.5±1.8 ng) ($p \leq 0.04$). Seminiferous tubule conditioned media previously treated with FSH increased Leydig cell testosterone 3.7 fold (115±7.3 ng) ($p \leq 0.0001$), while anti-BMPR-1B had no effect on FSH-modulated testosterone (101±7.1 ng).

Effects of anti-BMPR-1B on seminal vesicle and testis weight in pubertal and adult mice

Fig. 5 shows seminal vesicle weight in response to anti-BMPR-1B in basal and eCG stimulated conditions. The seminal vesicle weight of the anti-BMPR-1B group (0.011±0.002 g) of pubertal mice was similar to the control (0.013±0.001 g) while eCG increased seminal vesicle weight 2.9 fold (0.037±0.002 g) ($p \leq 0.0001$) and anti-BMPR-1B decreased eCG-mediated seminal vesicle weight gain by 1.4 fold (0.036±0.002 g) ($p \leq 0.002$). In adult mice seminal vesicle weight was elevated in the eCG group by (0.288±0.01 g) 1.3 fold compared to the control (0.223±0.009 g) ($p \leq 0.0001$), however anti-BMPR-1B did not alter basal (0.239±0.02 g) or eCG-stimulated seminal vesicle weight (0.293±0.009 g). In pubertal and adult mice anti-BMPR-1B and/or eCG had no effect on testis weight in basal or stimulated conditions, data not shown.

Discussion

We provide evidence that BMPR-1B augments aromatase activity in mouse testis, as anti-BMPR-1B decreased *Cyp19* mRNA expression in adult mice, while *in vitro* it blocked hCG mediated aromatization. Factors in media from Leydig cells previously treated with hCG inhibited the synthesis of Leydig cell testosterone production to below recordable levels, while anti-BMPR-1B obliterated the secondary response. This indicates that BMPR-1B may stimulate aromatization via its effect on *Cyp19* gene expression. However, aromatase is not known to have a presence in media and is more likely to stimulate the release of growth factors, which consequentially stimulate aromatase. In the zebra finch brain, the aromatase blocker Fadrozole decreased *bmp2* expression, indicating that aromatase enhanced *bmp2* expression [46], and our study provides some evidence that aromatase may also increase BMP signaling via BMPR-1B to inhibit testosterone in mouse testis. The observation that BMPR-1B is involved in aromatase activity offers an interesting concept that BMPR-1B already shown to be important for aromatase production by granulosa cells of ovarian follicles in females [15], may also be important for Leydig cell aromatase production in males.

In adult mice, BMPR-1B functions differentially in Leydig cells and seminiferous tubules as demonstrated by decreased testosterone in anti-BMPR-1B treated mature testis culture and seminiferous tubule conditioned Leydig cell culture, and increased basal testosterone in normal and Leydig cell conditioned Leydig cell culture. This finding aligns well with Belville et al. [16] who showed that BMPR-1B could indirectly down-regulate transcription of *P450scc* in Sertoli cell derived SMAT-1 cells. This suggests that BMPR-1B augments testosterone in seminiferous tubules containing Sertoli cells in a paracrine manner, while it inhibits testosterone synthesis in Leydig cells in an autocrine manner.

BMPR-1B has a developmental role in steroidogenesis, as anti-BMPR-1B increased testosterone levels only in adult males. Anti-BMPR-1B increased *Hsd3b1*, decreased *Hsd3b6*, and had no effect on *Hsd17b3* expression. As the up-regulation of *Hsd3b6* and *Hsd17b3* are gonadotrophin dependent [19,20] and anti-BMPR-IB had the opposite effect on *Hsd3b1* than

eCG, we found no evidence that BMPR-1B functions to increase pituitary gonadotrophins but rather plays a local role within the testis. While *Bmpr1b* has been listed in mouse pituitary in The Human Protein Atlas database, it may have significance in cell types other than the gonadotrophs [47].

In pubertal mice, BMPR-1B enhanced seminal vesicle growth partially independent of testosterone, as *in vivo* immunization decreased eCG-stimulated seminal vesicle weight gain but had no marked effect on steroidogenic enzyme expression or testosterone. In support of this finding, *Bmpr1b* mRNA was expressed in the seminal vesicle epithelium of immature mice [10] and we [8] also found *Bmpr1b* mRNA in adult seminal vesicles where anti-BMPR-1B had no effect. This suggests that BMPR-1B counter regulates seminal vesicle growth during puberty.

We conclude that in adult mice, BMPR-1B signaling acts locally within the testis to inhibit testosterone production via the modulation of HSD isoforms and aromatase while modifying seminal vesicle growth during puberty.

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Table 1. Primers used for RT-qPCR

Gene	NCBI reference	(5'→3')	Sequence	Amplicon length	Source
β-actin	NM_007393.3	Forward	CGTCGACAACGGCTCCGGCATG	150 bp	In house
		Reverse	TGGGCCTCGTCACCCACATAG		
Cyp19	NM_007810.3	Forward	TGTGTTGACCCTCATGAGACA	190 bp	[48]
		Reverse	CTTGACGGATCGTTCATACTTTC		
Hsd3b1	NM_008293.3	Forward	TGGACAAAGTATTCCGACCAGA	250 bp	[49]
		Reverse	GGCACACTTGCTTGAACACAG		
Hsd3b6	NM_013821.3	Forward	GGAGGAGATCAGGGTCCTGG	209 bp	[50]
		Reverse	CTAGGATGGTCTGCCTGGG		
Hsd17b3	NM_008291.3	Forward	ACAACGTTGGAATGCTCCCCAGC	147 bp	In house
		Reverse	GGCCTTTCCTCCTTGACTCCATGT		

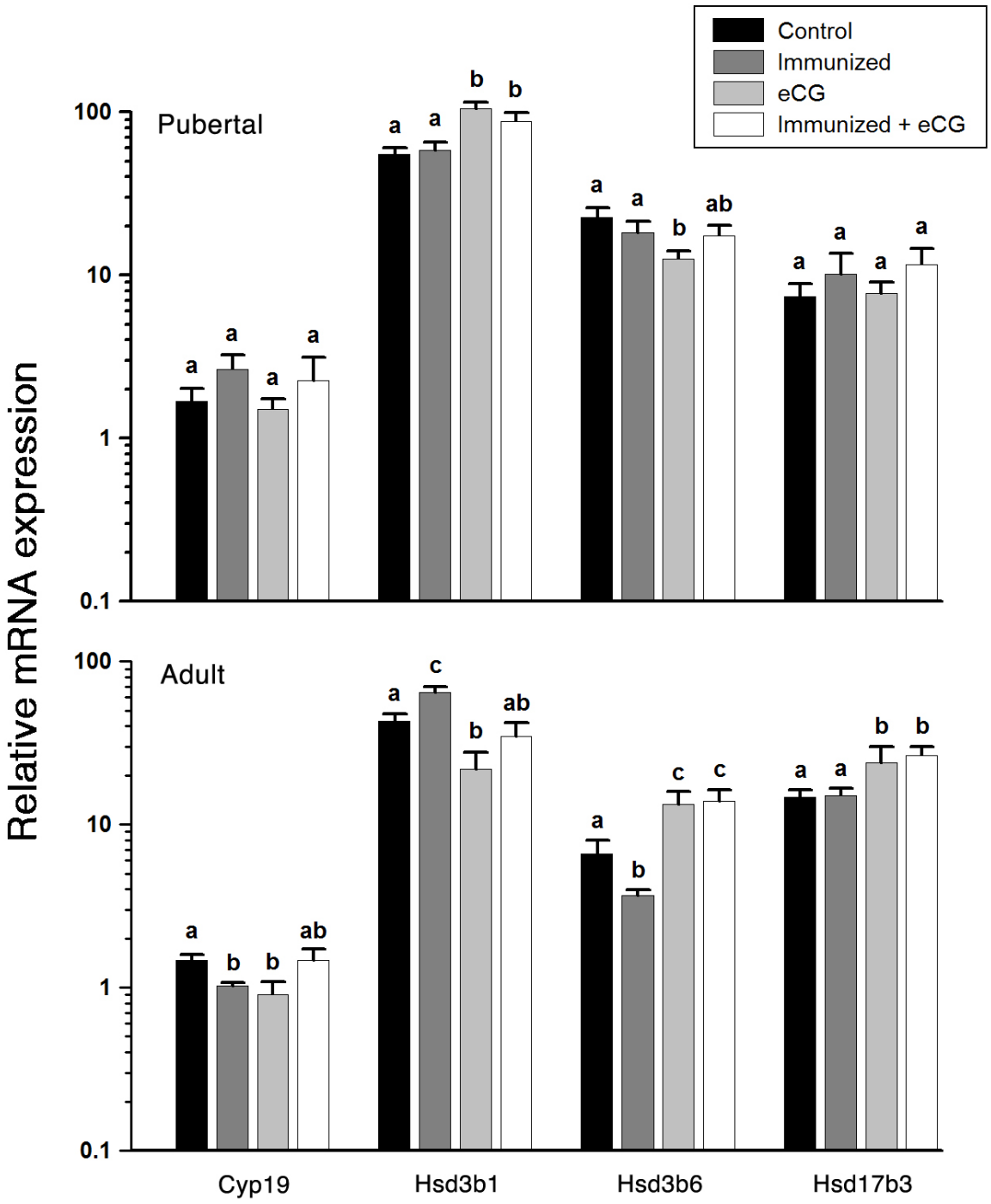

Fig. 1. Testicular *Cyp19*, *Hsd3b1*, *Hsd3b6* and *Hsd17b3* mRNA expression in control (black), anti-BMPR-1B (dark grey), eCG (light grey), and anti-BMPR-1B plus eCG (white) treated groups of pubertal and adult mice. Results were calculated relative to the housekeeping gene *β-actin* and presented as mean ± SEM. Different superscripts denote significant differences ($p<0.05$). n=5.

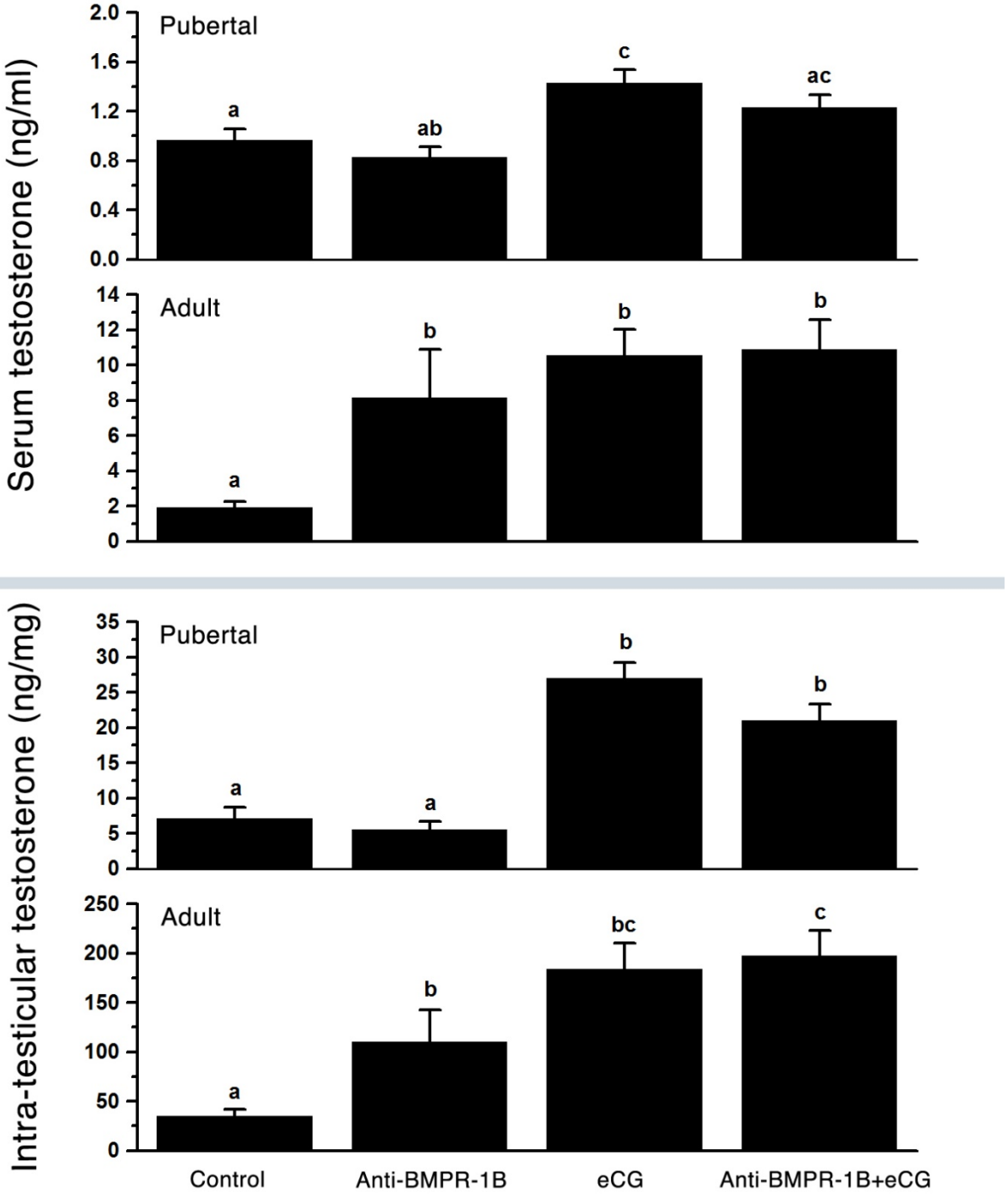

Fig. 2. Serum testosterone (ng/ml) (Top) and intra-testicular testosterone (ng/mg) (Bottom) in control, anti-BMPR-1B, eCG, and anti-BMPR-1B plus eCG treatment groups of pubertal and adult male mice. Results are presented as mean ± SEM. Different superscripts denote significant differences ($p<0.05$). n=15.

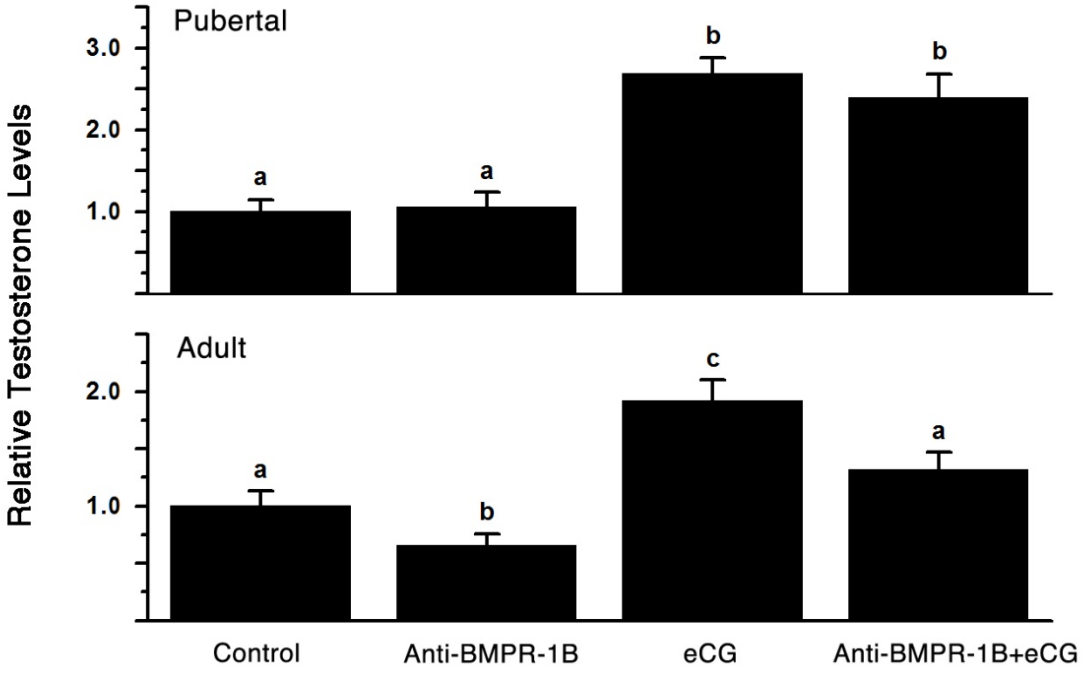

Fig. 3. Testosterone levels relative to the control in pubertal and adult testicular culture in response to treatment with anti-BMPR-1B, eCG, and anti-BMPR-1B plus eCG. Results are presented as mean ± SEM. Different superscripts denote significant differences ($p<0.05$). n=20.

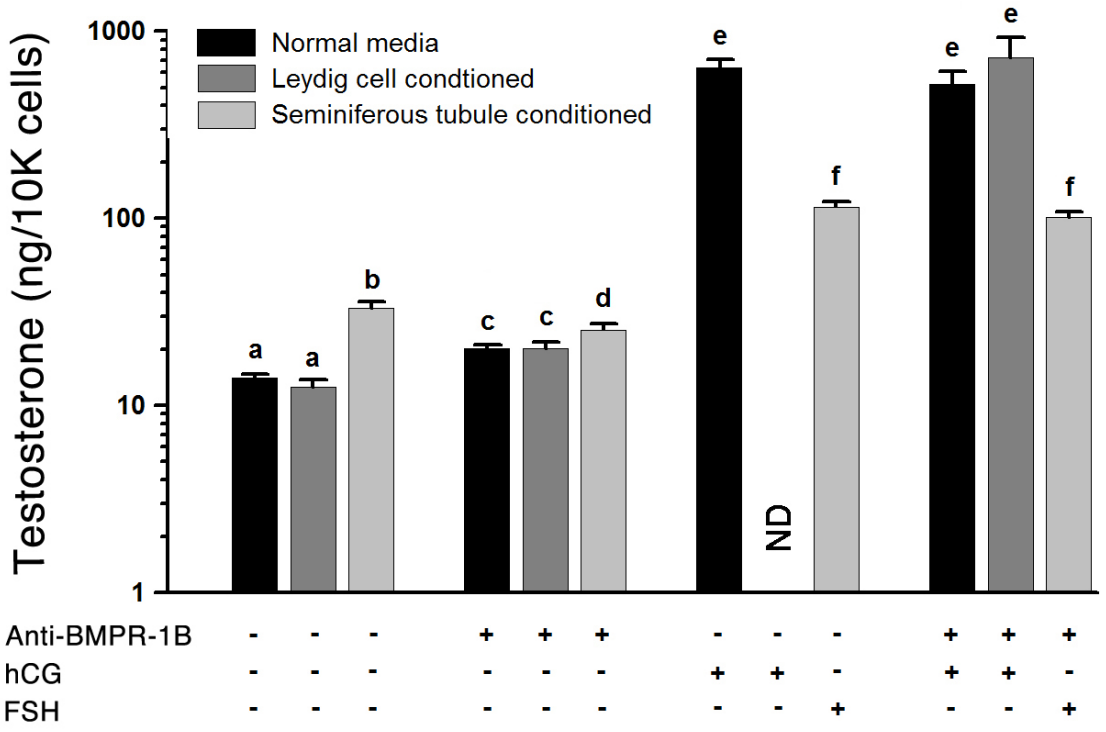

Fig. 4. Leydig cell testosterone in ng/10K cells in control, anti-BMPR-1B, gonadotrophin, and anti-BMPR-1B plus gonadotrophin treatment groups in unconditioned (normal) media (black), Leydig cell conditioned media (dark grey) and seminiferous tubule conditioned media (light grey). Results are presented as mean ± SEM. Different superscripts denote significant differences ($p<0.05$). n=20. Gonadotrophins: hCG = Human chorionic gonadotrophin; FSH = Follicle stimulating hormone. ND = Not detected.

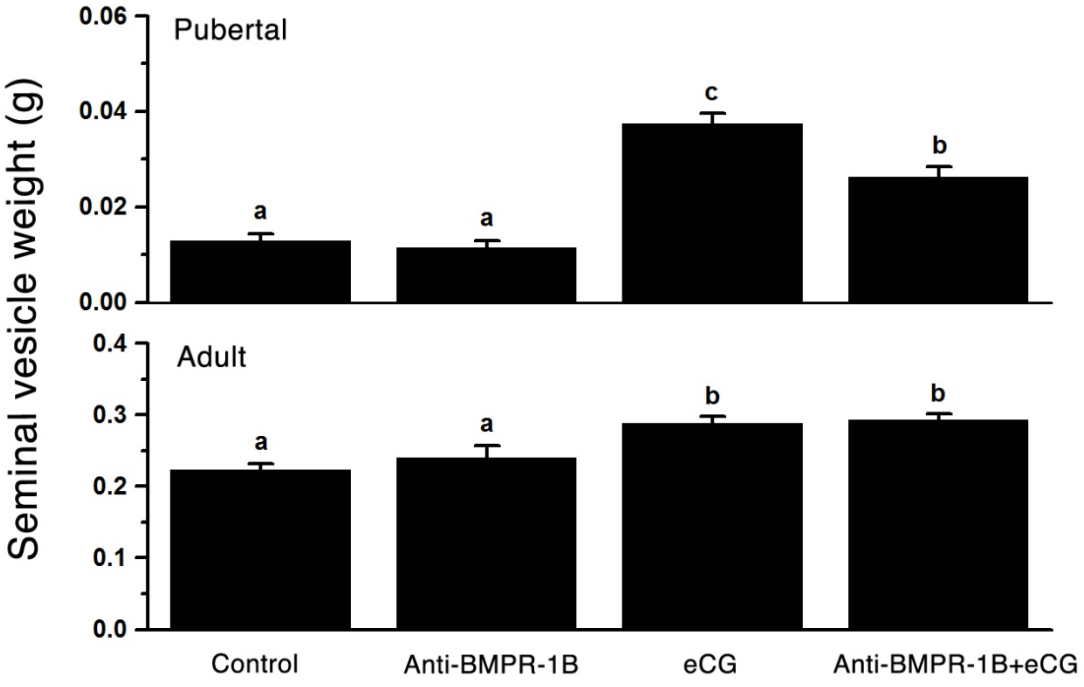

Fig. 5. Pubertal and adult seminal vesicle weight (g) in control, anti-BMPR-1B, eCG, and anti-BMPR-1B plus eCG treatment groups of male mice. Results are presented as mean ± SEM. Different superscripts denote significant differences ($p<0.05$). n=15.