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Vitamin A regulation of BMP4 expression in the male germ line

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Abstract

The molecular mechanisms leading to male infertility in vitamin A deficient (VAD) rodents have never been fully elucidated. Here, we report an interaction between BMP4 and retinoid signaling pathways in germ cells that may help clarify the biochemical basis of VAD. Adult germ cells, in particular spermatogonia, expressed BMP4 at both the mRNA and protein levels. BMP4 expression was significantly up-regulated in the testes of VAD mice and was down-regulated in freshly isolated germ cells and VAD testes by retinol, but not retinoic acid. The retinoid-responsive gene, RARβ, was not induced in germ cells following retinoid treatment. Examination of *BMP4* promoter usage in spermatogonia and the VAD testis revealed that germ cells utilize the recently characterized BMP4 intron 2 promoter, in addition to the classical 1A and 1B promoters. The observed decrease in BMP4 in response to retinol was mediated by the 1A and intron 2 promoters of the *BMP4* gene. Our results reflect a direct requirement for retinoids by germ cells for the resumption of spermatogenesis in VAD animals via mechanisms that involve the suppression of *BMP4* expression.

Keywords: Spermatogenesis; Retinoid signaling; BMP4; Vitamin A deficiency; Testis

Introduction

One of the essential roles of retinoids is in the regulation of mammalian spermatogenesis (Huang and Hembree, 1979; Packer and Wolgemuth, 1999). Spermatogenesis involves the differentiation of highly specialized haploid spermatozoa from a population of spermatogonial stem cells within the testis. Due to the complexity of the pathways involved, the molecular mechanisms that regulate this differentiation process remain poorly characterized. This process is a classic example of stem cell differentiation and it is completely, but not solely, reliant on adequate vitamin A.

Wolbach and Howe (1925) first observed that vitamin A deficient (VAD) rodents suffered various disorders including blindness and male infertility. Further research has since revealed that vitamin A withdrawal leads to an arrest in the transition of A spermatogonia to type A_1 spermatogonia (reviewed in Chung and Wolgemuth 2004). Supplementing the diet of these animals with retinoic acid (RA) alleviates all symptoms of vitamin A deficiency, with the exception of sight and spermatogenesis. It is not until VAD animals are readministered retinol (ROL) through the diet, or RA is injected in repeated high doses interperitoneally, that normal spermatogenic function is restored (Huang and Hembree, 1979; van Pelt and de Rooij, 1991). In addition, ablation of the RA receptors RARα and RXRβ in the mouse also results in male sterility, while female animals remain fertile (Kastner et al., 1996; Lufkin et al., 1993). *RARα*-null mice have testis and seminiferous tubule morphology similar to the VAD testis, while *RXRβ*-null mice exhibit seminiferous tubule degeneration (Kastner et al., 1996; Lufkin et al., 1993). It can be concluded from these studies that the mammalian testis is a major site of retinoid action. However, it is unclear from the VAD or knockout studies whether the germ cells are responding directly to the retinoids or if the effect is mediated by

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somatic cells, nor is it known which genes are regulated by retinoids during spermatogenesis. Thus, the exact mechanism of vitamin A action in the male germ line remains to be elucidated.

The transforming growth factor (TGF)-β superfamily is known to be important in germ cell differentiation and survival. The TGF-β superfamily consists of several ligand classes, including the bone morphogenetic proteins (BMPs), activins, inhibins, and the growth and differentiation factors (Zimmerman and Padgett, 2000). Specific members of the BMP family in particular have been implicated in various aspects of spermatogenesis and germ cell survival. (Ying et al., 2000; Zhao et al., 1996, 1998, 2001). One such example is BMP4. In the mouse, targeted inactivation of the *BMP4* gene results in a failure to form primordial germ cells (PGCs) (Lawson et al., 1999; Ying et al., 2000), and its presence is also necessary for PGC localization to the genital ridge and PGC survival (Fujiwara et al., 2001). The role of BMP4 in other stages of spermatogenesis is less well characterized and requires further investigation.

Given the important roles of both the retinoid and BMP signaling pathways in various cellular processes, it is not surprising that in several systems they interact in vitro and in vivo. For example, in P19 embryonal carcinoma cells, BMP2 and BMP4 have been shown to interact with RA signaling to induce apoptosis (Glozak and Rogers, 1996; Glozak and Rogers, 2001). RA-induced apoptosis mediated by BMP signaling is also known to occur in inter-digital cell death of the fetal vertebrate limb (Rodriguez-Leon et al., 1999). Several studies have revealed that some of the malformations resulting from RA-mediated teratogenicity, including cleft palate formation (Ho et al., 2004) and digestive tract abnormalities (Sasaki et al., 2004), are due to regulation of the BMP4 by RA. Treatment of 10.5 d.p.c. whole mouse embryos with RA also causes overall decreases in *BMP4* mRNA levels, indicating that BMP4 may mediate other aspects of RA teratogenicity (Zhu et al., 1999). Furthermore, during lung morphogenesis, RA regulation of *BMP4* mRNA levels is crucial for correct lung formation (Malpel et al., 2000). RA-mediated down-regulation of *BMP4* expression has been observed in the developing limb bud (Mic et al., 2003), and inner ear (Thompson et al., 2003). Together, these studies demonstrate that the effect of retinoids on BMP levels is cell type and tissue dependent, and that the interaction between these pathways is critical to many differentiative processes.

While it is evident that both retinoids and certain members of the TGF-β superfamily are necessary for male reproductive capacity, it is as yet unclear whether these signaling pathways interact in the germ line. Here, we report for the first time the characterization of BMP4 and retinoid receptor expression in murine spermatogonia, and describe an interaction between these two signaling systems that may indicate a potential mechanism through which they regulate spermatogenesis.

Materials and methods

Animals, antibodies, chemicals, and reagents

The use of animals for this study was approved by the University of Newcastle (Australia) Animal Care and Ethics Committee. Mouse anti-human BMP4 monoclonal antibody was obtained from R&D Systems (Minneapolis, MN, USA), rat anti-mouse GCNA1 monoclonal antibody supernatant was a kind gift from G. Enders (Kansas, USA), and mouse anti-bovine vimentin was obtained from BD Pharmingen (San Diego, CA, USA). All chemicals and reagents were obtained from Sigma (Sigma, St. Louis, MO, USA) or Research Organics (Research Organics Inc., Cleveland, Ohio, USA) unless otherwise stated. Cell culture reagents were from Sigma and Gibco (Gibco™ Invitrogen Corporation, Mt. Waverly Victoria, Australia). GC-1(spg) and P19 embryonal carcinoma cells were purchased from ATCC (Manassas, VA, USA).

Cell line culture and retinoid treatment of cell lines

GC-1(spg) and P19 embryonal carcinoma cells were cultured in DMEM supplemented with 100 μM sodium pyruvate, 200 μM L-glutamate, 100 U/ml penicillin, 10 μg/ml streptomycin, and 5% fetal bovine serum (FBS) for routine culture, and 0.5% FBS for all experiments. Retinoids were dissolved in 100% ethanol and diluted 1000-fold in DMEM media supplemented with 0.5% FBS to obtain final working concentrations. Stock solutions of all-*trans*-ROL and all-*trans*-RA were prepared fresh weekly and stored in the dark at −20°C.

Generation of vitamin A deficient mice

2-week-old male, Swiss, out-bred mice were weaned onto a vitamin A deficient (VAD) diet (Vitamin A deficient diet, modified. Catalogue number: 960220, MP Biomedicals). Mice were maintained on this diet until signs of vitamin A deficiency were observed, normally at around 8 weeks, which included listlessness, piloerection, blindness, diarrhea, and weight loss. Once deficiency was reached, animals were either culled and their tissues removed for histology and RNA isolation, or subjected to retinoid administration. Treatments involved injecting mice with either ROL, in the form of 0.5 mg retinyl acetate, interperitoneally (i.p.) (in 1:5, DMSO:sesame oil) and placing onto normal mouse food, or 0.5 mg all-*trans*-RA i.p. and placing on an RA supplemented diet (10 mg/kg all-*trans*-RA). At various times, mice were culled and one testis employed for histological analysis, while the contra-lateral testis was used for RNA extraction. For histological analysis, tissue was fixed overnight by incubation in ice-cold 4% paraformaldehyde in PBS at 4°C, then dehydrated and embedding in paraffin.

Immunohistochemistry (IHC)

Sections were de-paraffinized and rehydrated. Antigen retrieval was performed with 10 mM sodium citrate pH 6.0 at 95°C for 10 min. Slides were blocked in 1% H_2O_2 in PBS for 10 min. IHC was performed with DAKO Cytomaton Envision+ System (DAKO corporation, Carpinteria, CA, USA) according to the following protocol. Sections were blocked in 3% BSA in PBS for 1 h, and incubated with primary antibodies in 1% BSA in PBS overnight at 4°C (mouse anti-human BMP4 antibody dilution 1:50, rat anti-mouse GCNA1 monoclonal antibody supernatant dilution 1:10, mouse anti-bovine vimentin 1:25). Slides were washed in PBS with 0.05% Tween-20 (PBST), and incubated with HRP-labeled polymer anti-mouse (anti-BMP4 and anti-vimentin) or anti-rat (anti-GCNA1) secondary antibody solutions (DAKO) for 45 min. Slides were washed in PBST, then color development was performed using DAKO Liquid DAB+ Substrate-Chromagen System according to manufacturer's protocol. Sections were then counterstained with Gill's hematoxylin.

Germ cell isolation, culture, and vitality staining

For isolation of spermatogonia, testes from 20 8-day-old Swiss mice were pooled, while for pachytene spermatocytes and round spermatids, mice of a minimum of 8 weeks old were used. Testes were isolated, de-capsulated, and incubated for 15 min each in 0.5 mg/ml collagenase/DMEM with agitation and then in 0.25% trypsin/EDTA in DMEM. Tubules were dissociated manually by pipetting and washed in 0.5% BSA in DMEM by centrifugation. Cell pellets were resuspended in DMEM and filtered twice through a 70 μm membrane, then separated over a BSA gradient (Bellve et al., 1977). Purified germ cells were identified by GCNA1 staining for spermatogonia (Richards et al., 1999) and *Arachis hypogaea* lectin staining of the developing acrosome for spermatocytes and spermatids. Acrosome staining was performed as follows: cells were fixed for 10 min in ice-cold methanol and dried onto slides. Slides were incubated with 1 mg/ml lectin for 15 min, washed twice in PBS, and mounted in 10% Mowiol 4–88 with 30% glycerol in 0.2 M Tris (pH 8.5) with 2.5% 1,4-diazobicyclo-(2.2.2)-octane (DABCO). Cells were categorized based on both size and lectin staining pattern: spermatocytes exhibited relatively diffuse staining localized to a region representing the early stages of acrosome development, and spermatids were scored based on distinct labeling of the developing and elongating acrosome (data not shown). Fractions were found to be greater than 90% pure for spermatogonia based on GCNA1 positive cells, and with no obvious somatic cell contamination. Spermatocyte fractions were 65–70% pure with contaminating cells largely consisting of early spermatocytes. Spermatids were 85–95% pure with contaminating cells consisting of late spermatocytes.

Spermatogonia were either subjected to RNA or protein isolation, or further cultured for 6 h in DMEM containing 10% FBS, in the presence or absence of retinoids before extracting RNA. Cell vitalities were assessed by trypan blue exclusion, and were found to be greater than 75% (data not shown). Spermatocytes and spermatids were immediately subjected to RNA or protein extraction following separation. Each cell isolation experiment was repeated a minimum of 3 times.

RNA isolation

Total RNA was isolated from cells using two rounds of a modified acid guanidinium thiocyanate–phenol–chloroform protocol (Chomczynski and Sacchi, 1987): washed cells resuspended in lysis buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sarkosyl, 0.72% β-mercaptoethanol). RNA was isolated by phenol/chloroform extraction and isopropanol precipitated.

Reverse transcription polymerase chain reaction (RT-PCR) and cloning

Reverse transcription was performed with 2 μg of total RNA, 500 ng oligo-dT primer, 40 U of RNasin (MBI Fermentas), 0.5 mM dNTPs, and 20 U of RevertAid™ M-MuLV-Reverse Transcriptase (MBI Fermentas). PCR reactions contained cDNA equivalent to 100 ng of RNA, 80 ng of each primer, 0.5 mM dNTPs, 1 U of Taq polymerase (MBI Fermentas). Prior to analysis of gene expression, RT reactions were verified by β-*actin* PCR. Primer sequences are listed in Table 1, along with annealing temperatures. BMP4 promoter primer sequences and PCRs were carried out as previously described (Thompson et al., 2003). PCR fragments were cloned into pGEM-T Easy (Promega) according to manufacturer's instructions, and sequenced by Australian Genome Research Facility (AGRF, Brisbane, Australia) to confirm insert sequences. Each PCR was performed on 3 separate cell isolations, of which a representative PCR is shown.

Real-time PCR

Real-time PCR was performed on cDNA generated as described above, with the exception that prior to reverse transcription, RNA was treated with DNAse to remove genomic DNA contamination. Real-time PCR was performed using SYBR® Green JumpStart Taq ReadyMix (Sigma) according to manufacturer's instructions on an MJ Opticon 2 (MJ Research, Reno, NV, USA). Primer sequences are provided in Table 1. Reactions were performed on cDNA equivalent to 100 ng of total RNA and carried out for 40 amplification cycles. SYBR® Green fluorescence was measured after the extension step at the end of each amplification cycle and quantified using Opticon Monitor Analysis software

Table 1
Primer sequences and annealing temperatures

Gene	Primer sequences	Annealing temperature (°C)
RARα	5′-AGTTCCGAAGAGATAGTACC-3′ 5′-ATGATGCAGTTCTTGTCC-3′	55
RARβ	5′-CAGGAACAAGAAAAAGAAGG-3′ 5′-GCAAAGGTGAACACAAGG-3′	60
RARγ	5′-TCTTCAGACGCAGCATTC-3′ 5′-CTCCTCTTTTCAATCCTTTTTC-3′	60
RXRα	5′-ATCTTTGACAGGGTGCTAC-3′ 5′-CATACACCTCTCCCTCAAC-3′	58
RXRβ	5′-TTTCTTCAAGCGCACCATTC-3′ 5′-ATCTCCATCCCCGTCTTTGTC-3′	60
RXRγ	5′-TACGGTGACATGAACGTGG-3′ 5′-AGAAGGAGGCAATGAGCAG-3′	60
BMP4	5′-GAGGAGTTTCCATCACGAAGAA-3′ 5′-CAGGAACCATTTCTGCTGGG-3′	65
Exon 1A forward primer[a]	5′-GAAGGCAAGAGCGCGAGG-3′	60
Exon 1B forward primer[a]	5′-CAGGCCGAAAGCTGTTC- 3′	60
Intron 2 forward primer[a]	5′-GAGCCTGTCTGCTCCAGAGTCTC-3′	60
BMP4 reverse primer (common)[a]	5′-ACGACCATCAGCATTCGGTTAC-3′	60
Exon 1A forward primer (real-time PCR)	5′-GACCAGGTTCATTGCAGCTTTC-3′	60
β-Actin	5′-AGCCTTCCTTCTTGGGTATG-3′ 5′-CTTCTGCATCCTGTCAGCAA-3′	55–65

[a] Thompson et al. (2003).

Version 2.02 (MJ Research). For each sample, a replicate omitting the reverse transcription step was undertaken as a negative control. Reverse transcription reactions were verified by *β-actin* PCR, performed for each sample in all reactions in triplicate. Real-time data were analyzed using the equation $2^{-\Delta C(t)}$, where $C(t)$ is the cycle at which fluorescence was first detected above background fluorescence (Livak and Schmittgen, 2001). Data were normalized to *β-actin* and then to a reference sample (see figures) and are presented as the average of each replicate normalized to each reference sample (±SEM). The $2^{-\Delta\Delta C(t)}$ transformation (Livak and Schmittgen, 2001) was used for comparisons between retinoid-treated and vehicle control primary spermatogonial cultures. Each of these samples was examined in triplicate, and each data set consists of at least 3 separate experiments (Fig. 9). In this figure, values for each replicate were averaged, and relative expression levels between retinoid-treated samples are depicted as percentages of controls (±SEM). For experiments involving retinoid readministration to VAD animals, each data point is the average of at least 3 separate animals.

In situ hybridization

Testes were isolated and fixed in 4% paraformaldehyde in PBS at 4°C overnight, dehydrated, and embedded in paraffin, and 7 μm sections were processed for in situ hybridization. DIG-labeling of linearized probes was carried out using the Roche (Roche Molecular Biochemicals, Mannheim, Germany) DIG RNA Labelling Kit (SP6/T7) according to manufacturer's instructions. Probe detection was carried out using a Roche anti-DIG alkaline phosphatase-conjugated antibody and NBT/BCIP (Roche) was used for probe detection as per manufacturer's instructions. Sections were counterstained with Gill's hematoxylin and photographed under bright field microscopy using a Zeiss Axioplan 2 compound microscope.

Protein extraction and Western blotting

Cells were solubilized with lysis buffer (4% CHAPS, 7 M urea, 2 M thiourea in 30 mM Tris, pH 8.5). Protein concentration was estimated using a 2D Quant Kit (Amersham). Soluble proteins were then precipitated with ice-cold acetone and resuspended in SDS sample buffer (375 mM Tris–HCL, 2% (w/v) SDS, and 10% (w/v) sucrose, pH 6.8) containing 2% (v/v) β-mercaptoethanol, and boiled at 100°C for 15 min. 10 μg of each protein sample and 10 ng of human recombinant BMP4 were separated on a 12% polyacrylamide gel (Laemmli, 1970) and transferred onto a nitrocellulose hybond C-Extra membrane (Amersham) (Towbin et al., 1992) at 350 mA constant current for 1.5 h. Membranes were blocked for 1 h in 5% skim milk powder in TBST (0.1% Tween-20), then incubated with 2 μg/ml of mouse anti-BMP4 monoclonal antibody or 125 ng/ml anti-vimentin monoclonal antibody in 1% milk powder in TBST overnight at 4°C. Membranes were washed in TBST, then incubated with HRP-conjugated goat anti-mouse secondary antibody (Santa Cruz) at a 1:3000 dilution for 1 h at room temperature. Membranes were washed in TBST and visualized using an ECL Detection Kit (Amersham Biosciences) according to manufacturer's instructions. Membranes were then stripped in 100 mM β-mercaptoethanol, 2% SDS, and 62.5 mM Tris (pH6.7) at 60°C for 1 h, and reprobed using anti-α-tubulin (Santa Cruz) as a loading control. The Western blot image presented in Results is representative of 3 replicate experiments.

Data analysis

Experiments were replicated at least 3 times prior to statistical assessment by ANOVA. Post-hoc testing was performed using Fischer's PLSD. Samples with a P value of <0.05 were considered significant (*).

Results

BMP4 mRNA and protein are expressed in spermatogonia and early spermatocytes

Due to the lethal nature of the *BMP4* knockout, few studies exist that have examined BMP4 and its potential role in spermatogenesis. Since BMP4 is known to play an essential role in PGC formation, we examined the expression pattern of *BMP4* mRNA in the adult mouse testis using in situ hybridization. We detected *BMP4* messenger RNA expression at the earliest stages of spermatogenesis, predominantly in the cytoplasm of the spermatogonia and early spermatocytes (Fig. 1A), with decreased levels detected in mid-stage spermatocytes. Hematoxylin counterstaining was used to identify Sertoli cells, which did not exhibit any staining for *BMP4* (data not shown). No signal was detected with *BMP4* sense control probes (Fig. 1B).

Immunolocalization experiments using an anti-BMP4 antibody revealed that the expression pattern of the BMP4 protein corresponded with that of the mRNA (Figs. 1C, D). Staining tubules with anti-vimentin, a Sertoli cell-specific protein (Show et al., 2003; Wang et al., 2002; Zhang et al., 2004), confirmed that BMP4 expression was not apparent in Sertoli cells (Fig. 1E). Thus, while it is possible that a small amount of BMP4 levels could originate in Sertoli cells, we believe that the overall expression of BMP4 in Sertoli cells in the adult mouse testis is minimal.

Quantification of BMP4 levels in adult mouse testis and in isolated germ cells

To determine definitively whether the BMP4 expression we observed by in situ hybridization and immunohistochemistry was solely due to germ cell expression, Western

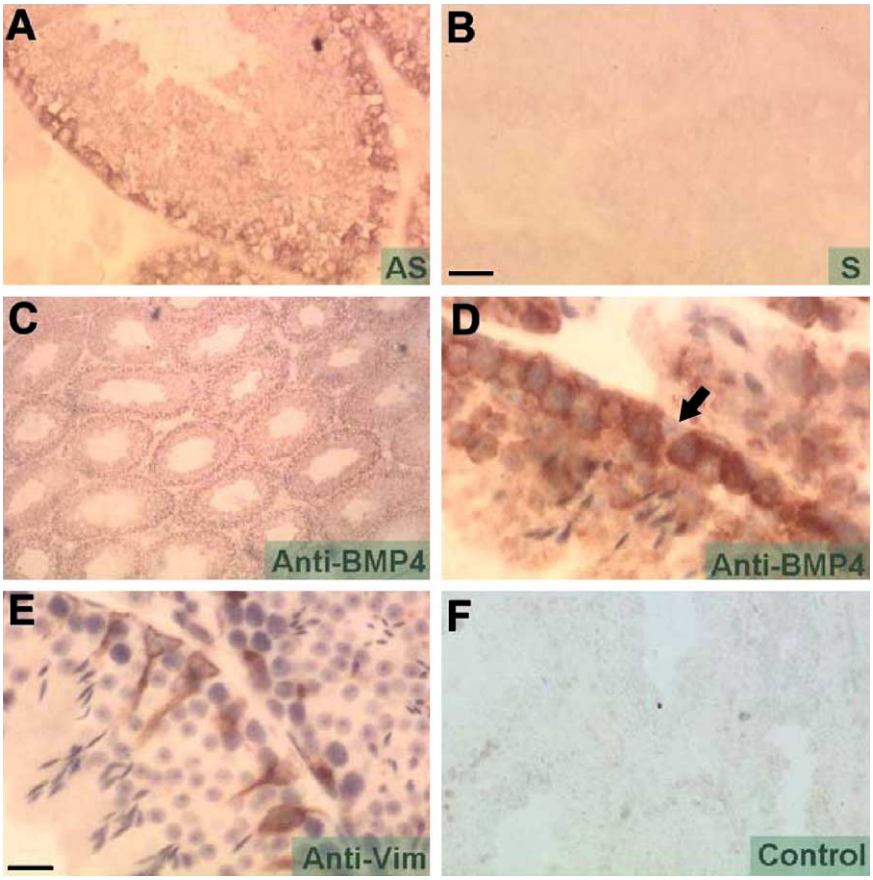

Fig. 1. Localization of *BMP4* mRNA and protein expression in adult mouse seminiferous tubules. Adult mouse testis sections were probed with DIG-labeled mRNA probes for either anti-sense (AS) or sense (S) *BMP4*, and visualized using an alkaline phosphatase-conjugated anti-DIG antibody. Immunohistochemical analysis was performed on adult mouse testis sections, using either anti-BMP4 or anti-vimentin monoclonal antibodies, and an HRP-conjugated secondary antibody. (A) Representative in situ hybridization analysis of *BMP4* gene expression in mouse seminiferous tubules. *BMP4* mRNA was detected primarily in the cytoplasm of spermatogonia and early spermatocytes, with decreased levels in late spermatocytes and spermatids. (B) No signal was detected in the *BMP4* mRNA sense control. (Magnification: 200×. Scale bar: 40 μm). Representative immunohistochemical experiments demonstrating expression characteristics of BMP4 protein in the adult mouse testis in (C) 40× magnification, and (D) 400× magnification counterstained with hematoxylin. Sertoli cells did not express detectable levels of BMP4 protein. (arrowhead = Sertoli cell). (E) Vimentin (Vim) localization in a section counterstained with hematoxylin. Vimentin staining is localized to Sertoli cell cytoplasm (Magnification: 400×. Scale bar: 15 μm). (F) Secondary antibody only control. The secondary antibody used for vimentin staining is the same as in panel D.

blot analysis was carried out on germ cell fractions. Through the use of an anti-human BMP4 antibody, we were able to detect mouse BMP4 in whole adult mouse testis and in isolated germ cells (Fig. 2). The membranes were subsequently reprobed with anti-vimentin to demonstrate the purity of germ cell populations, with no detectable levels of vimentin in our germ cell fractions. The expression of BMP4 in germ cells appeared highest in spermatogonia, and decreased significantly in pachytene spermatocytes and round spermatids. There were approximately equal levels of BMP4 in spermatogonia and whole testis, indicating that the BMP4 protein expression in the testis can be largely attributed to germ cell expression rather than that of any somatic cell expression. To obtain a quantitative measure of the relative levels of BMP4 expression between each germ cell fraction, we then utilized quantitative real-time PCR.

Real-time PCR analysis confirmed the gene and protein expression data, and is presented as the relative levels of *BMP4* mRNA for spermatogonia, pachytene spermatocytes, and round spermatids, normalized to β-actin (±SEM; Fig. 3). Significantly greater levels of *BMP4* mRNA expression were detected in spermatogonia relative to spermatocytes and spermatids ($P < 0.05$, $n = 4$). Collectively, the Western and real-time PCR data illustrate that the majority of the BMP4 expressed in adult mouse testis can be attributed to spermatogonial and early spermatocyte gene expression.

Regulation of BMP4 mRNA and protein levels during vitamin A deficiency (VAD)

As a result of VAD, the only remaining germ cells in the testes are spermatogonia (reviewed in Chung and Wolgemuth 2004). Since BMP4 mRNA and protein expression were highest in spermatogonia and BMP4 is regulated by retinoids in other cellular systems, we investigated whether retinoids were able to influence *BMP4* mRNA expression in the VAD testis (Fig. 4). We generated vitamin A deficient mice, and then quantitatively measured the levels of *BMP4* mRNA in testis samples from these mice compared to fertile control animals. The vitamin A deficient phenotype exhibited in these animals was typical, with seminiferous tubules containing only Sertoli cells and spermatogonia. Interestingly, we found a 7-fold increase in *BMP4* mRNA expression levels (as assessed by real-time PCR) in the testes of VAD mice compared with control

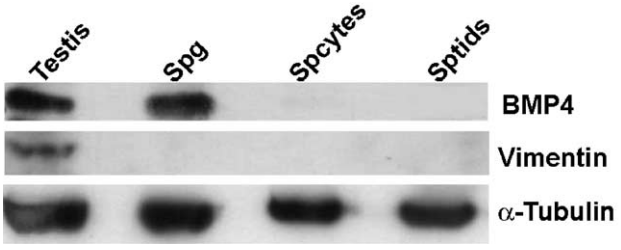

Fig. 2. Western blot showing localization of BMP4 protein in isolated germ cells. CHAPS extracted proteins from testes and isolated germ cells were subjected to Western blotting with anti-BMP4, anti-vimentin, and anti-α-tubulin antibodies. BMP4 is located primarily in spermatogonia (spg), with almost no levels detected in pachytene spermatocytes (spcytes) and round spermatids (sptids). A whole testis extract is provided for comparison. α-Tubulin was used as a loading control. Vimentin Western blotting was performed to determine Sertoli cell contamination in germ cell fractions, and shows no detectable levels of vimentin in germ cell fractions.

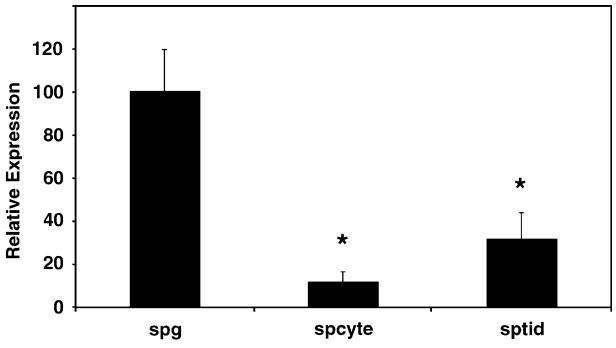

Fig. 3. Real-time PCR analysis of *BMP4* gene expression in spermatogonia, spermatocytes, and spermatids. Real-time PCR analysis was performed on cDNA generated from total RNA from isolated germ cells to examine relative expression levels of *BMP4* mRNA. Data are presented as relative levels of expression normalized to spermatogonia (±SEM). A statistically significant decrease in *BMP4* mRNA expression levels was observed from spermatogonia (spg) to pachytene spermatocytes (spcyte), and from spermatogonia to round spermatids (sptid) ($P < 0.05$).

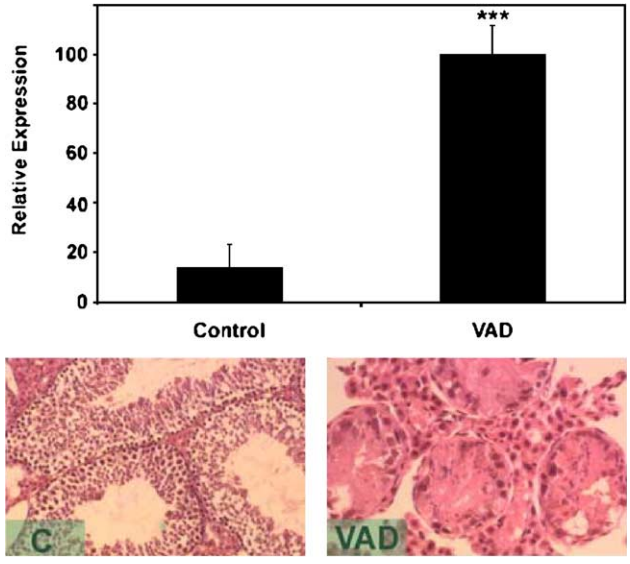

Fig. 4. Analysis of *BMP4* mRNA levels in the vitamin A deficient testis. Total RNA was collected from the testes of vitamin A deficient adult male mice, and real-time PCR on cDNA generated from these samples was used to examine the levels of *BMP4* mRNA in the absence of retinoids. Data are presented as the relative levels of expression between control testes samples and VAD samples, normalized to VAD (±SEM). A significant increase in *BMP4* mRNA levels was observed in the absence of retinoids ($P < 0.01$). (Control = complete spermatogenesis, VAD = vitamin A deficient). Representative images are provided of the testicular morphology of VAD animals, and control (C) animals for comparison (Counterstained with hematoxylin. Magnification: 200×).

animals (Fig. 4), thus demonstrating that the enrichment for spermatogonia resulted in a significant increase in BMP4 protein levels.

To examine whether these results were solely due to an enrichment of cells expressing BMP4, we examined BMP4 protein levels during progressive stages of vitamin A deficiency. When testicular samples were collected from mice in progressive states of VAD (from incomplete to complete VAD), it was observed that partially deficient mice experienced an increase in the number of cells staining positive for BMP4, as determined by immunolocalization studies (Fig. 5). As mice progressed towards more extreme cases of VAD, an increased number of apoptotic cells were observed. These can be seen in Fig. 5F, where they appear as large multinucleate cells, and stain positive in TUNEL assays (data not shown). In these cells, BMP4 is no longer detectable. As complete deficiency is finally reached, staining is localized to spermatogonia only (Figs. 5G–I). These results document an initial increase in BMP4 positive cells in the partially VAD testis which is then followed by the appearance of BMP4-negative apoptotic cells. The process culminates in the depletion of germ cells in the seminiferous tubules after prolonged VAD, leaving only spermatogonia, which retain BMP4 expression. These results suggest that BMP4 positive germ cells are the only germ cells remaining under conditions of VAD.

ROL but not RA preferentially initiates a rapid decrease in BMP4 in VAD

To determine whether testicular BMP4 was regulated by retinoids in vivo, VAD mice were treated with either RA or ROL. Mice received an initial inter-peritoneal injection of either RA or ROL, and were then maintained on a diet containing either RA or ROL, respectively. Real-time PCR analysis revealed that ROL administration for 24 h, led to a decrease in *BMP4* mRNA expression in the VAD testis. Similar treatments with RA did not affect *BMP4* mRNA levels. This down-regulation of *BMP4* gene expression by ROL was significant when compared to RA treatment (Fig. 6). Since a 24-h treatment with either retinoid is not sufficient to significantly alter the cellular composition of the VAD testis (Fig. 6), the observed differences in relative *BMP4* levels could not be attributed to enrichment for cells expressing *BMP4*.

VAD mice were also treated with RA or ROL for 21 or 19 days, respectively. It was apparent that 21 days of RA treatment were not sufficient to reinitiate spermatogenesis. However, in contrast, 19 days of ROL treatment were sufficient for the resumption of spermatogenesis (Fig. 7). It was apparent upon quantification of *BMP4* mRNA that, similar to 24 h of ROL administration, 19 days of ROL administration significantly down-regulated

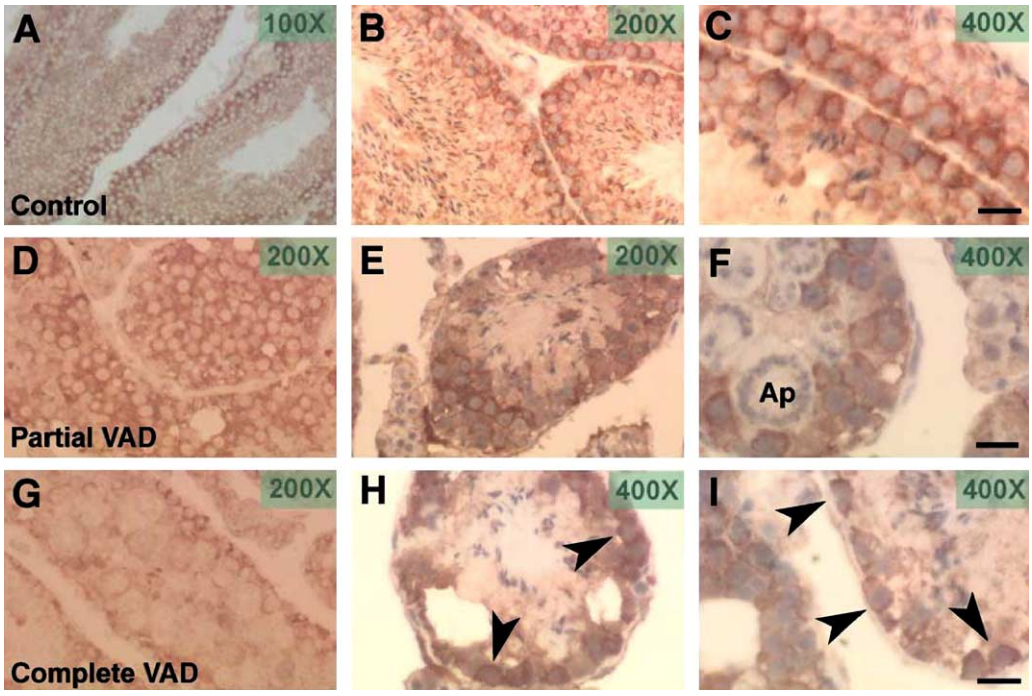

Fig. 5. BMP4 immunolocalization in seminiferous tubules at progressive stages of vitamin A deficiency. VAD mice were generated as described, and sampled at progressive stages of vitamin A deficiency. Tissue was processed for histology, and BMP4 was localized with anti-human BMP4. Immunohistochemical analysis revealed a progressive increase in the number and type of cells expressing BMP4 protein as VAD status increased. (A) Bright field image of BMP4 localization. (B, C) BMP4 staining (brown) in seminiferous tubules from control animals counterstained with hematoxylin. (D) Bright field image of BMP4 stained mouse testis at an early stage of VAD (6 weeks post-weaning). The number of cells staining positive for BMP4 increased. (E, F) Counterstained images showing apoptotic cells (Ap). (G) Bright field image showing BMP4 staining pattern of 7-week deficient VAD testis. The tubule no longer contains late stage BMP4 negative germ cells and BMP4 staining is now localized to cells around the basement membrane. (H, I) Counterstained images at increased magnification showing specific staining in spermatogonia (arrowheads). Scale bar: approximately 10 μm.

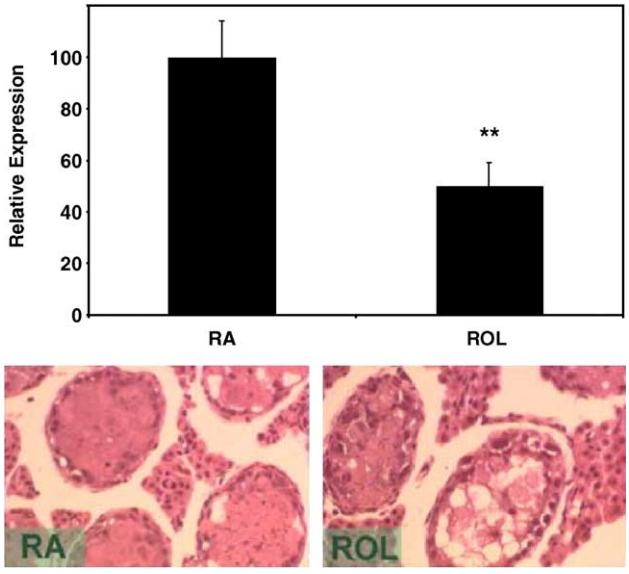

Fig. 6. Real-time PCR analysis of *BMP4* mRNA levels in adult VAD mouse testis 24 h after retinoid administration. VAD mice were readministered either RA or ROL and total RNA was collected from the testes of these animals 24 h post-retinoid administration. *BMP4* mRNA levels were examined through real-time PCR on cDNA from these samples. Data are presented as the relative levels of expression of *BMP4* mRNA normalized to RA (±SEM). Hematoxylin stained images of testes treated with either ROL or RA for 24 h (representative images) are provided for histological comparison. Results show a significant decrease in *BMP4* mRNA levels after ROL administration compared to RA ($P < 0.01$).

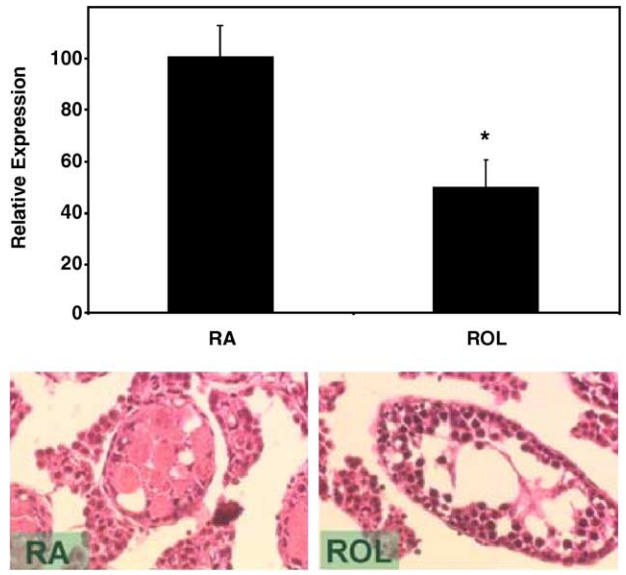

Fig. 7. Real-time PCR analysis of *BMP4* mRNA levels in adult VAD mouse testis after prolonged retinoid administration. VAD mice were administered either RA for 21 days or ROL for 19 days to instigate recovery of spermatogenesis, and relative levels of *BMP4* mRNA expression was examined through real-time PCR. Data are presented as the relative levels of expression of *BMP4* mRNA normalized to RA (±SEM). Results show that a significant decrease in *BMP4* mRNA levels is maintained 19 days after ROL administration, compared to 21 days with RA ($P < 0.05$). Hematoxylin stained representative images of RA or ROL administered testes are provided for histological analysis of spermatogenic recovery.

BMP4 gene expression in the testis (Fig. 7). Similarly, as with the 24-h time point, 21 days of RA treatment had no effect on *BMP4* gene expression. This confirmed that BMP4 expression in the adult mouse testis is retinoid dependent. However, interestingly, these results showed that ROL, and not RA, was the active retinoid metabolite.

Retinol regulates BMP4 mRNA in spermatogonia

To examine whether the effect of ROL on BMP4 levels in the VAD testis was mediated by Sertoli cells, or as a direct effect of ROL on germ cells, we examined the effect of retinoid treatment on *BMP4* gene expression in isolated spermatogonia. Primary cultures of spermatogonia were incubated for 6 h in the presence of 1 μM RA, 1 μM ROL, or a vehicle control (ethanol). Data are presented as the expression of *BMP4* mRNA in retinoid-treated spermatogonia relative to control levels (±SEM) (Fig. 8). We again found a marked down-regulation of *BMP4* gene expression, and this was statistically significant after ROL ($P < 0.05$, $n = 8$) but not RA treatment. These results illustrate that isolated spermatogonia are more responsive to ROL than RA in terms of the regulation of the expression of BMP4 and possibly other genes, and the effects observed above are a result of ROL acting directly on germ cell gene expression, rather than an indirect effect mediated by Sertoli cells.

Retinoid receptor expression in spermatogenic cells at various stages of differentiation

Since we had demonstrated a regulation of BMP4 levels in germ cells in response to retinoids, we examined gene expression for the retinoid receptors using RT-PCR. Messenger RNA for *RARα*, *RARγ*, and all of the *RXRs*

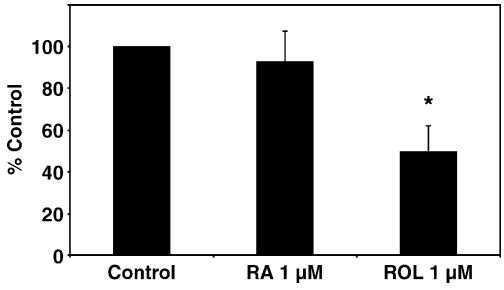

Fig. 8. Effect of retinoid treatment on *BMP4* mRNA expression in isolated spermatogonia. Spermatogonia were isolated from 8-day-old testes and cultured for 6 h in the presence of either 1 μM RA, 1 μM ROL, or ethanol vehicle control. Total RNA was then isolated and real-time analysis was performed on cDNA generated from these samples to examine *BMP4* mRNA levels. Treatment of spermatogonia with of 1 μM ROL for 6 h decreased *BMP4* gene expression in spermatogonia significantly ($P < 0.05$). Treatment with 1 μM RA for 6 h induced a slight decrease in *BMP4* mRNA levels relative to untreated spermatogonia; however, this was not statistically significant. Data were presented as percentage of control levels (±SEM).

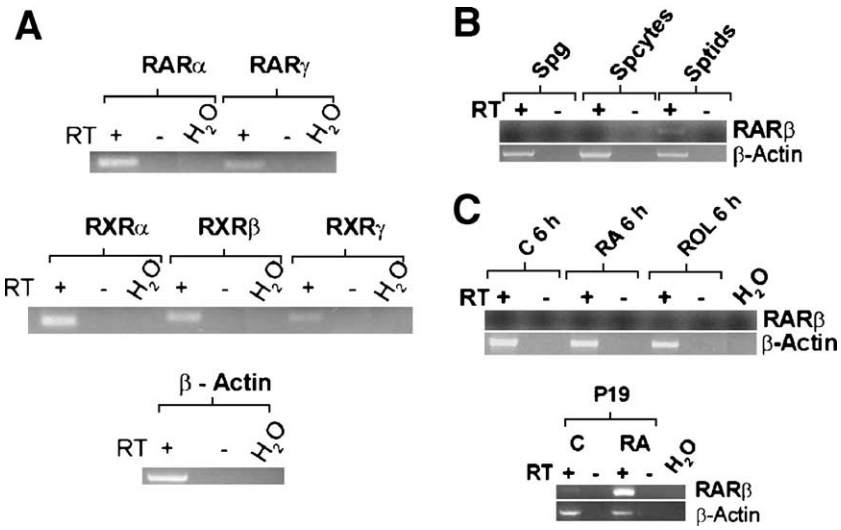

Fig. 9. Retinoid receptor expression in spermatogonia. Reverse transcription (RT)-PCR was performed on cDNA generated from total RNA isolated from spermatogonia to examine the expression of the retinoid receptors. Negative control samples where reverse transcriptase was omitted are included (−RT). (A) Spermatogonia express all the RXRs, RARα, and RARγ. β-Actin was used as an RT control. (B) *RARβ* mRNA was not detectable in spermatogonia (spg), spermatocytes (spcytes), or spermatids (sptids). (C) RARβ mRNA expression could not be induced after treatment with either 1 μM RA or ROL for 6 h (6 h). RA-treated P19 embryonal carcinoma cells were used as a positive control for all RARβ PCRs.

were detected in spermatogonia (Fig. 9A) by RT-PCR; however, *RARβ* mRNA could not be easily detected in spermatogonia.

We further analyzed the expression of *RARβ* in isolated spermatogonia, spermatocytes, and spermatids, and found extremely low levels of *RARβ* mRNA, at the limits of detection by PCR (Fig. 9B). To verify this result, the P19 embryonal carcinoma cell line was used as a positive control as an example of RARβ induction upon retinoid treatment, and as an RARβ PCR control (Fig. 9C). The paradigm response to retinoid treatment is *RARβ* induction, as the gene encoding RARβ contains an RA response element (RARE) in the promoter region, and RARβ expression is classically up-regulated in response to RA treatment (de The et al., 1990; Kobayashi et al., 2000; Sucov et al., 1990). Thus, we expected *RARβ* mRNA to increase in response to RA treatment. However, *RARβ* was not inducible by retinoid treatment in spermatogonia treated for 6 h with either RA or ROL (Fig. 9C).

BMP4 promoter expression in mouse germ cells and the VAD testis

As described previously, retinoid down-regulation of *BMP4* gene expression is not unique to the mouse germ line. In one particular study, Thompson et al. (2003) described a mechanism by which *BMP4* gene expression could be down-regulated by RA in the mouse inner ear. They determined the existence of a novel promoter in the second intron of the *BMP4* gene, which mediated this *BMP4* response to RA in both an inner ear-derived cell line (2B1) and in isolated otocysts. Previously, the existence of two promoters (1A and 1B) had been documented (Feng et al., 1995), with 1A being predominantly active in most cell types (Feng et al., 1995). We used RT-PCR with the promoter-specific transcript primers used by Thompson et al. (2003) to examine whether these *BMP4* promoters were active in isolated spermatogonia. For this study, the immortalized mouse spermatogonial cell line GC-1(spg) was also used as a control. This cell line is thought to exhibit an intermediary phenotype between type A and type B spermatogonia, and has been found to express several germ cell markers, including the germ-cell-specific BMP, *Bmp8b* (unpublished results). We found promoter 1A and the intron 2 promoter product (termed i2) to be expressed in both spermatogonia and GC-1(spg) (Fig. 10). The 1B transcription product was also detected in germ cells, although they were found to have a shortened exon 1B, based on the size of the 1B transcription product. Thompson et al. (2003) similarly observed this in 2B1 cells, where the 1B exon could be expressed as either 21 or 279 base pairs in length.

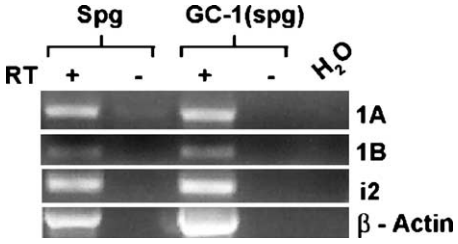

Fig. 10. BMP4 promoter utilization in mouse germ cells. Total RNA was isolated from spermatogonia from 8-day-old testes, and from GC-1 (spg). RT-PCR for the 1A, 1B, and i2 promoter-specific transcripts was used to examine promoter usage in germ cells. PCR results indicate the presence of BMP4 promoter-specific transcripts arising from transcription from the 1A and 1B promoters, and from the i2 promoter. The GC-1(spg) cell line also utilized all three BMP4 promoters. β-Actin was used as an RT control (spg = spermatogonia). Samples omitting reverse transcriptase are included as negative controls (−RT).

Germ cells clearly express the 21 base pair exon; however, we were not able to detect the presence of the 279 base pair exon through RT-PCR.

At present, few studies exist where germ cells have been successfully transfected, and germ cells are notoriously difficult to culture for extended periods of time. Thus, *BMP4* promoter analysis in germ cells through the use of reporter construct assays is problematic. Therefore, in order to analyze *BMP4* promoter utilization in the VAD testis, we carried out real-time PCR analysis on the promoter PCR products shown in Fig. 10. For 1A, another primer to that used by Thompson et al. (2003) was designed to amplify a smaller transcript within the same region, so the resulting 200 base pair product could be measured by Sybr® Green analysis (primer sequence is provided in Table 1). We performed our studies on testicular tissue from mice sampled 24 h after the administration of either RA or ROL, to determine if the decrease in *BMP4* mRNA levels after ROL treatment of VAD mice was attributable to the activity of a particular BMP4 promoter. Based on the relative expression levels of these promoter-specific transcripts, we found that the activity of both the 1A and i2 promoters was reduced in response to treatment by ROL, but not by RA (Fig. 11). The activity of the 1B promoter was found to be so low in both RA- and ROL-treated tissue, that a functional analysis of this particular promoter was not possible. This conclusion is based on the high $C(t)$ values of the PCR product downstream of the 1B promoter and the difficulty in visualizing a 1B product after touchdown PCR (as described in Thompson et al., 2003). Thus, it can be assumed that the majority of BMP4 signaling in response to retinoids in the adult mouse testis is due to the activity of both the 1A and i2 promoters, and that both promoters are responsible for the down-regulation of *BMP4* mRNA levels in response to ROL during spermatogenesis.

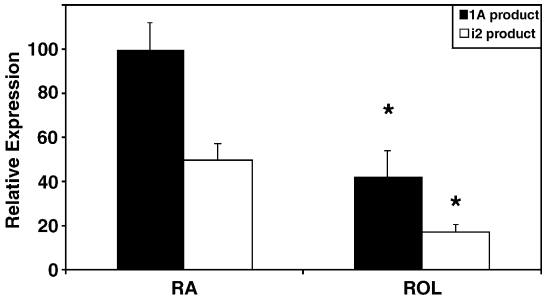

Fig. 11. Analysis of promoter utilization in VAD testis using real-time PCR. VAD mice were administered either RA or ROL for 24 h, and total RNA was collected from testes at the end of each treatment. Real-time PCR on promoter-specific transcripts was used to analyze BMP4 promoter activity 24 h post-retinoid administration. 1B promoter-specific transcript expression levels were below the limits of accurate detection with this assay. Results are presented as the relative levels of expression normalized to 1A transcript levels in RA administered testes (±SEM). A significant decrease in relative transcript levels was seen for both the 1A and i2 promoters after ROL treatment when compared to corresponding RA administered animals ($P < 0.05$).

Discussion

The molecular mechanisms underlying the induction of infertility in VAD rodents have never been fully elucidated. In addition, while several genes have been implicated in spermatogenesis, relatively little is known regarding the regulation of gene expression in the mammalian testis. Several studies have attempted to characterize the genes involved in spermatogenesis (Schultz et al., 2003) but, at present, little is known regarding the function of such genes or the significance of the signal transduction pathways they mediate. Here, we report an interaction between two signaling pathways in germ cells, that is supported by observations in other differentiation processes, and that provides insights into the role of retinoid metabolism in spermatogenesis.

It has been well documented that BMP4 is necessary for the generation and survival of PGCs and might therefore be involved in the developmental regulation of spermatogenesis. We found that adult germ cells expressed BMP4 at both mRNA and protein level. In the adult testis, *BMP4* mRNA expression was found to be predominant in the early germ cells, including spermatogonia and early spermatocytes, with expression being absent from Sertoli cells. We found this pattern of expression by in situ hybridization, immunohistochemistry, Western blotting, and immunohistochemical analysis. Hu et al. (2004) found *BMP4* mRNA to be localized to spermatocytes, and to a lesser extent other germ cells. While we also found germ cell localization, our in situ hybridization data describe a different pattern of expression, in that BMP4 was localized to earlier stages of spermatogenesis than found by Hu et al. (2004). Our studies using freshly isolated germ cells confirm maximal BMP4 expression in spermatogonia.

Recently, a paper by Pellegrini et al. (2003) detailed a functional SMAD signaling network in isolated germ cells, which was triggered by BMP4 treatment. These authors described the expression of the BMP4 receptors, Alk-3 and BMP receptor II, in spermatogonia but not spermatocytes. Using Northern blot analysis, *BMP4* mRNA was found to be expressed in Sertoli cells from 4- and 7-day-old mice, and then down-regulated in the 10- and 17-day-old mouse testis. However, no *BMP4* gene expression was detectable in spermatogonia, spermatocytes, or spermatids. Our results contradict this, as we were able to detect *BMP4* expression in germ cells through the use of several sensitive techniques, and as previously mentioned, Hu et al. (2004) also detected *BMP4* mRNA in spermatocytes. The disparity between these data could be due to different temporal patterns of expression in different cell types whereby Sertoli cells express BMP4 at post-natal days 4–7, with a subsequent decline. However, Hu et al. (2004) describe *BMP4* mRNA expression in 1, 2, and 3-week-old testis sections and state that germ cells in these tubules express *BMP4*. Moreover, at day 8, we detect germ cell expression of BMP4 and, in the adult, BMP4 appears to be exclusively expressed in the

germ cells of the testes. In our experiments, the probes used for in situ hybridization were all confirmed through DNA sequencing, and the in situ staining patterns are in accordance with immunohistochemical results. While we cannot definitively state that Sertoli cells do not express any BMP4, we believe that the amount of BMP4 in these cells, if any, is difficult to detect above background using conventional techniques.

As a consequence of VAD, there is an increase in apoptotic cell numbers ultimately resulting in depletion of all germ cells with the exception of BMP4 positive spermatogonia. Subsequent readministration of ROL to VAD animals caused a rapid decrease in *BMP4* mRNA levels after 24 h, which was maintained after 19 days of treatment, which coincided with a resumption of spermatogenesis. A similar exposure to RA did not affect overall BMP4 mRNA levels in the VAD testis, nor could it support spermatogenesis. The finding that ROL is more effective than RA in suppressing BMP4 is in keeping with a wealth of data indicating that ROL can reinitiate spermatogenesis, while a similar dose of RA cannot. The only reported case where RA was able to restore spermatogenesis was when it was injected in large repeated doses into VAD rats (van Pelt and de Rooij, 1991). The conclusion is, therefore, that RA is metabolized rapidly leading to lower effective testis concentrations than when ROL is provided. It is clear from our studies that the down-regulation in *BMP4* mRNA in vitro correlates with the reinitiation of spermatogenesis. The observed decrease in *BMP4* gene expression in ROL-treated isolated spermatogonia confirms that the germ cells themselves are responsible for this down-regulation of BMP4, and that they themselves can respond directly to retinoids.

A core observation in this study was that the expression of BMP4 is regulated by ROL in freshly isolated germ cells. Previously, it had been suggested that Sertoli cells metabolized ROL to RA for delivery to the germ cells (reviewed in Chung and Wolgemuth 2004). This paper documents the direct response of germ cells suggesting that the necessary metabolic activity may reside in these cells. Our results indicate that the relative efficacy of ROL over RA in terms of BMP4 regulation is evident even when the germ cells are exposed directly to these retinoids. This would suggest that retinoid metabolism is not restricted to somatic cells but can occur in germ cells as well.

In the developing mouse ear, BMP4 is similarly down-regulated by retinoids; however, in this case, the active retinoid metabolite is all-*trans*-RA (Thompson et al., 2003). Down-regulation of BMP4 gene expression by RA was mediated by a *direct* interaction of RARα and RARγ on a novel BMP4 promoter (termed the i2 promoter) in both an otocyst cell line and in the embryonic inner ear in vivo, indicating that the retinoid receptors directly regulate BMP4 gene expression in this system. We have now found the i2 promoter to be active in germ cells, and that germ cells contain a shortened 1B exon, as described in otocyst cells by Thompson et al. (2003). In order to examine promoter activity in germ cells, which are difficult both to culture long term and to transfect, we used real-time PCR to analyze the relative expression levels for the three promoter-specific transcripts of the 1A, 1B, and intron 2 promoters. We found the 1B promoter to be expressed at extremely low levels in the control testis, the VAD testis, and in isolated germ cells. However, analysis of the 1A and i2 promoter-specific transcripts revealed that both promoters were involved in the down-regulation of *BMP4* mRNA levels in response to ROL supplementation of VAD mice. Thus, the ROL-mediated down-regulation of BMP4 in germ cells involves the regulation of both the 1A and i2 promoters.

Whether the down-regulation of BMP4 levels by ROL is a direct effect mediated by the interaction of retinoid receptors and the promoter region of the BMP4 gene, or whether other retinoid-regulated proteins mediate this effect remains to be examined. Sequence analysis of the *BMP4* gene has revealed the existence of several direct repeats and potential RAREs in the 1A intron region (Feng et al., 1995; Helvering et al., 2000). However, the ability of retinoid receptors to bind directly to these sites and regulate gene transcription has not been determined. In an inner ear cell line, Thompson et al. (2003) provided evidence that RARs mediate RA regulation of BMP4 gene expression. The applicability of these results to the germ line remains to be elucidated.

Our observations regarding retinoid receptor gene expression in germ cells show not only that this regulation is not mediated by RARβ, but also provide evidence that the response of germ cells to retinoids is atypical. *RARβ* mRNA was barely detectable in spermatogonia, and nor could the *RARβ* message be induced by RA treatment after 6 h of culture. However, 6 h of retinoid treatment were sufficient to effect a profound change in *BMP4* levels. This result was unusual, since in all other retinoid-responsive cells, *RARβ* mRNA is induced in response to retinoid treatment (de The et al., 1990; Hoffmann et al., 1990; Leroy et al., 1991a,b; Lin et al., 2000; Mendelsohn et al., 1991; Roy et al., 1995; Sucov et al., 1990). The presence of the other retinoid receptors in isolated spermatogonia corresponds with previous studies showing the expression of all the known retinoid receptors in both mouse and rat germ cell lineages (Boulogne et al., 1999; Dufour and Kim, 1999; Gaemers et al., 1998). The presence of these receptors indicates that a functional retinoid signaling network exists in germ cells; however, the absence of induction of *RARβ* expression highlights the possibility that a unique retinoid signaling pathway exists in mouse germ cells.

In conclusion, this study suggests that retinoid signaling in male germ cells is atypical. We have provided novel evidence indicating that in vitro mouse spermatogenic cells are able to respond directly to ROL. Germ cells respond to this retinoid by down-regulating *BMP4* mRNA expression, via mechanisms that are not mediated by RARβ. In vivo BMP4 positive cells are all that remain once VAD is

achieved. This would suggest that the effects described for germ cells in vitro also apply to germ cells in vivo. This down-regulation of BMP4 in mouse spermatogonia may be associated with their passage through a developmental gateway, allowing them to continue differentiation into more mature cell types.

Acknowledgments

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