The gene encoding bone morphogenetic protein 8B is required for the initiation and maintenance of spermatogenesis in the mouse

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Bone morphogenetic protein 8B (BMP8B) is a member of the TGFβ superfamily of growth factors. In the mouse, Bmp8b is expressed in male germ cells of the testis and trophoblast cells of the placenta, suggesting that it has a role in spermatogenesis and reproduction. To investigate these possibilities, we have generated mice with a targeted mutation in Bmp8b. Here, we show that homozygous Bmp8b^"""" mutant males exhibit variable degrees of germ-cell deficiency and infertility. Detailed analysis reveals two separable defects in the homozygous mutant testes. First, during early puberty (2 weeks old or younger) the germ cells of all homozygous mutants either fail to proliferate or show a marked reduction in proliferation and a delayed differentiation. Second, in adults, there is a significant increase in programmed cell death (apoptosis) of spermatocytes, leading to germ-cell depletion and sterility. Sertoli cells and Leydig cells appear relatively unaffected in mutants. This study therefore provides the first genetic evidence that a murine germ cell-produced factor, BMP8B, is required for the resumption of male germ-cell proliferation in early puberty, and for germ-cell survival and fertility in the adult.

[Key Words: BMP8b; targeted gene inactivation; male germ cell; spermatogenesis; testis degeneration; fertility]

Received April 9, 1996; revised version accepted May 13, 1996.
Zhao et al. al. 1991; Han et al. 1993; Boujrad et al. 1995b; Tsirigotis and O'Brien 1995; Bitgood et al. 1996). Up to now, several members of the TGFβ superfamily of growth factors and their receptors have been shown to be expressed in the mammalian testis. However, inactivation of the inhibinα, activinβB, MIS, and TGFβ1 genes does not lead to primary defects in spermatogenesis, indicating that they do not have essential roles in this process (Matzuk et al. 1992, Behringer et al. 1994; Vassalli et al. 1994; T. Doetschman, pers. comm.). The absence of activinβA subunit leads to perinatal lethality, therefore, its role in spermatogenesis cannot be evaluated (Matzuk et al. 1995). The expression of several serine/threonine kinase receptors, such as ActRIIA and ActRIIB in meiotic and postmeiotic germ cells (Kaipia et al. 1992, 1993; Manova et al. 1991; Han et al. 1993; Boujrad et al. 1995b; Tsuruta et al. 1995), suggests that members of the TGFβ superfamily of growth factors bind directly to germ cells and regulate spermatogenesis. Because the above-mentioned members of the TGFβ superfamily do not appear to be directly required for spermatogenesis, it is possible that other TGFβ-related members bind to these receptors in vivo. As we reported recently (Zhao and Hogan 1996), the murine Bmp8a (Opr2) and Bmp8b (Opr3) genes are closely related in their protein-coding exons and genomic organization, and are expressed in the placenta during embryogenesis and in male germ cells during postnatal development in a tightly regulated temporal and spatial manner. Before 3.5 weeks of age, both genes are ubiquitously expressed at low levels in spermatogonia and primary spermatocytes. After 3.5 weeks of age and during adult life, high levels of expression of the Bmp8 genes are observed, specifically in stage 6–8 round spermatids. To investigate the role of Bmp8b during spermatogenesis, we have inactivated the gene by homologous recombination in embryonic stem (ES) cells. Here, we report the phenotype of mice homozygous for the mutant allele, Bmp8b<sup>−/−</sup>. From this study we conclude that in the mouse a functional Bmp8b gene is required for the initiation and maintenance of spermatogenesis. First, it is needed for the full resumption of germ-cell proliferation during early postnatal development. Second, in the adult, Bmp8b is required for the survival of male germ cells and hence for the maintenance of spermatogenesis.

Results

**Targeted mutagenesis of the mouse Bmp8b gene**

The mouse Bmp8b gene has a genomic organization similar to that of the human Bmp8/op2 gene, which contains seven coding exons (Ozkaynak et al. 1992; Zhao and Hogan 1996). In the Bmp8b<sup>−/−</sup> allele, a 7-kb genomic fragment including exon 4, which encodes amino acids 221–287 encompassing the conserved dibasic prolylcleavage site between the pro- and mature regions, is deleted and replaced by the MClneo' cassette (Fig. 1) (Rudnicki et al. 1992). Therefore, it is predicted that no functional BMP8B protein will be expressed from the Bmp8b<sup>−/−</sup> allele. Moreover, in situ hybridization studies using an antisense RNA probe against the Bmp8b 3' untranslated region (UTR), which is specific to Bmp8b (Zhao and Hogan 1996), detected no signals above background in sections of homozygous mutant testis, whereas Bmp8a expression appears unchanged (data not shown). Five different targeted ES cell lines were obtained (5B11, A5, D1, F4, and F7). Three of these

![](https://genesdev.cshlp.org/content/10/9/1658/F1.large.jpg)

**Figure 1.** Targeted mutagenesis of the mouse Bmp8b locus. (A) Schematic representation of the Bmp8b wild-type allele on the top, targeting vector in the middle, and targeted Bmp8b<sup>−/−</sup> allele at the bottom. Genomic DNA fragments used as the long arm and short arm of homology in the targeting vector are indicated by thick lines. Coding exons are shown as solid boxes. Expression cassettes PGK TK, MClneo', and MCI DT-A are indicated as boxes. 5' and 3' probes used for Southern blots are shown underneath the genomic loci. Exons 2–6 are indicated as E2–E6. Restriction enzyme abbreviations: [E] EcoRI, [Xh] Xhol, (B) BamHI, (S) Sall, and (X) Xbal. (B) Southern blot of the Bmp8b<sup>−/−</sup> mice. Genomic DNA isolated from tail was digested with EcoRI, size-fractionated on a 1% agarose gel, and blotted onto a positively charged nylon membrane. Hybridization was performed with a 1.4-kb 3' probe. The wild-type allele generated a fragment of 4.5 kb, whereas the targeted allele generated a fragment of 5 kb.
(SB11, A5, and D1) were injected into C57BL/6 blastocysts and the \textit{Bmp8b}^{m1bhi} allele was transmitted through the germ line of resulting chimeras. Homozygous mutant animals of all three lines show the same phenotype. SB11 was used for the studies on adult reproduction and testis histology reported here, whereas all three lines were used for the remainder of the studies. Mutant mice were maintained on a mixed genetic background of [129 × Black Swiss] for this study.

\textbf{Reproduction phenotype of Bmp8b\textsuperscript{m1bhi} mutant mice}

We have shown previously that \textit{Bmp8b} is expressed in trophoblast cells of the placenta and in stage 6–8 round spermatids of the adult testis [Zhao and Hogan 1996]. Therefore, abnormal reproduction phenotypes is expected. Interbreeding male and female heterozygous mutants yielded the predicted ratio of wild-type \(n = 72\), heterozygous \(n = 165\), and homozygous \(n = 69\) mutant offspring, indicating that in the absence of fetal BMP8B protein, the placenta still functions normally. Homozygous mutant males and females appear normal and healthy. However, in two of three initial mating pairs of homozygous \textit{Bmp8b}\textsuperscript{m1bhi} mutants, females never became pregnant or had offspring after 3.5 months. The third pair eventually showed a relatively normal reproduction performance after a 30-day delay. To further pinpoint the reproductive defect in \textit{Bmp8b}\textsuperscript{m1bhi} mice, mating pairs were set up with different combinations of male and female mice. As shown in Table 1, 10 of 18 homozygous \textit{Bmp8b}\textsuperscript{m1bhi} male mice were sterile when mated with females of different genotypes. Another two males were initially fertile, with a litter of two to three pups, but then became sterile. Heterozygous animals and homozygous \textit{Bmp8b}\textsuperscript{m1bhi} females did not show obvious reproduction defects.

\begin{table}[h]
\centering
\caption{Reproduction phenotype of Bmp8b mutant mice} \label{tab:reproduction}
\begin{tabular}{lllll}
\hline
Male \(n\) & Female \(n\) & Fertile & Testis & degeneration \\
\hline
\(+/+\) (6) & \(-/-\) (5) & yes & 100 ± 15 & none \\
\(-/-\) (6) & \(+/-\) (5) & yes & 117 ± 15 & none \\
\hline
\end{tabular}
\end{table}

Males and females of different genotypes (7–10 weeks of age) were mated for a period of 45 days or longer. Females that became pregnant or gave birth were scored as fertile. After mating, animals aged 17–25 weeks were sacrificed and their reproductive organs were examined.

\textbf{Bimodal expression of Bmp8 genes in male germ cells during testicular development}

As shown previously [Zhao and Hogan 1996], \textit{Bmp8a} and \textit{Bmp8b} genes are expressed at high levels in stage 6–8 round spermatids and at lower levels in 20%–30% of pachytene spermatocytes in stage VI–VIII seminiferous tubules. The expression of \textit{Bmp8} genes therefore appears to be tightly controlled in a stage-specific manner after mid-puberty (3.5 weeks old) and during adult life. However, careful examination of \textit{Bmp8} expression in male germ cells during the early postnatal period revealed low levels of expression of both \textit{Bmp8} genes in almost all spermatogonia and early meiotic germ cells [Fig. 3C,D, 2 weeks]. This early expression of \textit{Bmp8} in male germ cells was not observed in newborn animals [Fig. 3A,B] but was obvious 1 week after birth [data not shown]. The expression of \textit{Bmp8} genes in spermatogonia is shut off once the stage-specific expression is established after mid-puberty [Zhao and Hogan 1996]. Such a bimodal expression pattern of \textit{Bmp8} genes during testis development suggests that different mechanisms are used to regulate \textit{Bmp8} expression in the early and late stage testis and that the

**Progressive germ-cell apoptosis leads to sterility in homozygous Bmp8b\textsuperscript{m1bhi} males**

As shown in Table 1, sterile \textit{Bmp8b}\textsuperscript{m1bhi} males had testes \(\sim 15\%\) the size of wild-type or heterozygous testes. Fertile homozygous mutant males also had smaller testes than wild-type and heterozygous males. Histological examination revealed almost complete testis degeneration or germ-cell depletion in all sterile mutants, whereas partial germ-cell depletion or degeneration was observed in all fertile homozygous mutants [Table 1, Fig. 2]. None of the heterozygous or wild-type males showed obvious testis degeneration. The remaining reproductive organs and accessory glands appeared normal in all mutant animals.

In completely degenerated testes, most of the seminiferous tubules were occupied mainly by Sertoli cells with only very few germ cells [Fig. 2C,F; data not shown]. In partially degenerated testes, seminiferous tubules in the process of degeneration were found. They lacked primary spermatocytes and had irregular elongated spermatids in the center and some spermatogonia and Sertoli cells at the periphery [Fig. 2B,E]. Local accumulation of interstitial Leydig cells was present in the completely degenerated areas of the testis [Fig. 2B,C,F]. Terminal deoxynucleotide transferase-mediated dUTP nick end labeling [TUNEL] [Gavrieli et al. 1992] of the testis sections revealed increased apoptosis of germ cells in the degenerating seminiferous tubules [Fig. 2I] compared with normal tubules [Fig. 2G]. In addition, as shown in Fig. 2H, some morphologically normal homozygous mutant tubules contained numerous labeled primary spermatocytes, indicating that these cells are the first germ-cell population to be affected in the absence of a functional \textit{Bmp8b} gene.
Figure 2. Histology and TUNEL labeling of adult wild type and homozygous Bmp8b<sup>m168</sup> testes. [A–F] Testis sections stained with periodic acid-Schiff's (PAS) reagent and hematoxylin. [A] Section through a wild-type testis (105 mg); [B] section of a partially degenerated homozygous Bmp8b<sup>m168</sup> testis (42 mg) [note the morphologically normal tubules (asterisks) and tubules in the process of degeneration (arrowheads) as well as the completely degenerated tubules above the boxed region]; [C] section of a completely degenerated homozygous Bmp8b<sup>m168</sup> testis (15 mg); [D] high power magnification of a wild-type stage XII seminiferous tubule; [E] high power magnification of the area boxed in B showing a degenerating seminiferous tubule [arrowhead], which contains numerous irregular elongated spermatids in the center and spermatogonia and Sertoli cells at the periphery; [F] high power magnification of completely degenerated seminiferous tubules that contain numerous Sertoli cells [SC]. Accumulation of intertubular Leydig cells (LC) is noted between the degenerated tubules; [G] TUNEL labeling of a wild-type seminiferous tubule showing absence of cells undergoing apoptosis; [H] TUNEL labeling of a section through a morphologically normal seminiferous tubule of a homozygous Bmp8b<sup>m168</sup> testis showing numerous apoptotic primary spermatocytes (arrow); [I] TUNEL labeling of an adjacent section to E showing numerous germ cells undergoing apoptosis. G–I were counterstained with eosin. Bar, 320 μm in A–C and 80 μm in D–I.

genes have two distinct functions during spermatogenesis.

The onset of germ-cell deficiency in homozygous Bmp8b<sup>m168</sup> males coincides with early Bmp8b expression and initiation of spermatogenesis

The expression of Bmp8 genes in male germ cells during early puberty led us to investigate the roles of Bmp8b during early spermatogenesis. As shown in Figure 4, from 1 to 7 weeks after birth, there was a continuous increase in testis weight in animals of all genotypes. However, at each age, the average weight of homozygous mutant testes was significantly lower than that of wildtype and heterozygous testes, and the difference was most pronounced at 2–3 weeks. From 4 to 7 weeks, ~50% of the homozygous mutant testes reached a size comparable with that of some heterozygous testes, but ~20–30% of the homozygous mutant testes never grew >20 mg in weight. The remaining testes were of intermediate size. During adult life (17 to 25 weeks old) many of the homozygous mutant testes degenerated and weighed <20 mg.

The histology of testes at different ages is shown in Figure 5. Even 1 week after birth, heterozygous and homozygous mutant testes showed a reduction in the diameter of the seminiferous tubules and a decrease in the number of cells within them [Fig. 5B,C]. At this time, early meiotic germ cells were found in many wild-type seminiferous tubules [Fig. 5A] but not in heterozygous and homozygous mutant animals. At 2 weeks, the histology of most heterozygous testes was similar to that of wild type, with a slight decrease in the diameter of the seminiferous tubules [Fig. 5D,E] in contrast, most homozygous mutant testes continued to show reduced seminiferous tubule diameter and cell number, and contained only a single cell layer in the seminiferous epithelium [Fig. 5F]. At 3 weeks of age, many seminiferous tubules still contained a single-layered epithelium (asterisk in Fig. 5I), but a number of mutant tubules now contained a stratified seminiferous epithelium (arrowhead in Fig. 5I). By 5 weeks, 50% of the homozygous mutant testes exhibited a relatively normal histology.

Figure 3. Bmp8a and Bmp8b expression in newborn and 2-week-old testes. Antisense RNA probes corresponding to the 3' UTRs of each gene were used for in situ hybridization. All sections were counterstained with hematoxylin. Only bright-field photomicrographs are shown. No obvious hybridization signals were detected in the gonocytes of newborn testes (NB) arrows in A and B]. Low levels of hybridization were detected in most germ cells of 2-week-old testes (2 WK) arrows in C and D]. Bar, 25 μm.
Bmp8b in spermatogenesis and fertility

Figure 4. Comparison of the weight of wild-type and Bmp8b<sup>tm1bh</sup> testes at different ages during postnatal development. For each age several litters of animals were used and the mean ± s.e. is indicated for each group of animals. [N] The number of animals in each group. Because of big litter sizes, the 2-week-old animals and their testes were slightly smaller than average. However, animals of all three genotypes had comparable body weights. Testis weight differences among wild-type (+/+), heterozygous (+/-), and homozygous (-/-) mutants are statistically significant at different ages (using Student’s t-test) except between wild-type and heterozygous animals at 2 weeks. Note the relatively large standard error in the weight of homozygous mutant testes at 3 weeks and later, which reflects the wide variation of testis weight in these groups. At 2 weeks, all homozygous testes were significantly smaller than wild-type and heterozygous mutant testes. Between 3 and 7 weeks, 50% of the homozygous mutant testes gradually recovered to sizes comparable with some heterozygous testes after a lag of 1-2 weeks. About 20%-30% of the homozygous mutant testes never grew beyond 20 mg. When homozygous animals reached 4-6 months old, two-thirds of the testes were 20 mg or less.

(Fig. 5L) but with delayed germ-cell differentiation in comparison with wild-type and heterozygous animals. For example, in 5-week-old wild-type or heterozygous testes, stage 16 elongated spermatids (Russell et al. 1990) or mature sperm were found in many seminiferous tubules or even in the epididymis. However, at this age the most differentiated germ cells in the homozygous mutant testes were stage 12–13 elongated spermatids that could be found at 4 weeks of age in the normal testis. A slight delay in meiosis and a decrease in the size of the homozygous spermatocytes (Fig. 4) may reflect some degree of haploinsufficiency in these mice.

Defects in germ-cell proliferation rather than increased apoptosis cause germ-cell deficiency in homozygous Bmp8b<sup>tm1bh</sup> testes during early puberty

To investigate the cause of the germ-cell deficiency in Bmp8b<sup>tm1bh</sup> mutant animals during early puberty, bromodeoxyuridine (BrdU) labeling (Sakai et al. 1994) was performed to examine cell proliferation, and TUNEL labeling was used to detect programmed cell death. As shown in Figure 6, at 2 weeks of age, homozygous mutant testes showed a marked decrease in the number of BrdU-labeled cells in the seminiferous epithelium, which were mainly spermatogonia and early primary spermatocytes [Fig. 6H,K] compared with wild-type and heterozygous animals [Fig. 6B,E]. However, BrdU incorporation in the interstitial cells seemed to be unaffected in the homozygous mutants [Fig. 6H,K] compared with control. Germ cells labeled by TUNEL were detected in wild-type and heterozygous mutant testes [Fig. 6C,F], but there appeared to be fewer labeled cells in the homozygous mutant testes [Fig. 6L]. Analysis of testes from 1- to 4-week-old animals revealed similar defects in germ-cell proliferation in homozygous mutants [Fig. 7]. Therefore, based on these data, we conclude that abnormal germ-cell proliferation and a subsequent delay in differentiation, rather than increased apoptosis, are responsible for the paucity of mitotic and meiotic germ cells in mutant testes during the early postnatal period.

Local accumulation of interstitial cells in the homozygous Bmp8b<sup>tm1bh</sup> testes is not caused by increased cell proliferation

As shown in Figures 6 and 7, there was no obvious difference in BrdU incorporation in the interstitial cells of mutant [Figs. 6H,K and 7B,D,F,H] and wild-type testes [Figs. 6B and 7A,C,E,G] during early and mid-puberty. To investigate the cause of the accumulation of interstitial cells in the degenerating testis of the adult, we performed BrdU labeling of 8-week-old young adult testes. As shown in Figure 8, although partially degenerated testes contained abnormal tubules surrounded by interstitial cells (asterisk in Fig. 8A), there was no increase in the number of labeled cells in the degenerating area.
Figure 5. Histology of wild-type (+/+), heterozygous (+/-), and homozygous (-/-) Bmp8b<sup>1m1/bb</sup> testes. All sections were stained with PAS. Note the different magnification of A-F, G-I, and J-L. [A–C] Sections through testes of 1-week-old animals. A multilayered seminiferous epithelium containing meiotic germ cells is present in the wild-type seminiferous tubules (A). In heterozygous and homozygous Bmp8b<sup>1m1/bb</sup> testes most tubules have only a single basal layer of cells (B,C). [D–F] Sections through testes of two-week-old animals. Note the reduction in the diameter and number of cells in most seminiferous tubules of the homozygous Bmp8b<sup>1m1/bb</sup> testis (asterisk). However, some tubules do contain multilayered seminiferous epithelium (arrowhead). [G–I] Sections through testes of 3-week-old mice. Similar histology is noted in the homozygous Bmp8b<sup>1m1/bb</sup> testis as in F, but more seminiferous tubules contain multiple layers of germ cells (arrowhead). [J–L] Sections through testes of 5-week-old animals. About 50% of the homozygous Bmp8b<sup>1m1/bb</sup> testes now contain seminiferous tubules with relatively normal histology, but a delayed differentiation (stage 12–13 elongated spermatids are the most differentiated germ cells) is noted in comparison with wild-type or heterozygous testes (stage 16 elongated spermatids or mature sperm are present in seminiferous tubules or epididymis). Bar, 80 μm in A–F, 160 μm in G–I, and 320 μm in J–L.

Effect on Sertoli cell gene expression in the absence of a functional Bmp8b gene

To investigate the possible effect of BMP8B protein on Sertoli cell function, in situ hybridization was performed with antisense RNA probes derived from two Sertoli cell-specific markers, desert hedgehog (Dhh) (Bitgood and McMahon 1995; Bitgood et al. 1996) and cyclic protein 2 (Cp2) (Wright and Luzarranga 1986; Joseph et al. 1988). As shown in Figure 9, low levels of expression of both Dhh and Cp2 were observed in the morphologically normal seminiferous tubules of the homozygous mutant testes (Fig. 9, lower parts of A and B), comparable with levels in the wild type (data not shown). However, much higher levels of expression of both genes were observed in the degenerated seminiferous tubules (Fig. 9, upper parts of A and B). Higher magnification of the degenerated seminiferous tubules revealed that the Sertoli cells attached to the basement membrane expressed high levels of both genes. However, clusters of cells in the center of the tubules did not express either gene (arrows in Fig. 9E,F). These were most likely early spermatogonia that had detached from the basal lamina. From these data, we conclude that in the absence of a functional Bmp8b gene, before seminiferous tubule degeneration occurs, the levels of Dhh and Cp2 expression are not altered, but after germ-cell degeneration, expression of both genes is dramatically up-regulated. This may either be a result of removal of a specific inhibitory effect of BMP8B on Dhh and Cp2 gene expression, or, more likely, be a consequence of removal of a general inhibition of germ cells on Sertoli cell gene expression.

Discussion

Numerous studies have indicated that cell–cell interactions are critical for initiating and maintaining spermatogenesis in the mammalian testis (for review, see Orth 1993; Wright 1993). For example, Sertoli cell-derived Steel factor acts on spermatogonia through c-kit receptor to maintain spermatogenesis (Yoshinaga et al. 1991). Inactivation of the mouse Dhh gene, which is also expressed in Sertoli cells, leads to germ-cell degeneration (Bitgood et al. 1996). Conversely, a deficiency of germ cells can cause changes in Sertoli cell gene expression, such as the up-regulation of Dhh, Cp2 (Fig. 9), and GATA1 (Yamogida et al. 1994; Bitgood et al. 1996) in degenerated seminiferous tubules. Leydig cells are responsible for the production of testosterone, which supports spermatogenesis through effects on the Sertoli cells. In turn, Sertoli cell-derived proteins, such as the complex of TIMP1-procathepsin L, regulate testosterone production by Leydig cells (Boujrad et al. 1995a).

Although germ cells and somatic cells are physically...
and functionally associated in the testis, their proliferation and differentiation are thought to be controlled by different mechanisms (for review, see McCarrrey 1993; Orth 1993). For example, in the mouse, Sertoli cell proliferation initiates during late fetal development, peaks perinatally, and decreases gradually, so that it has ceased completely by 2 weeks after birth. In contrast, male gonocytes cease proliferation around 14.5 days post-coitum (p.c.) and do not resume cell division until several days after birth. By late puberty (5 weeks of age), a steady state of spermatogenesis is reached, and the proliferation and differentiation of the self-renewable spermatogonial stem cell population is tightly controlled and maintained. All of the progeny of a stem cell are connected through intercellular bridges, presumably to promote synchronous development and differentiation. At present, rather little is known about the mechanisms controlling the initiation and maintenance of spermatogenesis and whether these are interdependent or separable processes. A mouse mutation, juvenile spermatogonia depletion [jsd], throws some light on these questions. In the homozygous jsd mutants, the first wave of spermatogenesis takes place, but spermatogenesis fails to be maintained, leading to germ-cell degeneration after mid-puberty and sterility (Beamer et al. 1988; Barton et al. 1989; Mizunuma et al. 1992). This seems to indicate that the initiation and maintenance of spermatogenesis are controlled by different mechanisms.

**Growth and differentiation factors identified previously in the testis**

Up to now, a number of growth and differentiation factors have been shown to be expressed in the testis where they could mediate interactions among different cell populations. Most of these factors, including IGF-I, TGFα, epidermal growth factor [EGF], basic fibroblast growth factor [bFGF], TGFβ1, INHIBINα, ACTIVINβA, INHIBINβB, Müllerian inhibiting substance [MIS] (for review, see Bardin et al. 1993; Robertson et al. 1993), and DHH (Bitgood and McMahon 1995) are produced by Sertoli cells, whereas germ cells express WNT1, bFGF, nerve growth factor [NGF], BMP8A, and BMP8B (Shackleford and Varmus 1987; Parvinen et al. 1992; Han et al. 1993; Zhao and Hogan 1996). Cross-regulation among some of these factors has been shown in the testis; for example, the production of IGF-I by Sertoli cells is stimulated by FGF and EGF (Chatelain et al. 1987; Hansson et al. 1989). Previous studies have suggested that some of these growth factors have either direct or indirect roles in spermatogenesis, as their putative receptors are expressed in either somatic cells or germ cells or both (for review, see Bardin et al. 1993; Orth 1993; Robertson et al. 1993). However, in only a few cases have their precise in vivo roles been established with molecular genetic approaches, using targeted gene inactivation and/or misexpression in testicular cells in transgenic mice. Inactivation of inhibinα and activinβB genes does not lead to primary defects in spermatogenesis, supporting the idea that these proteins function primarily through regulating follicle-stimulating factor [FSH] and luteinizing hormone [LH] production in the pituitary gland. Inactivation of MIS causes the incomplete suppression of female reproductive organ differentiation during development and some degrees of Leydig cell over proliferation in a small number of male animals, confirming that the major function of MIS is to suppress the development of the female reproductive system. Homozygous-null TGFα
Bmp8b is expressed in male germ cells and is required for the initiation and maintenance of spermatogenesis

As reported recently (Zhao and Hogan 1996) and in this study, we have shown that Bmp8a and Bmp8b, encoding two closely related BMPs, are expressed in male germ cells in a tightly controlled, bimodal manner. The initiation of their expression in early germ cells coincides with the initiation of spermatogenesis in early puberty. After mid-puberty, their expression shifts specifically to the stage 6–8 round spermatids where transcripts are found at high levels. Such a bimodal expression pattern suggests that the transcriptional regulation of Bmp8 genes is controlled by two distinct mechanisms during spermatogenesis. In this study we have shown that the targeted inactivation of the mouse Bmp8b gene results in two separable phenotypes during spermatogenesis. During early puberty, the absence of a functional Bmp8b gene blocks germ-cell proliferation in 20%–30% of the homozygous mutant testes and results in delayed and reduced germ-cell proliferation and differentiation in the 

mutants show normal reproductive performance and presumably have normal spermatogenesis (Luetteke et al. 1993). The surviving homozygous TGFB mutant male mice also have apparently normal spermatogenesis (T. Doetschman, pers. comm.). IGF-I homozygous mutant mice die perinatally; therefore, it is not possible to determine the in vivo role of IGF-I during spermatogenesis (Powell-Braxton et al. 1993). The observation that inactivation of the Dhh gene results in germ-cell degeneration and the putative hedgehog signaling component patched (ptc) is expressed predominantly in Leydig cells (Bitgood et al. 1996) suggests that DHH and PTC proteins mediate interactions between somatic Sertoli cells and Leydig cells. Most homozygous null Wnt1 mutants die perinatally because of defects in the cerebellum and midbrain, but the few males surviving to adulthood are fertile, showing that Wnt1 is not essential for spermatogenesis (for review, see Nusse and Varmus 1992). The in vivo functions of other factors still need further investigation.

Figure 7. Comparison of BrdU labeling of wild-type and Bmp8b^{mut/mut} testes at different ages. Note the different magnification of testis sections of different ages. (A,B) Sections through 1-week-old testes; (C,D) sections through 2-week-old testes; (E,F) sections through 3-week-old testes. Note the difference in the diameter of seminiferous tubule and the number of labeled cells in them between wild-type and homozygous mutant (arrows indicate the labeled cells). Many of the seminiferous tubules with single-layered seminiferous epithelium in the mutant testes are not labeled by BrdU (asterisk). (G,H) sections through 4-week-old testes. Note the smaller diameter of the seminiferous tubules in the mutant testis. However, in most tubules of most mutant testes the number of BrdU-labeled cells is comparable between wild-type and mutant animals. Bar, 80 μm in A–D; 160 μm in E–H.

Figure 8. BrdU labeling of an 8-week-old homozygous mutant testis with partial degeneration. Eight hours after BrdU injection, the testis was dissected out and processed. (A) Low power magnification of a section through a partially degenerated testis. The upper portion contains seminiferous tubules with relatively normal morphology; the lower portion contains degenerated tubules surrounded by locally accumulated interstitial cells. There is no obvious increase in the number of BrdU-labeled cells in the degenerated area (asterisk) in comparison with an area containing normal seminiferous tubules. (B) A higher magnification of the boxed area in A. (Arrow) Degenerating tubule containing numerous BrdU-labeled germ cells. Bar, 320 μm in A; 80 μm in B.
suggestion that lower levels of BMP8 proteins are sufficient for germ-cell proliferation in the adult, whereas higher levels of BMP8 are required for the survival of pachytene spermatocytes.

Potential elements of a BMP8 signal transduction pathway are expressed in the testis

Two models can be proposed for the mechanism by which BMP8 proteins exert their regulatory roles during spermatogenesis. First, they may work primarily through an endocrine pathway, as in the case of activins and inhibins, by influencing gonadotropin production in the pituitary gland. The fact that the phenotype of the homozygous Bmp8b mutant does not show any significant changes in morphology, proliferation, or apoptosis in the somatic Leydig and Sertoli cells (Figs. 2, 4, 5, 6, and 8), which are the primary target cells for gonadotropins, does not support a long-range paracrine model for BMP8 function. The up-regulation of Sertoli cell gene expression in the degenerated tubules of homozygous mutants is probably caused by the absence of differentiating germ cells rather than a systemic effect, because some tubules with normal germ associations in the same testsis do not show such up-regulation. The male Wolfian duct-derived tissues, including vas deferens, epididymis, and accessory glands, appear normal morphologically in all homozygous mutant males, indicating that androgen production by Leydig cells is sufficient. According to the second model (schematized in Fig. 10) the BMP8 proteins function locally within the seminiferous tubule, either through an autocrine effect on germ cells and/or through a short-range paracrine effect on Sertoli cells. This model seems more feasible for the following reasons. (1) At least two receptors that bind BMPs, namely ActRIIA and ActRIIB, are expressed in the seminiferous epithelium; ActRIIA mainly in pachytene spermatocytes and round spermatids, and ActRIIB mainly in spermatogonia, early meiotic germ cells, and Sertoli cells (Kaipia et al. 1992, 1993; Manova et al. 1995). BMP2 and BMP7 have been shown to bind ActRIIA and ActRIIB and to signal biological responses (Yamashita et al. 1995; J. Wrana, pers. comm.). It is reasonable to speculate that BMP8 proteins will bind to the same receptors and exert their biological functions through them because BMP8A and BMP8B (OP2 and OP3) are in the same subfamily as BMP7 (Griffith et al. 1996). (2) We have found that two mouse homologs of the Drosophila mothers against dpp (mad) gene (Raftery et al. 1996). (2) We have found that two mouse homologs of the Drosophila mothers against dpp (mad) gene (Raftery et al. 1995; Sekelsky et al. 1995) are expressed in the seminiferous epithelium of the testis. One gene, which we have called Madr1, is identical to human MadR-1 [J. Wrana, pers. comm.] and is expressed mainly in the pachytene spermatocytes and early round spermatids, whereas the other, which we have named Madr2, is mainly expressed in spermatogonia, early meiotic germ cells, and Sertoli cells (data not shown). Recent studies have shown that human MADR-1 and Drosophila MAD serve as downstream signaling targets for BMP2 and ActRIs in a cell culture system [J. Wrana, pers. comm.]. Therefore, MADR1 and MADR2 may.

Figure 9. Expression of Sertoli cell-specific genes in a partially degenerated homozygous Bmp8b<sup>+/+;+/+</sup> testis. In situ hybridization was carried out with antisense probes to Dhh and Cp-2 on sections of a homozygous Bmp8b<sup>+/+;+/+</sup> testis of 2.5-month-old (60 mg). [A,B] Dark-field photomicrographs showing that both Dhh and Cp-2 are expressed at much higher levels in the degenerated seminiferous tubules (top) than in the tubules with normal morphology [bottom] (C,D) Dark-field and [E,F] bright-field photomicrographs of the degenerated tubules at higher magnification. Arrows in E and F show clusters of darkly stained cells in the center of the seminiferous tubules do not express Sertoli cell-specific markers, suggesting that they are probably early spermatogonia that have detached from the basal lamina. Bar, 320 μm in A and B, 80 μm in C-F.
serve as downstream targets for BMP8 signaling in the testis. It is important to note that in adult homozygous Bmp8b mutants, the pachytene spermatocytes that express Mad1 at very high levels are the first cell population showing increased apoptosis and depletion (Fig. 2H, I), indicating they are most sensitive to BMP8 availability.

In conclusion, the initiation of Bmp8 gene expression during testis development coincides with the onset of the first wave of spermatogenesis, and the absence of a functional Bmp8b gene results in either a block or a delay in germ-cell proliferation and differentiation during early puberty. During adulthood, increased apoptosis of pachytene spermatocytes in homozygous mutants leads to testis degeneration and sterility. Therefore, BMP8B appears to be required both for the initiation and maintenance of spermatogenesis. The early low levels of Bmp8 expression and the high levels of stage-specific Bmp8 expression established after mid-puberty are probably regulated by different mechanisms, in agreement with the idea that the initiation and maintenance of spermatogenesis are controlled by different mechanisms. At present we cannot rule out the possibility that BMP8 proteins influence spermatogenesis through long-range endocrine effects, rather than through local effects within the seminiferous tubule (see Fig. 10). However, the fact that putative receptors of BMP8 proteins, ActRIIA and ActRIIB, and downstream signaling molecules, MADR1 and MADR2, are expressed in seminiferous epithelium supports an autocrine model or short-range paracrine model for BMP8 function during spermatogenesis.

Materials and methods

Construction of targeting vector

Bmp8b genomic DNA clones were isolated from a 129/SvJ mouse X FixII genomic library (Stratagene) using a Bmp8a cDNA probe spanning exons 2–7. Nine phage clones were obtained. Two overlapping clones correspond to the BmpSa genomic locus as revealed by restriction mapping and sequencing. The remaining clones belong to the Bmp8b locus, and a partial restriction map is shown in Figure 1. A replacement targeting vector was constructed using a 7-kb 5’ homology region (BamHI fragment) and a 1-kb 3’ homology region (BamHI and XbaI fragment). A 7-kb segment containing exon 4 was replaced by a MC1neo cassette (Rudnicki et al. 1992) resulting in the deletion of the predicted cleavage site and part of the pro-region as well as part of the mature region. A phosphoglycerokinase thymidine kinase (PGK TK) cassette (Rudnicki et al. 1992) and a MC1DT-A cassette (Yagi et al. 1990) were attached to the 5’ and 3’ ends, respectively, for negative selection. This Bmp8b mutant allele is designated as Bmp8b<sup>null</sup> based on standard nomenclature (Davisson 1995).

Generation of recombinant ES cell clones and mouse chimeras

R1 (passage 15, kindly provided by Drs. Janet Rossant and András Nagy, Mt. Sinai Hospital, Toronto, Canada) and TL1 (passage 11, derived from 129/SvEv strain by P.A. Labosky) ES cell lines were electroporated with 20–50 μg of NotI-digested replacement vector DNA. A single pulse was delivered to the ES cells in a volume of 0.8 ml of phosphate-buffered saline with a Bio-Rad gene pulser at 800 V, 3 μF. After electroporation, the ES cells were plated onto irradiated neo<sup>+</sup> mouse primary embryonic fibroblasts. ES cell culture and drug selection were performed essentially as described (Winnier et al. 1995). One targeted ES cell clone was injected into a C57BL/6J blastocyst.
line (SB11) from R1 cells and four targeted cell lines (A5, D1, F4, and F7) from TLL cells were obtained with a frequency of 3% of the double-resistant clones. SB11, A5, and D1 were used for host blastocyst (C57BL/6J) injection essentially as described (Hogan et al. 1994b), and all three clones gave germ-line transmission of the mutant allele. Chimeras were crossed with Black Swiss females (Taconic), and agouti offspring were analyzed by Southern blotting for the presence of the Bmp8b<sup>rn11b</sup> allele. Homozygous mutant animals were obtained by crossing heterozygotes, and all analyses were carried out with the mixed genetic background of 129 and Black Swiss. All three lines show the same phenotype.

Southern blotting
Genomic DNA isolated from double-resistant ES clones and mouse tail tips was digested with EcoRI. Hybridization was performed with a 1.4-kb 3′ probe (Fig. 1A). A 4.5-kb fragment corresponding to the wild-type allele and a 5-kb fragment corresponding to the Bmp8b<sup>rn11b</sup> allele were detected on Southern blots (Fig. 1B).

Histology, cell proliferation, and apoptosis analyses
Male mice were injected intraperitoneally with a cocktail of BrdU and 5-fluoro 2′-deoxyuridine (FEdU) (Sigma) at a concentration of 50 and 10 mg/kg, respectively, in PBS. After 2 or 8 hr, testes were dissected out, weighed, and immersed either in 4% paraformaldehyde PBS or in Bouin’s fixative at 4°C for 2–24 hr (the time depending on the size of the testis). Dehydration was done in series of ascending concentrations of ethanol for a period of 2–5 hr. After the testes were embedded in paraplast, 7-μm sections were cut and mounted onto Superfrost Plus slides (Fisher Scientific). Sections were stained with periodic acid–silver methenamine (PAS) reagent–hematoxylin for routine histology. Staining for BrdU incorporation was carried out essentially as described (Sakai et al. 1994).

Programmed cell death was analyzed by TUNEL labeling using the ApoTag detection kit (Oncor) essentially following manufacturer’s instruction with some modifications. In brief, sections were dewaxed and rehydrated with a series of decreasing concentrations of ethanol and rinsed in PBS. Proteinase K digestion was empirically optimized at 7 min. Sections were incubated with terminal deoxynucleotidyl transferase (TdT) and digoxigenin–UTP at 37°C for 90 min. Sections were then quenched in 2% hydrogen peroxide for 10 min. The incorporated digoxigenin–UTP was detected using an anti-digoxigenin peroxidase coupled antibody with Sigma Fast DAB tablets (Sigma). Sections were counterstained with either eosin or hematoxylin.

In situ hybridization
In situ hybridization with α<sup>35S</sup>-labeled UTP RNA probes was performed essentially as described (Zhao and Hogan 1996) with slight modifications. In brief, the sections from testes fixed in Bouin’s fixative were dewaxed and soaked in 70% ethanol for at least 12 hr to remove picric acid. Bmp8a 3′ UTR and Bmp8b 3′ UTR probes (Zhao and Hogan 1996) were used to detect Bmp8a and Bmp8b transcripts in male germ cells. A 900-bp mouse cyclic protein 2 (Cp-2) cDNA fragment corresponding to the coding region was obtained by RT–PCR, cloned into PCRscript (Stratagene), and used for synthesis of antisense RNA probes. Mouse Dhh plasmid pSP73.Dhhex3 (Bitgood et al. 1996) was used to synthesize antisense probes to mouse Dhh.

Acknowledgments
We thank Linda Hargett and M. Rosanna Gray-Swain for excellent technical assistance, Drs. Richard Behringer and Claude Nagamine for critically reading and helpful comments on the manuscript; and Richard Behringer, Mary Ann Handel, Claude Nagamine, and Gary Olson for valuable advice. We thank Dr. Lillian Nanny and the Tissue Analysis Core Laboratory (supported by AR41943) for analysis of BrdU labeling. This work was supported by National Institutes of Health (NIH) grant CA48799 to B.L.M.H. L.L. was supported by NIH training grant CA09592. G.-Q.Z. and P.L. are Associates and B.L.M.H. is an Investigator of the Howard Hughes Medical Institute.

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*Genes Dev.* 1996, 10:
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