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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*^{tm1blh} 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]

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Bone morphogenetic proteins (BMPs) constitute a large group of secreted signaling molecules of the transforming growth factor-β (TGFβ) superfamily (for review, see Hogan et al. 1994a; Kingsley 1994; McPherron and Lee 1996). Evidence from a number of different organisms shows that BMPs are multifunctional regulators of embryonic development, controlling processes as diverse as cell proliferation, apoptosis, specification of cell fate, and differentiation. For example, in *Drosophila, decapentaplegic* (*dpp*) has an essential role in dorsal–ventral patterning of the blastoderm embryo, cardiac morphogenesis, midgut development, and imaginal disc patterning (for review, see Gelbart 1989; Wall and Hogan 1994). BMP2 and BMP4, the vertebrate homologs of DPP, have also been shown to exert important patterning functions during vertebrate embryogenesis. Targeted inactivation of the murine genes encoding BMP4 and a type I BMP2/4 receptor (*Bmpr*) results in defects in mesoderm formation and patterning during gastrulation (Mishina et al. 1995; Winnier et al. 1995). Later in embryogenesis, the temporal and spatial expression of BMPs coincides with sites of inductive epithelial–mesenchymal interactions during organogenesis (Lyons et al. 1989, 1990; Jones et al. 1991; Bitgood and McMahon 1995; Roberts et al. 1995). This has been particularly well investigated in tooth development, where exogenous BMP2 or BMP4 protein mimics the signal from the epithelial dental placode that induces expression of the *Msx1* and *Msx2* homeo box genes, as well as *Bmp4*, in the underlying mesenchyme (Vainio et al. 1993). Moreover, *Bmp2*, *Bmp4*, and *Bmp7* are expressed in localized, nested domains in the enamel knot, a region that has a key role in regulating proliferation and spatial patterning of the tooth (Vaahtokari et al. 1996). Targeted inactivation of *Bmp7* results in abnormal kidney development in homozygous mutant mice. In this case, it appears that the primary defect is not in the early inductive interactions between the ureteric bud and nephrogenic mesenchyme, but rther in subsequent cellular interactions required for continued proliferation, survival, and/or differentiation of the nephrogenic mesenchymal cells (Dudley et al. 1995; Luo et al. 1995).

Normal spermatogenesis requires numerous reciprocal interactions between somatic and germ cells mediated by growth factors and their receptors (Yoshinaga et

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al. 1991; Han et al. 1993; Boujrad et al. 1995b; Tsuruta 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. 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* (*Op2*) and *Bmp8b* (*Op3*) 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 recombinaion in embryonic stem (ES) cells. Here, we report the phenotype of mice homozygous for the mutant allele, *Bmp8b*^{tm1blh}. 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*^{tm1blh} allele, a 7-kb genomic fragment including exon 4, which encodes amino acids 221–287 encompassing the conserved dibasic proteolytic cleavage site between the pro- and mature regions, is deleted and replaced by the MC1neo^r cassette (Fig. 1) (Rudnicki et al. 1992). Therefore, it is predicted that no functional BMP8B protein will be expressed from the *Bmp8b*^{tm1blh} 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

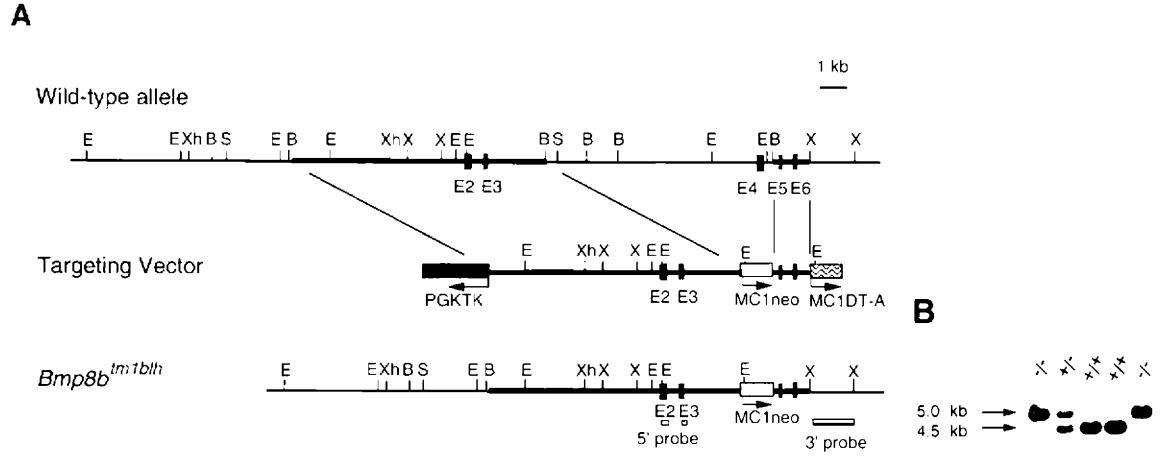

Figure 1. Targeted mutagenesis of the mouse *Bmp8b* locus. (*A*) Schematic representation of the *Bmp8b* wild-type allele on the *top*, targeting vector in the *middle*, and targeted *Bmp8b*^{tm1blh} allele at the *bottom*. Genomic DNA fragments used as the long arm and short arm of homology in the targeting vector are indicated by thick lines. Coding exons are shown as solid boxes. Expression cassettes PGK TK, MC1neo^r, and MC1DT-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) *Eco*RI; (Xh) *Xho*I; (B) *Bam*HI; (S) *Sal*I; and (X) *Xba*I. (*B*) Southern blot of the *Bmp8b*^{tm1blh} mice. Genomic DNA isolated from tail tips was digested with *Eco*RI, 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.

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(5B11, A5, and D1) were injected into C57BL/6 blastocysts and the *Bmp8b*^{tm1blh} allele was transmitted through the germ line of resulting chimeras. Homozygous mutant animals of all three lines show the same phenotype. 5B11 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.

Reproduction phenotype of Bmp8b^{tm1blh} mutant mice

We have shown previously that *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 *Bmp8b*^{tm1blh} 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 *Bmp8b*^{tm1blh} mice, mating pairs were set up with different combinations of male and female mice. As shown in Table 1, 10 of 18 homozygous *Bmp8b*^{tm1blh} 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 *Bmp8b*^{tm1blh} females did not show obvious reproduction defects.

Table 1. *Reproduction phenotype of* Bmp8b *mutant mice*

Male (n)	Female (n)	Fertile?	Testis weight (mg)	Testis degeneration
−/− (12)	+/− (3) +/+ (5) −/− (12)	no[a]	17 ± 4.9	complete
−/− (6)	+/− (2) +/+ (7)	yes	80 ± 17	partial
+/− (5)	−/− (5)	yes	100 ± 15	none
+/+ (6)	−/− (8)	yes	117 ± 15	none

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.

[a]Two of the males in this group had litters of two to three pups in the beginning, and no more pregnancies were scored thereafter.

Progressive germ-cell apoptosis leads to sterility in homozygous Bmp8b^{tm1blh} *males*

As shown in Table 1, sterile *Bmp8b*^{tm1blh} males had testes ~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 *Bmp8b* gene.

Bimodal expression of Bmp8 *genes in male germ cells during testicular development*

As shown previously (Zhao and Hogan 1996), *Bmp8a* and *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 *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 *Bmp8* expression in male germ cells during the early postnatal period revealed low levels of expression of both *Bmp8* genes in almost all spermatogonia and early meiotic germ cells (Fig. 3C,D, 2 weeks). This early expression of *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 *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 *Bmp8* genes during testis development suggests that different mechanisms are used to regulate *Bmp8* expression in the early and late stage testis and that the

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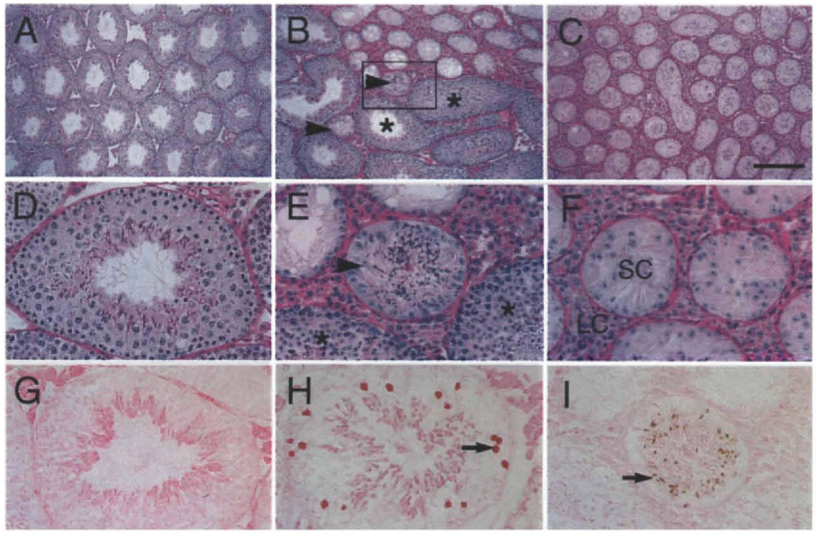

Figure 2. Histology and TUNEL labeling of adult wild-type and homozygous *Bmp8b*^{tm1blh} testes. (*A–F*) Testis sections stained with periodic acid-Schiff's (PAS) reagent and hematoxylin. (*A*) Section through a wild-type testis (105 mg); (*B*) section of a partially degenerated homozygous *Bmp8b*^{tm1blh} 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*^{tm1blh} 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*^{tm1blh} 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^{tm1blh} *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 wild-type 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.

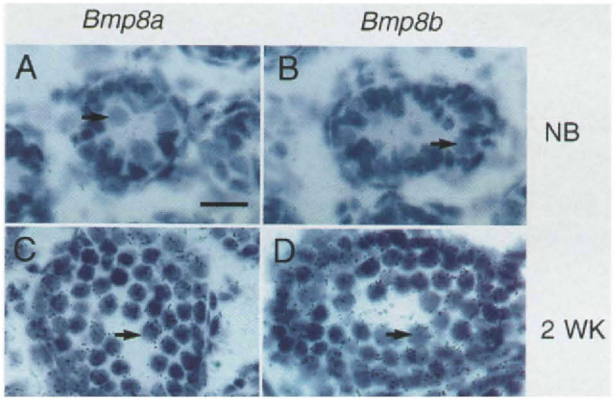

Figure 3. *Bmp8a* and *Bmp8b* expression in newborn and 2-week-old testes. Antisense RNA probes corresponding to the 3′ UTRs of each gene were used for in situ hybridization. All sections were counterstained with hematoxylin. Only bright-field photomicrographs are shown. No obvious hybridization signals 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.

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

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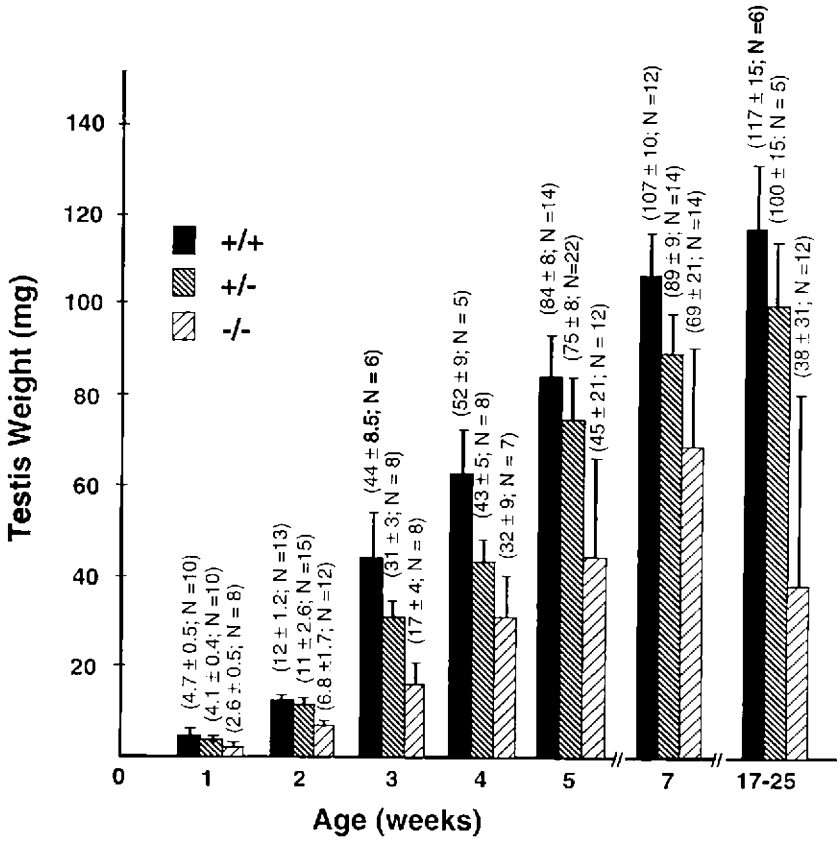

Figure 4. Comparison of the weight of wild-type and *Bmp8b*^{tm1blh} testes at different ages during postnatal development. For each age several litters of animals were used and the mean ±S.E. is indicated for each group of animals. (*N*) The number of animals in each group. Because of big litter sizes, 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 heterozygous testes (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^{tm1blh} *testes during early puberty*

To investigate the cause of the germ-cell deficiency in *Bmp8b*^{tm1blh} 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. 6I,L). 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^{tm1blh} *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 testis 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 8 hr

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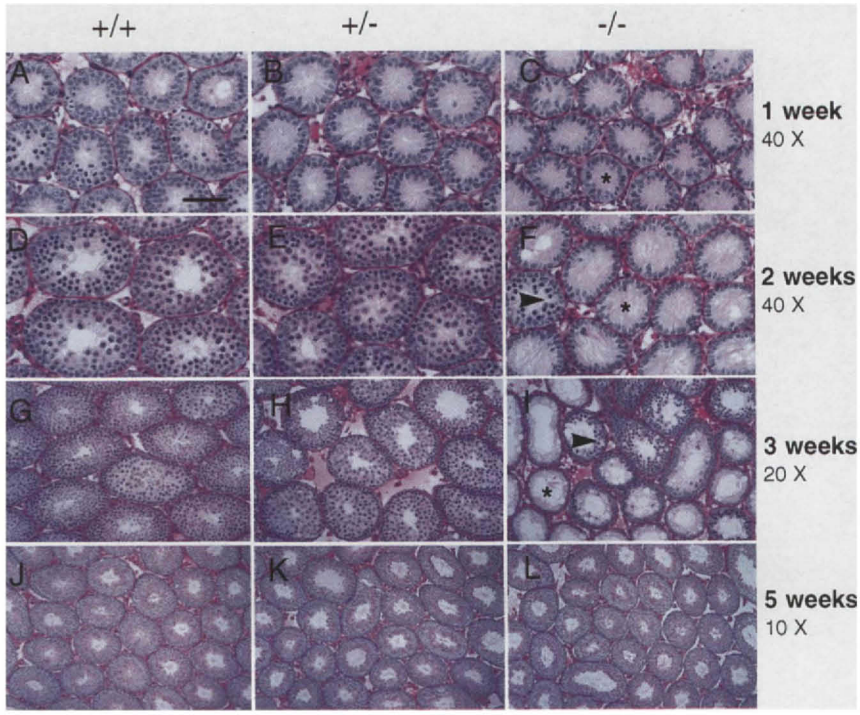

Figure 5. Histology of wild-type (+/+), heterozygous (+/−), and homozygous (−/−) *Bmp8b*^{tm1blh} testes. All sections were stained with PAS. Note the different magnification of *A–F*, *G–I*, and *J–L*. (*A–C*) Sections through testes of 1-week-old animals. A multilayered seminiferous epithelium containing meiotic germ cells is present in the wild-type seminiferous tubules (*A*). In heterozygous and homozygous *Bmp8b*^{tm1blh} 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*^{tm1blh} 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*^{tm1blh} 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*^{tm1blh} 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*.

after BrdU injection. Therefore, we conclude that the local accumulation of interstitial cells is a secondary effect caused by the decreased volume of the seminiferous tubules.

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 (Yomogida 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

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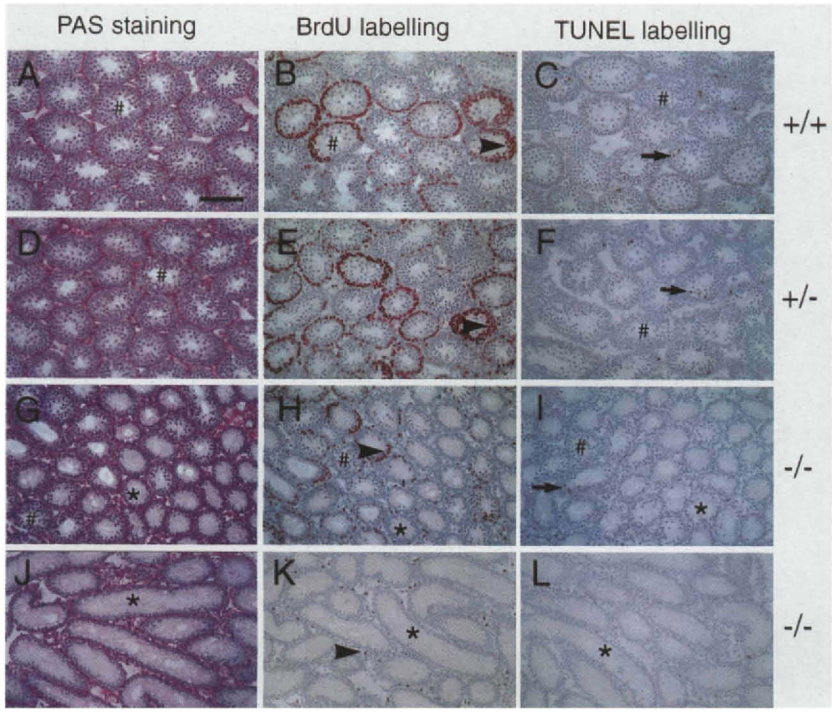

Figure 6. Comparison of histology, BrdU labeling, and TUNEL labeling of testes of 2-week-old animals. (*A–C*) Sections of a wild-type testis; (*D–F*) sections of a heterozygous *Bmp8b*^{tm1blh} testis; (*G–I*) sections of a homozygous *Bmp8b*^{tm1blh} testis with moderate germ cell deficiency; (*J–L*) sections of a homozygous *Bmp8b*^{tm1blh} testis with severe seminiferous tubule defects. In wild-type and heterozygous *Bmp8b*^{tm1blh} testes, heavy BrdU labeling is noted in many seminiferous tubules (arrowheads in *B* and *E*). However, in homozygous *Bmp8b*^{tm1blh} testes there is a significant reduction (arrowhead in *H*) or absence (*K*) of BrdU labeling in the seminiferous epithelia. In *K* only the interstitial cells are labeled by BrdU (arrowhead) at a comparable frequency to wild-type and heterozygous testes. In wild-type and heterozygous *Bmp8b*^{tm1blh} testes, germ cells of some tubules are labeled by TUNEL (arrows in *C* and *F*). In homozygous *Bmp8b*^{tm1blh} testes, a reduction (*I*) or absence (*L*) of TUNEL-labeled cells is noted. (#) Normal or relatively normal seminiferous tubules. (Asterisk) Seminiferous tubules with severely reduced numbers of germ cells. Bar, 160 μm.

and functionally associated in the testis, their proliferation and differentiation are thought to be controlled by different mechanisms (for review, see McCarrey 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*

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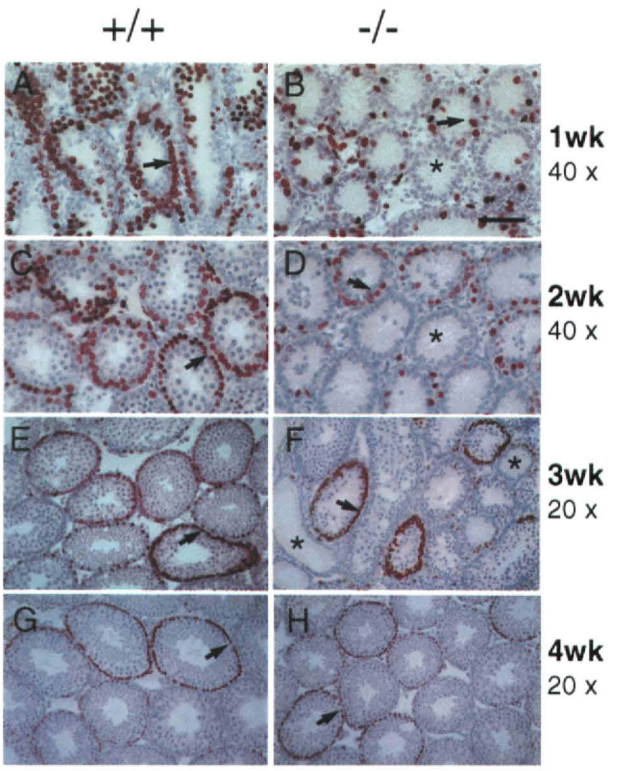

Figure 7. Comparison of BrdU labeling of wild-type and *Bmp8b*^{tm1blh} testes at different ages. Note the different magnification of testis sections of different ages. (*A,B*) Sections through 1-week-old testes; (*C,D*) sections through 2-week-old testes; (*E,F*) sections through 3-week-old testes. 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*.

mutants show normal reproductive performance and presumably have normal spermatogenesis (Luetteke et al. 1993). The surviving homozygous *TGFβ* 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.

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

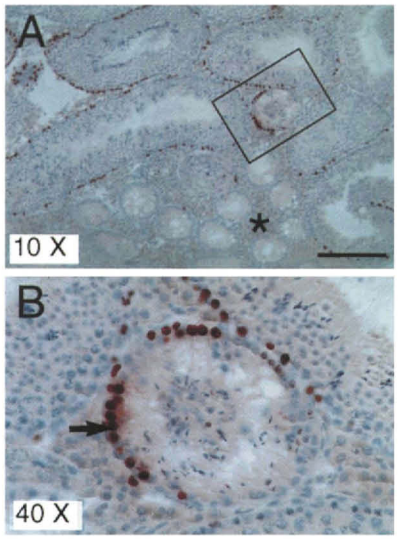

Figure 8. BrdU labeling of an 8-week-old homozygous mutant testis with partial degeneration. Eight hours after BrdU injection, the testis was dissected out and processed. (*A*) Low power magnification of a section through a partially degenerated testis. The upper portion contains seminiferous tubules 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*.

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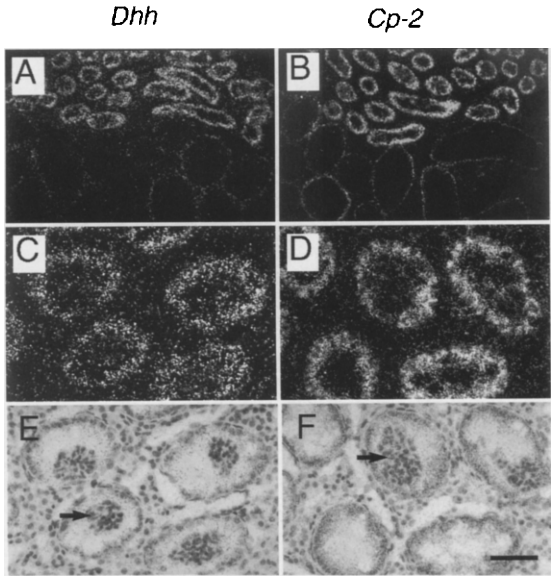

Figure 9. Expression of Sertoli cell-specific genes in a partially degenerated homozygous *Bmp8b*^{tm1blh} testis. In situ hybridization was carried out with antisense probes to *Dhh* and *Cp-2* on sections of a homozygous *Bmp8b*^{tm1blh} testis of 2.5-month-old (60 mg). (*A*,*B*) Dark-field photomicrographs 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*.

remainder of the testes. This suggests that the BMP8B protein has a major role in the initiation of spermatogenesis. During mid- and late puberty, 50% of the homozygous mutant testes gradually reach a size comparable with that of some heterozygous mutants but with an apparently delayed first wave of spermatogenesis (Figs. 5L and 7H). Such a recovery is presumably caused by the presence of BMP8A, which compensates for the absence of BMP8B. This hypothesis will be tested by generating mice homozygous for mutations in both genes.

According to our model, during early adulthood, increased apoptosis of pachytene spermatocytes in homozygous mutant testes leads to a decreased number of round spermatids in the next round of meiosis and consequently to reduced levels of BMP8A protein and an acceleration of degeneration of other germ-cell populations. This adult phenotype of *Bmp8b* homozygous mutants suggests that the BMP8B protein is also required for the maintenance of spermatogenesis and that BMP8A is insufficient to compensate for the absence of BMP8B. Therefore, it is reasonable to conclude that the BMP8B protein is required for both the initiation and maintenance of spermatogenesis. The presence of BrdU-labeled spermatogonia in the degenerating tubules (Fig. 8A,B) suggests 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 testis do not show such up-regulation. The male Wolffian 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. 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 ActRIIs in a cell culture system (J. Wrana, pers. comm.). Therefore, MADR1 and MADR2 may

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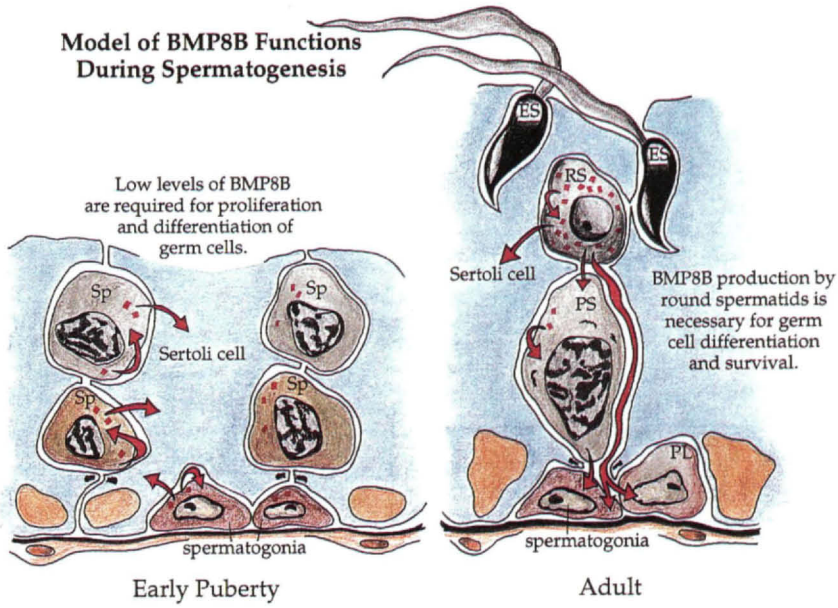

Figure 10. Schematic representation of an autocrine and/or short range paracrine model of BMP8B function. Diagrams represent a cross section through the seminiferous tubule of an early pubertal (*left*) or adult (*right*) testis, with the lumen of the tubule at the *top*. At the periphery of the tubules are myoid cells that share a basement membrane (thick black line) with Sertoli cells (blue), spermatogonia, and preleptotene spermatocytes (PL). Germ cells remain embedded between Sertoli cells as they differentiate into primary spermatocytes (Sp) in early puberty or pachytene spermatocytes (PS), round spermatids (RS), and elongated spermatids (ES) in the adult. During early puberty, BMP8B (red squares) is produced by all germ cells at low levels. In the adult, stages 6–8 round spermatids express high levels of BMP8B, and low levels of BMP8B are also produced by a small number of pachytene spermatocytes in stage VI–VII seminiferous tubules. Red arrows indicate action of BMP8B action on target cells. In this model, BMP8B acts on germ cells through autocrine and/or short-range paracrine effects and on Sertoli cells through a short-range paracrine effect.

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 *Madr1* 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 λ FixII genomic library (Stratagene) using a *Bmp8a* cDNA probe spanning exons 2–7. Nine phage clones were obtained. Two overlapping clones correspond to the *Bmp8a* 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 (*Bam*HI fragment) and a 1-kb 3′ homology region (*Bam*HI and *Xba*I 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 $Bmp8b^{tm1blh}$ 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 Andras 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 *Not*I-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^r mouse primary embryonic fibroblasts. ES cell culture and drug selection were performed essentially as described (Winnier et al. 1995). One targeted ES

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line (5B11) from R1 cells and four targeted cell lines (A5, D1, F4, and F7) from TL1 cells were obtained with a frequency of 3% of the double-resistant clones. 5B11, A5, and D1 were used for host blastocyst (C57BL/6) 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*^{tm1blh} 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 *Eco*RI. 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*^{tm1blh} 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 (FrdU) (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-Schiff's (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 ApopTag 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 deoxynucleotide transferase (TdT) and digoxygenin–dUTP at 37°C for 90 min. Sections were then quenched in 2% hydrogen peroxide for 10 min. The incorporated digoxygenin–dUTP was detected using an anti-digoxygenin-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-^{35}S-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*.

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