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The TGF-beta Pseudoreceptor Gene *Bambi* Is Dispensable for Mouse Embryonic Development and Postnatal survival

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Summary

The *Bambi* (Bmp and activin membrane-bound inhibitor) gene encodes a transmembrane protein highly similar in amino acid sequence to TGF-beta type-I receptors. Unlike the TGF-beta receptors, however, the *Bambi* intracellular domain is short and lacks a serine/threonine-kinase domain that is essential for transducing TGF-beta signaling. Previous biochemical assays showed that *Bambi* interacts directly with BMP receptors and antagonizes BMP signaling. Interestingly, the expression of *Bambi* largely overlaps, both temporally and spatially, with that of *Bmp4* during early embryonic development in *Xenopus*, zebrafish, and mice, which led to the hypothesis that *Bambi* may function to regulate BMP signaling during embryogenesis. To directly analyze the roles of *Bambi* during embryonic development, we generated mice carrying a conditional allele of *Bambi*, *Bambi^{flox}*, with *loxP* sequences flanking the first exon that encodes the N-terminus and signal peptide region of the *Bambi* protein. Mice homozygous for this targeted conditional allele appear normal and fertile. We crossed the *Bambi^{flox/+}* mice to the *Elia-Cre* transgenic mice and generated mice carrying deletion of the first exon of the *Bambi* gene. Surprisingly, mice homozygous for the deleted allele were viable, fertile and didn't exhibit any discernible developmental defect. Our data exclude an essential role for *Bambi* in mouse embryonic development and postnatal survival.

Keywords

Bambi; BMP signaling; Conditional knockout; Cre/*loxP*

The transforming growth factor-beta (TGFβ) superfamily of secreted signaling molecules, including TGFβs, bone morphogenetic proteins (BMPs), and activins, plays essential roles throughout animal development. These molecules initiate cellular signaling by binding to and bringing together two types of receptor serine/threonine kinases. Upon ligand binding, the type-II receptor phosphorylates and activates the type-I receptor kinase, which in turn phosphorylates and activates a set of transcriptional co-activators called Smad proteins and leads to their nuclear translocation and transcriptional activation of downstream target genes (reviewed in Shi and Massague, 2003; Nohe et al., 2004). In an expression screen for components involved in BMP4 signaling in *Xenopus* embryos, Onichtchouk et al. (1999) identified a gene encoding a transmembrane protein highly related to type-I TGFβ receptors. They named the gene product BAMBI (BMP and activin membrane-bound inhibitor) because it lacked an intracellular kinase domain and its overexpression antagonized BMP and activin signaling in *Xenopus* embryos (Onichtchouk et al., 1999). The *Bambi* gene is evolutionarily conserved and its homologs have

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been identified in human (Degen et al., 1996), Zebrafish (Tsang et al., 2000), mouse (Grotewold et al., 2001), and rat (Loveland et al., 2003).

Ectopic expression of *Bambi* mRNA in *Xenopus* and zebrafish embryos resulted in phenotypes resembling inhibition of BMP signaling (Onichtchouk et al., 1999; Tsang et al., 2000). In vitro biochemical assays showed that the Bambi protein associated with type-I and type-II BMP/TGF β receptor complexes in the presence of ligands, suggesting that Bambi inhibits BMP/TGF β signaling by interfering with receptor complex formation or phosphorylation of type-I receptors (Onichtchouk et al., 1999; Tsang et al., 2000). Interestingly, *Bambi* is coexpressed with *Bmp4* in many developmental processes during embryogenesis in *Xenopus* (Onichtchouk et al., 1999), zebrafish (Tsang et al., 2000), and mouse (Grotewold et al., 2001). The co-expression and the ability of Bambi to antagonize BMP/TGF β signaling suggest that Bambi may play important roles in the regulation of BMP4 signaling during embryonic development.

BMP4 signaling is essential for many developmental processes. Mouse embryos lacking BMP4 function die at midgestation with severe defects in mesoderm formation (Winnier et al., 1995). BMP4^{+/-} heterozygous mice exhibit variable defects in craniofacial, eye, kidney, and limb development (Dunn et al., 1997). Moreover, mice lacking chordin and/or noggin, endogenous inhibitors of BMP signaling, have multiple developmental defects (Brunet et al., 1998; McMahon et al., 1998; Bachiller et al., 2000; Bachiller et al., 2003), indicating that the levels of BMP signaling are highly regulated during normal embryonic development. Since Bambi is a unique inhibitor of Bmp signaling at the receptor level and since *Bambi* mRNA is co-expressed with *Bmp4* during craniofacial, eye, kidney, and limb development (Grotewold et al., 2001), it may play important roles in these developmental processes by fine-tuning BMP signaling.

To investigate the roles of Bambi in mammalian development, we generated mice carrying a conditional null allele of the *Bambi* gene, *Bambi*^{fllox}, through homologous recombination in mouse embryonic stem cells and subsequent production of chimeric mice (see MATERIALS AND METHODS, and FIG. 1a). Both *Bambi*^{fllox/+} and *Bambi*^{fllox/fllox} mice were viable, fertile and didn't display any obvious phenotypic abnormality.

We crossed *Bambi*^{fllox/+} mice to either *Elia-cre* or *CMV-cre* transgenic mice, both of which could mediate deletion of *loxP*-flanked sequences in germ cells (Lakso et al., 1996; Dupe et al., 1997). *Bambi*^{del/+} mice were then intercrossed. The deletion of *loxP*-flanked sequence was confirmed by Southern hybridization analysis of *EcoRI*-digested genomic DNA using a probe corresponding to the deleted region (FIG. 1a). A 7.7 Kb band representing the wildtype allele was detected in both wildtype and *Bambi*^{del/+} heterozygous mice, but absent in *Bambi*^{del/del} homozygotes (FIG. 1c). The presence of genomic DNA in all lanes was confirmed by hybridizing the same membrane to a probe corresponding to genomic DNA 5' to the deletion (data not shown). The deletion was also verified by PCR genotyping. The wildtype allele gives a 215 bp PCR product using the primer pair F2/R2 and the deleted allele results in a 316 bp PCR product using the primers F2 and R4 (FIG. 1d). Furthermore, we performed RT-PCR analysis of total RNA from wildtype and *Bambi*^{del/del} mutant mouse embryos using primers RF1 and RR1. As expected, a 329 bp product was amplified from wildtype but not homozygous mutant littermate RNA samples (FIG. 2b). Similarly, a 586 bp product was amplified from wildtype but not homozygous mutant littermate RNA samples using primers flanking the exons 1 and 2 (FIG. 2c); indicating that the *loxP*-flanked sequence had been successfully deleted by the Cre recombinase. Since the 1075 bp genomic deletion includes the *Bambi* gene promoter and the first exon encoding the signal peptide, the deleted allele should represent a null allele of *Bambi*.

Of 125 pups from heterozygous intercross genotyped at two weeks of age, 30 (24.0%) were of wildtype, 59 (47.2%) were heterozygous, and 36 (28.8%) were homozygous for the *Bambi^{del}* allele. Chi-square analysis indicated that the distribution of these three genotypes was not significantly different from the expected Mendelian ratio. Morphological and histological examination of *Bambi^{del/del}* homozygous mice didn't detect any overt defects, indicating that the *Bambi* gene was not essential for normal mouse embryogenesis and postnatal survival.

To assess whether loss of *Bambi* function has any effect on postnatal growth, the body weights of homozygous mutant, heterozygous and wild type littermates were measured at regular intervals up to two months after birth (FIG. 3). Independent student's t-test indicated that the body weights of *Bambi^{del/del}* female mice were not significantly different from those of wildtype and heterozygous littermates before weaning (pups were usually weaned at 21 – 22 days after birth). However, the *Bambi^{del/del}* homozygous mutant female mice exhibited a small (9%–10%) but statistically significant ($P < 0.05$) reduction in body weight, compared to their wildtype littermates. The *Bambi^{del/+}* heterozygous female mice had body weights in between and not statistically significant from the wildtype and homozygous mutant littermates. For male mice, body weights of *Bambi^{del/del}* homozygous mutants were not significantly different from those of wildtype and heterozygous littermates at all the stages examined.

We further investigated whether loss of *Bambi* function affected male and female fertility. *Bambi^{del/del}* homozygous males and females were intercrossed and litter sizes were monitored. Pups were born with normal litter sizes and were normally nursed by their mothers, suggesting that deletion of *Bambi* has no significant effect on fertility. These data also exclude any maternal or paternal effect on the *Bambi* gene.

Since BMP signaling plays important roles in skeletal development, and genetic inactivation of other endogenous Bmp inhibitors resulted in skeletal defects (e.g., Balemans et al., 2002), we examined skeletal preparations of newborn pups. Compared with wildtype littermates, *Bambi^{del/del}* mutant mice didn't exhibit any obvious defect in the skeleton (data not shown).

Bambi is co-expressed with *Bmp4* during craniofacial and limb development (Grotewold et al., 2001). We carefully examined these structures in newborn mice by histological and skeletal preparations. *Bambi^{del/del}* mutant mice didn't display any obvious craniofacial or limb defects (Fig. 4).

In conclusion, we have generated mice carrying a floxed conditional allele of *Bambi*. These mice were crossed to Cre-transgenic mice and mice carrying the deleted null *Bambi* allele were generated. Analysis of *Bambi^{del/del}* mutant mice indicated that *Bambi* function is not essential for embryonic development and postnatal survival although it has some minor effect on the growth of female mice after weaning.

MATERIALS AND METHODS

Generation of *Bambi^{flox}* and *Bambi^{del}* mice

A BAC clone containing the *Bambi* genomic region was obtained by screening the RPCI-22 129/SvEvTac mouse BAC library (BACPAC Resources, Children's Hospital of Oakland, Oakland, CA). An 7.7 Kb EcoRI fragment containing exons 1 and 2 as well as an 13.5 Kb *BamHI* fragment containing the entire gene were subcloned into pBluescript plasmid vectors for construction of the targeting vector. The targeting vector contained the following fragments in the 5' to 3' order: (1) a diphtheria toxin (DTA) expression cassette, (2) an 2.9 Kb EcoRI/PmlI fragment from 5' to the *Bambi* gene promoter as the 5' homologous arm, (3) a *loxP* sequence, (4) the 1.07 Kb PmlI fragment containing the *Bambi* gene promoter and first exon,

(5) another loxP sequence followed by an FRT-flanked PGKneo expression cassette, and (6) the 3.6 Kb PmlI-SacI fragment from Intron1 to exon3 region as the 3' homology arm (FIG. 1a). The final targeting vector was linearized at a unique KpnI restriction site 3' to the 3' homology arm and electroporated into CJ7 mouse embryonic stem (ES) cells.

Following electroporation, ES cells were subjected to G418 selection as described previously (Swiatek and Gridley, 1993). G418-resistant ES clones were screened by Southern hybridization analysis of BglII-digested ES genomic DNA using a 5' external probe. Three independent targeted ES clones were microinjected into C57BL/6J blastocysts that were subsequently transferred into the uterus of pseudopregnant C57BL/6J female mice to generate chimeric mice. Chimeric mice from two of the three ES clones gave germ-line transmission of the targeted allele and were crossed to C57BL/6J female mice to generate *Bambi^{lox/+}* heterozygous mice, which were then intercrossed to generate and test whether there is any obvious defects in the *Bambi^{lox/lox}* mice.

To generate mice with loss of *Bambi* gene function, *Bambi^{lox/+}* heterozygous mice were crossed to either *Ell1a-Cre* or *CMV-Cre* transgenic mice (purchased from The Jackson Laboratory, Bar Harbor, ME). Mice carrying the targeted allele and the *Cre* transgene were backcrossed to C57BL/6J mice to generate mice heterozygous for the deletion of the floxed first exon (*Bambi^{del/+}*) but without the *Cre* transgene. The *Bambi^{del/+}* heterozygous males and females were intercrossed for generation and analysis of *Bambi^{del/del}* homozygous mutant mice.

All animal protocols were approved by the University of Rochester Committee on Animal Resources in accordance with National Institute of Health guidelines.

Genotyping

For genotyping, DNA was isolated from either tail or embryonic yolk sac samples. To distinguish wildtype from the *Bambi^{lox}* allele, forward primer F2 (GGGATCCGGGTCTTGAGAC) and reverse primer R2 (TCGGAGTTTGCTACGAGACC) were used for PCR, amplifying an 215 bp product from the wildtype allele and an 336 bp product from the *Bambi^{lox}* allele. The *Bambi^{del}* allele was detected by PCR using the forward primer F2 and reverse primer R4 (TTCGGAATAGGAAGTTCGTCG), which yielded an 316 bp product. The program used for PCR genotyping reactions is as follows: 1 cycle of 94°C for 4 minutes; followed by 40 cycles of 30 seconds at 94°C, 30 seconds at 62°C, and 40 seconds at 72°C. PCR products were resolved on 1.5% agarose gels.

RT-PCR

Total RNAs were extracted from E10.5 whole embryos using Trizol reagents (Invitrogen), treated with RNase-free DNase I (Promega) to remove any residual genomic DNA, and purified further using the RNeasy kit (QIAGEN). 3 µg of each purified total RNA sample was reverse-transcribed to generate first-strand cDNAs using the Superscript II reverse transcription kit (Invitrogen). The following primers were used for RT-PCR analysis of *Bambi*: RF1, GTGTAGGCGTTGCTCTCTGT; RR1, CTTTGGTGAGCAGCACAGCC; and RR2, CGTCATGCAGTCCTCGATAA. The following PCR program was used for the PCR reactions: 1 cycle of 94°C for 4 min; followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 58°C, and 40 seconds at 72°C.

Skeletal Preparation, Growth Curve and Statistical Analysis

Skeletal preparation was performed as described previously (Martin et al., 1995). For growth curve analysis, the body weights of mice were measured every 10 days for a period of 60 days

starting from the 10th day after birth. Male and female growth curves were generated separately. For statistical analysis, independent Student's *t* test was used, and a *P* value less than 0.05 was regarded as significant.

Chi-square test was used for determining whether distribution of genotypes of progeny from *Bambi*^{del/+} heterozygous intercrosses reflected the expected Mendelian ratio, and *P* value less than 0.05 was considered significant.

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FIG. 1. Generation of *Bambi*^{flox} and *Bambi*^{del} mice. (a) Schematic representation of the *Bambi* genomic locus, the gene targeting construct and the targeted (*Bambi*^{flox}) allele. *LoxP* sequences are indicated by black triangles. D marks the deletion probe; DTA, diphtheria toxin A expression cassette; E1, exon 1; E2, exon 2; E3, exon 3; Neo, Frt-flanked PGK-Neo cassette. The restriction enzyme recognition sites are: B, Bgl I; E, EcoRI; P, PmlI; S, SacI. (P) marks PmlI site destroyed during subcloning. (b) Southern hybridization analysis of BglI-digested genomic DNA from representative G418-resistant ES clones using the 5' external probe. The upper band (11 Kb) represents the targeted allele and the lower band (9.6 Kb) represents the wildtype allele. (c) Southern hybridization analysis of EcoRI-digested tail genomic DNA from *Bambi*^{+/+}, *Bambi*^{del/+}, and *Bambi*^{del/del} mice using the deletion probe. A 7.7 Kb band was only detected in the wildtype and heterozygous mouse DNA samples, but not in DNA from the homozygous *Bambi*^{del/del} mice. (d) PCR analysis of tail DNA of *Bambi*^{+/+}, *Bambi*^{del/+}, and *Bambi*^{del/del} mice using primers F2, R2 and R4. The upper fragment (215 bp) represents the wildtype allele, and the lower band (316 bp) represents the *Bambi*^{del} allele.

FIG. 2.

RT-PCR analysis of RNAs isolated from E10.5 wildtype and *Bambi*^{del/de} embryos. (a) The approximate positions of primers used in (b) and (c) are indicated by arrows. E1, exon 1; E2, exon 2. (b) A 329 bp fragment corresponding to the entire exon 1 was only amplified from wildtype but not *Bambi*^{del/de} embryo RNA samples. As internal control, RT-PCR product corresponding to the *Hprt* mRNA was amplified from both wildtype and *Bambi*^{del/de} embryo RNA samples. RT, with reverse transcription; -RT, without reverse transcription. (c) A 586 bp fragment containing Exon 1 and Exon 2 sequences was amplified from wildtype but not mutant RNA samples using primers RF1 and RR2 as indicated in (a). RT-PCR amplification of a fragment from *Hprt* mRNA was used as internal control.

FIG. 3.

Growth curves of wild type mice, *Bambi*^{del/+} heterozygous, and *Bambi*^{del/del} homozygous mice. (a) Growth curves of female wildtype (n=7), *Bambi*^{del/+} heterozygous (n=12), and *Bambi*^{del/del} homozygous (n=7) mice. From 30 days onwards, the body weights of the *Bambi*^{del/del} female mice were 9%–10% lower than those of wildtype mice ($P<0.05$). (b) Growth curve shows the average body weights of male wildtype (n=9), *Bambi*^{del/+} heterozygous (n=18), and *Bambi*^{del/del} homozygous (n=10) mice taken on the days indicated for a period of 60 days. At all the stages examined, body weights of *Bambi*^{del/del} male mice were not significantly different from those of wildtype and heterozygous male mice.

FIG. 4.

Bambi is not essential for limb and craniofacial development. Skeletal preparations of the forelimb (a, b), hindlimb (c, d), and skull (e, f) were compared between wildtype (a, c, e) and *Bambi*^{del/del} homozygous (b, d, f) newborn littermates. No significant difference was found. (e) and (f) show the ventral view of the skulls.