Subfertility, Uterine Hypoplasia, and Partial Progesterone Resistance in Mice Lacking the Krüppel-like Factor 9/Basic Transcription Element-binding Protein-1 (Bteb1) Gene*

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Progesterone receptor (PR), a ligand-activated transcription factor, is a key regulator of cellular proliferation and differentiation in reproductive tissues. The transcriptional activity of PR is influenced by co-regulatory proteins typically expressed in a tissue- and cell-specific fashion. We previously demonstrated that basic transcription element-binding protein-1 (BTEB1), a member of the Sp/Krüppel-like family of transcription factors, functionally interacts with the two PR isoforms, PR-A and PR-B, to mediate progestin sensitivity of target genes in endometrial epithelial cells in vitro. Here we report that ablation of the Bteb1 gene in female mice results in uterine hypoplasia, reduced litter size, and increased incidence of neonatal deaths in offspring. The reduced litter size is solely a maternal genotype effect and results from fewer numbers of implantation sites, rather than defects in ovulation. In the early pregnant uterus, Bteb1 expression in stromal cells temporally coincides with PR-A isoform-dependent decidual formation at the time of implantation. Expression of two implantation-specific genes, Hoxa10 and cyclin D3, was decreased in uteri of early pregnant Bteb1-null mutants, whereas that of Bteb3, a related family member, was increased, the latter possibly compensating for the loss of Bteb1. Progesterone responsiveness of several uterine genes was altered with Bteb1-null mutation. These results identify Bteb1 as a functionally relevant PR-interacting protein and suggest its selective modulation of cellular processes that are regulated by PR-A in the uterine stroma.

Progesterone (P) is a pleiotropic hormone whose genomic actions are mediated by two progesterone receptor (PR) isoforms, PR-A and PR-B, which in the human and mouse originate from two distinct PR promoters (1, 2). The PR proteins form homo- or heterodimers upon binding P, and as a consequence of ligand-induced conformational changes, interact with specific response elements in target genes (3, 4). Recent evidence has demonstrated that the transcriptional activity of the PRP complex is dependent on a large part on its selective interaction with nuclear co-regulatory proteins that can be classified as co-activators or co-repressors (5, 6). These proteins form large complexes with PR and modify transcriptional events by distinct mechanisms, including chromatin remodeling, enzymatic modification of histone tails, and interaction with components of the preinitiation RNA polymerase complex and by serving as scaffolds for the assembly of multicomponent protein units (5–7). Agonist-bound PR recruits several PR-interacting coactivators; these include members of the steroid receptor coactivator (SRC) family of proteins such as SRC-1, SRC-2 (TPG2/GRIP1/N-CoA2), and SRC-3 (AIB1/pCIP/RAC3/ACTR) (7); CBP/p300 (8, 9); and AP-1 family members such as Jun dimerization protein 2 (10) and Jun activation domain-binding protein-1 (11). These proteins can act synergistically to enhance PR transactivation (12), may be recruited in a sequential fashion by ligand-bound PR (13), and selectively determine PR-mediated transcriptional response (14). Transcriptional co-repressors such as the structurally related proteins NCoR and the silencing mediator for retinoid and thyroid hormone receptor similarly associate with and alter the activity of PR (15). These proteins are recruited to the PR complex only when PR is bound to an antagonist, a consequence of the distinct conformations induced by agonists and antagonists on this protein. Preferential formation of antagonist-bound PR-A/PR-B heterodimers with reduced DNA binding activity also occurs (16). Mice deficient in a number of nuclear coactivator genes (e.g. Src-1, Src-3, CBP, p300) have been generated; the collective data from careful analyses of their phenotypes indicate distinct functional roles in vivo, including in reproduction, consistent with their involvement in PR-dependent responses (17–19). Decreases in uterine levels of CBP and SRC family members are associated with onset of human labor (20), and cyclic changes in the expression of SRC-1 and p300/CBP in the normal endometrium accompany the menstrual cycle (21), suggesting the importance of nuclear co-activators in PR-mediated regulation of proliferation and differentiation in the human uterus.

We recently identified a novel PR-interacting protein, basic transcription element-binding protein-1 (BTEB1), whose expression has been documented in reproductive and non-reproductive tissues (22, 23). BTEB1, a member of the Sp/Krüppel-like factor family, is expressed in reproductive tissues. Previous studies have shown that BTEB1 interacts with the PR, functioning as a coactivator (24). In addition, BTEB1 regulates the expression of genes involved in the implantation process, including those that are essential for proper decidualization (25). These findings suggest that BTEB1 plays a crucial role in the regulation of genes that are important for the establishment of a successful pregnancy. In this study, we investigated the role of BTEB1 in the regulation of uterine gene expression during pregnancy. We found that BTEB1 expression is downregulated in the uteri of Bteb1-null mutant mice, which results in uterine hypoplasia, decreased litter size, and increased neonatal mortality. These findings indicate that BTEB1 plays a critical role in the regulation of uterine gene expression during pregnancy.
pel-like family (KLF) of transcription factors (24, 25), preferentially binds PR-B in vitro and enhances PR-mediated transactivation in endometrial epithelial cells (26). Although BTEB1 does not appear to directly physically interact with PR-A in these cells, it can modulate PR-A transactivity of P-responsive promoters and cooperates with CBP in enhancing both unliganded PR-A and agonist-bound PR-B transcriptional activities (27). Because PR-A and PR-B have cell- and promoter-specific transcriptional regulatory properties (28–30), we have previously shown that homozygous Bteb1-null (Bteb1<sup>−/−</sup>) female mice have altered uterine, but not ovarian, phenotypes from those of wild type (WT) mice. We also identified endometrial stromal cells as a major site of Bteb1 synthesis during early pregnancy and showed that in these cells, expression is temporally coincident with initial events in decidual formation, a process requiring PR-A regulation (33, 34). Further, we have provided a molecular rationale for uterine hypoplasia and subfertility in Bteb1<sup>−/−</sup> mice by the identification of uterine growth-associated and P-responsive genes whose patterns of expression are altered in uteri of Bteb1<sup>−/−</sup> mice. Taken together, these findings suggest the functional contribution of BTEB1 to PR-A action in uterine endometrial stromal cells and provide support to the hypothesis that tissue-specific modulation of progesterin activity by PR isoforms may involve their selective interaction with BTEB1.

**EXPERIMENTAL PROCEDURES**

**Experimental Animals**—Animal experiments were conducted following protocols approved by the Institutional Animal Care and Use Committee of the University of Florida (Gainesville, FL) and the University of Arkansas for Medical Sciences (Little Rock, AR). Klf9/Bteb1 mutant mice (Bteb<sup>−/−</sup>) were generated by insertion of the bacterial g-galactosidase (LacZ) gene in-frame into the first exon of the mouse Bteb1 gene as described previously (28). Mice were genotyped by PCR of genomic DNA prepared from mouse tails by overnight digestion at 55 °C in Tris-HCl buffer containing Triton X-100 (0.5%) and proteinase K (50 μg/ml). Two primer pairs were used for PCR. The first pair detected the wild type allele (forward, 5′-ACATGACTCTGGCGTGCCTCC-3′; reverse, 5′-CGCT-GTCGAGATCTCATATCC-3′) by the amplification of a 200-bp region within the first exon of the mouse Bteb1 gene, whereas the second pair detected the mutant allele (forward, 5′-ATGAACTGAGGACAGGCACGCAGCAGCAGC-3′; reverse, 5′-GGCCGATAGAAGGCGATGCGCTG-3′) by the amplification of a 600-bp region within the neomycin (neo) resistance gene. PCR cycling parameters for Bteb1 and neo were 94 °C for 12 min (“hot start”), 94.5 °C for 45 s, 55 °C for 45 s, and 72 °C for 1 min for 35 cycles (30 cycles for neo), with extension at 72 °C for 7 min. The Bteb1<sup><small>+/−</small></sup> colony was maintained on a hybrid background of 129Sv and C57Bl/6J strains by crossing heterozygous mutant mice (Bteb1<sup>−/−</sup>) in a continuous mating scheme. Mice were maintained on a 12-h light, 12-h dark schedule calculated at threshold cycles (C<sub>T</sub>) for detection of template contamination, if any. The mRNA quantity was measured by Lowry reagent using bovine serum albumin as standard protein. Uteri were rapidly removed, homogenized in TRIzol reagent (Invitrogen), and stored at −80 °C prior to isolation of RNA following the manufacturer’s instructions. Integrity of the RNA was confirmed using the RNA 6000 Nano LabChip kit with the Agilent 2100 bioanalyzer system (Agilent Technologies, Palo Alto, CA). cDNA was synthesized from total RNA (1 μg) in a total reaction volume of 10 μl using random hexamers and MultiScribe reverse transcriptase in a two-step RT-PCR reaction (PE Applied Biosystems, Foster City, CA). Primers that span intron/exon junctions were designed to prevent amplification of residual genomic DNA, using PrimerExpress software (Applied Biosystems); these are summarized in Table I. QPCR was performed with the Light-Cycler-based SYBR Green I detection system (Applied Biosystems) using an ABI Prism 7000 sequence detector (Applied Biosystems). Amplification conditions were: preincubation at 50 °C for 2 min, DNA polymerase activation at 95 °C for 1 min, followed by 40 PCR cycles of 95 °C for 15 s and 60 °C for 1 min. Expression levels were normalized to that for a housekeeping gene, cyclophilin, to control for input RNA and are presented as arbitrary units and expression fold changes were calculated using the ΔΔCT method.

**X-Gal Staining**—Isolated tissues were soaked in phosphate-buffered saline containing 20% sucrose, embedded in Tissue-Tek OCT medium (Fisher Scientific), and frozen in liquid nitrogen. Sections were cut (7 μm) using a cryostat, postfixed in phosphate-buffered saline containing 2% paraformaldehyde and 0.2% glutaraldehyde for 5 min, and then etched in 0.1% diethyl-pyrocatechol (1 mg/ml) at 37 °C overnight, followed by counterstaining with neutral red (1%) in sodium acetate (50 mM, pH 3.3).

**Statistical Analysis**—All reported values represent mean ± S.E. Differences were considered significant at p < 0.05 using analysis of variance.

**RESULTS**

**Loss of Bteb1 Gene Expression in Bteb1<sup>−/−</sup> Females**—The deletion of exon 1 of the Bteb1 gene and loss of Bteb1 protein expression in Bteb1<sup>−/−</sup> females was confirmed by PCR analysis...
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**Table I**

| Gene       | Forward primer                                      | Reverse primer                                      | Accession no. |
|------------|-----------------------------------------------------|-----------------------------------------------------|---------------|
| Axl        | 5′-GTGAAATCTAGCCGGGACT-3′                           | 5′-TCTCTGGAGAGCCCGGACAGT-3′                        | BC008551.1    |
| Bteb3      | 5′-ACAGAGTTGAGAGCCGCCTG-3′                          | 5′-ACAGAGTTGAGAGCCGCCTG-3′                         | NM_021366.2   |
| Calcitonin | 5′-CCAGCGTTGACCCCATCGT-3′                           | 5′-CCAGCGTTGACCCCATCGT-3′                          | BC030071.1    |
| Cyp11D1     | 5′-CCAGTTGTCGCGGTCGCTTA-3′                          | 5′-ACACCTGGTGCTATAGATGC-3′                         | NM_07632.1    |
| Cyclin D3  | 5′-GAGTTTCCTCCCTGCTGATGACAT-3′                      | 5′-GACAGGGTGGCGCTATAGATGC-3′                       | NM_07632.1    |
| Gapdh      | 5′-GCTGTTGGTGTGCTATAGATGC-3′                        | 5′-GACAGGGTGGCGCTATAGATGC-3′                       | NM_07632.1    |
| Hoxa10      | 5′-GGCTCAAGGTGGAGGAGGATG-3′                         | 5′-GGCTCAAGGTGGAGGAGGATG-3′                        | BC023196      |
| Hoxa11      | 5′-TGGCGGTCTCCCTTCTTGGT-3′                          | 5′-GGCTCAAGGTGGAGGAGGATG-3′                        | BC050839.1    |
| PR         | 5′-GAGTTTCCTCCCTGCTGATGACAT-3′                      | 5′-GACAGGGTGGCGCTATAGATGC-3′                       | NM_010450     |
| SLPI       | 5′-AGTGCCTCTGCGGTCTTAC-3′                           | 5′-GACAGGGTGGCGCTATAGATGC-3′                       | BC028509      |

of genomic DNA and by Western blot analysis of nuclear extracts prepared from uteri of 6-week-old animals, respectively (Fig. 1). The PCR fragment corresponding to the Bteb1 gene was detected in WT and Bteb1−/− females but, as expected, was absent in Bteb1−/− littermates. Conversely, the PCR fragment corresponding to the neomycin resistance gene neo was absent in DNA from WT but was present in DNA of Bteb1−/− and Bteb1+/− animals. Further, although a strong immunoreactive band corresponding to the neomycin resistance gene was present in nuclear extracts from WT and Bteb1−/− uteri, this band was absent from Bteb1−/− extracts (Fig. 1). Results demonstrate ablation of uterine Bteb1 expression with targeted deletion of the Bteb1 gene.

**Growth Phenotype of Bteb1-deficient Mice—Bteb1−/− mutants exhibited no obvious external phenotype and appeared to mature normally (23).** Because BTEB1 has been shown to influence proliferation of cells in vitro (38), the growth rates of WT and Bteb1−/− offspring were evaluated. For studies of early postnatal growth (postnatal day (PN) 4, PN7, and at weaning (PN21)), both male and female littermates were obtained from matings between WT males × WT females or between Bteb1−/− males × Bteb1−/− females as used. As shown in Fig. 2, inset, both WT and Bteb1−/− pups exhibited progressively increased body weights with age (PN4 < PN7 < PN21). Male and female pups of the same genotype did not differ (p > 0.05) in body weight at these ages (data not shown). However, Bteb1−/− pups lagged slightly, albeit significantly (p < 0.05), behind WT littermates in body weight. To determine whether the slower growth rates of Bteb1−/− pups persisted through early adulthood, age-matched WT, Bteb1+/−, and Bteb1−/− females were evaluated for changes in body weight from PN21 until PN140. Females of all three genotypes showed normal growth rates that did not differ significantly (Fig. 2). Thus, although Bteb1 deficiency appeared to negatively affect growth rate prior to weaning, this effect was lost during early adulthood, suggesting possible compensatory somatic growth at postweaning with loss of Bteb1 expression.

**Bteb1-deficient Females Are Subfertile—** In previous studies (26, 27), BTEB1 was demonstrated to function as a PR-interacting partner in the uterine endometrium in vitro; thus, the loss of Bteb1 expression in vivo could conceivably compromise a number of reproductive parameters, including pregnancy success. To assess the effect of uterine Bteb1 deficiency on litter size, WT, Bteb1+/−, and Bteb1−/− females between the ages of 6 weeks and 10 months were bred with males of all three genotypes, and the average litter size was determined for each breeding pair. As shown in Table II, Bteb1−/− females were subfertile, with an average of 4–5 pups/litter regardless of the male genotype. By contrast, WT and Bteb1+/− females had an average of 7–8 and 6 pups/litter, respectively. As observed for Bteb1−/− females, the litter sizes of WT and Bteb1−/− females were also unaffected by male genotype (Table II). These results indicate that decreased litter size is likely a maternal genotype effect and suggest that male fertility is not demonstrably compromised by loss of Bteb1 expression.

**Increased Incidence of Neonatal Lethality for Bteb1−/− Males—** Mortality in PN1 pups was observed with greater frequency for pups born from Bteb1−/− than from WT dams. In homozygous matings, WT pups (n = 20 litters) had 4.05 ± 2.64% incidence of neonatal deaths compared with 18.43 ± 7.41% for Bteb1−/− mutants (n = 22 litters). Moreover, Bteb1−/− pups (n = 15 litters) that were born of Bteb1−/− dams died at a frequency (22.67 ± 8.87%) greater than those born of WT dams (9.09 ± 9.09; n = 11 litters) and comparable with those of Bteb1-null mutants.

**Bteb1 Deficiency Does Not Alter Steroid Hormone Levels and Ovarian Capacity—** Serum steroid hormone levels were determined for WT and Bteb1−/− females at 5–6 and 8–10 weeks of age. Progesterone and estrogen levels were not significantly different (p > 0.1) between the two groups at both developmental ages (Fig. 3). Moreover, normal superovulation was observed in WT and Bteb1−/− mice, with comparable numbers of oocytes produced by both genotypes (WT: 41.86 ± 6.2; Bteb1−/−: 40.43 ± 5.3; n = 7 mice/genotype). Because Bteb1−/− mice also exhibited estrous cycle duration, pregnancy length, and ovarian morphology analogous to those of their WT littermates (data not shown), results suggest that the subfertility phenotype of Bteb1−/− females is a defect intrinsic to the uterus.

**Bteb1-deficient Mice Exhibit a Uterine Phenotype—** To determine whether alterations in uterine growth might underlie, in part, the subfertility phenotype of Bteb1−/− females, uteri from WT and Bteb1−/− littermates were evaluated for differences in tissue weight, morphology, and histologic appearance as a function of age. Age-matched WT and Bteb1−/− mice exhibited increased uterine weights with age (10 weeks > 6 weeks > 3 weeks) (p < 0.05), although across this developmental period
Fig. 2. Growth curves for mice with different Bteb1 genotypes. Body weight is presented as average (n = 20–25 female mice/ genotype) for each age beginning at postweaning (postnatal day 21). Inset, body weights of neonatal male and female pups (n = 20–30/genotype) prior to weaning. All data are presented as mean ± S.E. Asterisk denotes significant difference at p < 0.5.

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Average litter size for mouse breeding pairs

| Genotype | M/WT | M(+/-) | M(-/-) |
|----------|------|--------|--------|
| F(WT)    | 7.26 ± 0.40 | 7.16 ± 0.60 | 7.80 ± 0.51 |
| n = 14   | n = 6  | n = 10 |
| F(+/-)   | 6.40 ± 1.21 | 6.63 ± 0.33 | 6.00 ± 0.43 |
| n = 5    | n = 34 | n = 16 |
| F(-/-)   | 4.27 ± 0.66* | 4.32 ± 0.40* | 4.94 ± 0.77* |
| n = 16   | n = 25 | n = 16 |

The uteri of WT mice were heavier (p < 0.05) than those of Bteb1+/- (Fig. 4). Because body weight did not significantly differ between WT and Bteb1+/- females after 3 weeks of age (Fig. 2), these results suggest that the effect of Bteb1 on uterine weight is independent of somatic growth.

To determine the basis of uterine hypoplasia in homozygous Bteb1 mutant mice, we evaluated WT and Bteb1+/- uteri for diameter (cross-sectional area), luminal epithelium height, and number of endometrial glands. Although endometrial gland numbers in WT uteri did not differ from those in Bteb1+/- for all ages examined, luminal epithelial height was greater (p < 0.05) for WT than for Bteb1+/- at 6 weeks of age. On the other hand, uterine diameter was greater for WT than for Bteb1+/- mice at 3 (p < 0.1) and 6 (p < 0.05) weeks of age, although this difference was lost when the animals reached 10 weeks of age (Fig. 4). These findings suggest growth effects of Bteb1 on specific uterine compartments at particular developmental windows.

To characterize alterations in uterine gene expression between WT and Bteb1+/- females, we used QPCR to measure the relative mRNA abundance for a number of genes that we had previously demonstrated to be under BTEB1 regulation in endometrial cells (36–38) and that are implicated in cell growth (38–40). Uteri from WT and Bteb1+/- mice at 5–6 and 8–10 weeks of age were compared because the effect of Bteb1 ablation on uterine diameter and luminal epithelial height differed during these times (Fig. 4). At 5–6 weeks of age, Bteb1+/- mice had lower (p < 0.05) uterine transcript levels, relative to WT counterparts, for cyclin D1, Axl tyrosine kinase receptor, and secretory leukocyte protease inhibitor (S SPi) (Fig. 5). By contrast, the expression of Bteb3, a closely related Bteb1 family member (41), showed a tendency to increase (p = 0.1) in Bteb1+/- uteri. At 8–10 weeks of age, there was no difference in the uterine expression of these genes in WT and Bteb1+/- mice, the exception being Bteb3, whose uterine mRNA levels were higher (p < 0.05) in Bteb1+/- than WT. These results are consistent with a molecular basis for the uterine phenotype of adult Bteb1+/- females and suggest a compensatory role for Bteb3 in Bteb1-mediated uterine growth control.

Stromal Expression of Bteb1 in Peri-implantation Endometrium—To determine the site of Bteb1 action in the uterus during early pregnancy, the loss of which might underlie the subfertility condition observed in Bteb1+/- females, we evaluated the expression of Bteb1 in specific uterine endometrial cell types. Because currently available anti-BTEB1 antibodies, including ours, have so far given high nonspecific staining in immunohistochemical studies of mouse tissues (data not shown), we took advantage of the functional β-galactosidase gene that was inserted into exon 1 of the Bteb1 gene to determine endogenous sites of Bteb1 expression in the early pregnant uterus. A previous study (23) demonstrated that X-gal staining of various tissues from Bteb1+/- lacZ mice recapitulates the endogenous expression pattern of the Bteb1 gene. X-gal stained-cryosections of uterine tissues isolated from Bteb1+/- lacZ mice at 0.5, 3.5, and 6.5 dpc revealed the distinct cell type localization of this gene (Fig. 6). Bteb1 expression was predominantly observed in endometrial stromal cells with detectable but low and no expression, respectively, in glandular and luminal epithelial cells (Fig. 6, A–C). The smooth muscle cells of the myometrium also exhibited robust expression of Bteb1 (Fig. 6, D–E). In stromal cells, the pattern of expression was 3.5 dpc > 6.5 dpc > 0.5 dpc, with no expression detected in decidualized cells (Fig 6D). Uterine sections from WT mice at 0.5 and 3.5 dpc did not show any X-gal staining, demonstrating the specificity of the staining procedure (data not shown). Results indicate that Bteb1 expression in the mouse uterine endometrium during early pregnancy is predominant in stromal cells and temporally coincides with important events associated with PR-A-dependent stromal cell proliferation that occurs between 3.5 and 6.5 dpc (42, 43).

Mice Deficient in Bteb1 Have Reduced Numbers of Implantation Sites—Because Bteb1 expression is temporally and spa-
null mutants thus implies that one mechanism by which Bteb1 is involved in parallel, non-redundant pathways to regulate PR/P-mediated responses in vivo would lead to reduction in PR/P-mediated transactivation in the uterus endometrium.

DISCUSSION

Here we report that Bteb1-deficient female mice have compromised reproduction that is most likely because of pleiotropic effects of the null mutation on the uterus. Disruption of the Bteb1 gene resulted in uterine growth retardation, fewer numbers of embryo implantation sites, decreased litter size, and, for a number of uterine genes, partial or total resistance to P. By contrast, ovarian function was essentially unaltered with Bteb1 ablation, as manifested by comparable puberty onset, estrous cycle length, production levels of E and P, and ovulatory capacity of WT and Bteb1−/− females. Because loss of PR-A rather than PR-B underlies the gross reproductive failure in mice due to defects in P-dependent responses in the ovary and the uterus (33, 34), the observed uterine phenotype of Bteb1-null mutant females suggests the selective contribution of Bteb1 to PR-A action in this tissue. More importantly, these data demonstrate that Bteb1 is involved in parallel, non-redundant pathways with other steroid receptor co-activators in mediating uterine sensitivity to P.

As noted above, Bteb1-null females exhibited no obvious ovarian phenotype; hence, the observed smaller litter sizes of Bteb1−/− relative to WT are likely related to defects in uterine function. Indeed, the observations that: 1) decreased litter size is predominantly a maternal genotype effect; 2) heterozygous embryos exhibit higher implantation success in WT than in Bteb1−/− uteri; and 3) Bteb1−/− females have reduced uterine weights at early adult stages compared with WT counterparts, independent of body weight, are consistent with the uterus as a major target of Bteb1 action. In this regard, loss of Bteb1 was found to alter the molecular phenotypes of pregnant and non-pregnant uteri, with modifications related to the quantitative expression of cell cycle (cyclin D1, cyclin D3) and growth-associated (Slpi, Hoxa11) genes, all of whose expression are regulated by P. Of note is our demonstration of Axl tyrosine kinase receptor as a Bteb1-induced gene in vivo, consistent with our initial report on this gene in in vitro studies of human endometrial cells overexpressing BTEB1 (37). Recently, Axl expression was demonstrated in both the stromal and glandular epithelial cells of the uterine endometrium (40) and shown to be dramatically increased in endometriotic endometria (56), a pathological condition characterized by increased levels of PR-A relative to PR-B (57). Thus, Axl is a likely downstream target of BTEB1, alone and in concert with PR-A. Indeed, data reported here support the BTEB1- and P-regulated expression of Axl, although the PR isoform mediating this P regulation is unknown. The compromised reproductive phenotype of Bteb1-null mutants thus implies that one mechanism by which Bteb1 deficiency likely impairs PR function is through the absence of

![Graph showing serum estrogen (E) and progesterone (P) concentrations in WT and Bteb1−/− females](image)

**FIG. 3.** Serum estrogen (E) and progesterone (P) concentrations in WT and Bteb1−/− females. The values are presented as mean ± S.E. The numbers of animals used for each assay are: Estrogen: WT (5–6 weeks, n = 7; 8–10 weeks, n = 17), (−/−) (5–6 weeks, n = 5; 8–10 weeks, n = 6); Progesterone: WT (5–6 weeks, n = 5; 8–10 weeks, n = 10), (−/−) (5–6 weeks, n = 7; 8–10 weeks, n = 8). No significant differences in serum levels were found for either hormone as a function of Bteb1 genotype.

Potentially coincident with decidual formation, the numbers of embryos in uteri of WT and Bteb1−/− females at 6.5 dpc were determined. WT females were time-mated with homozygous Bteb1−/− males, and conversely, Bteb1−/− females were time-mated with WT males to generate heterozygote embryos in a WT or Bteb1-null uterine environment. This mating strategy precluded genotype-associated embryo death, if any, as a contributory factor to implantation differences. WT females had an average of 8.00 ± 0.44 embryos (n = 7 dams) compared with 5.56 ± 0.32 embryos (n = 9 dams) for Bteb1−/− (difference at p < 0.01). The number of embryos obtained from WT females crossed with Bteb1−/− males was comparable with the number of newborn pups from WT females mated to Bteb1−/− males (8.00 ± 0.44 embryos versus 7.80 ± 0.51 offspring; p = 0.78) (Table II). By contrast, the number of embryos obtained from mating Bteb1−/− females with WT males tended to be greater by one from the number of pups born from such mating (5.56 ± 0.32 embryos versus 4.27 ± 0.66 offspring; p = 0.10) (Table II). These results suggest that the subfertility phenotype of Bteb1−/− females is largely because of reduced numbers of implanting embryos.

To examine whether alterations in expression of specific uterine genes at 6.5 dpc accompany the reduced number of implantation sites noted in Bteb1−/− mice, uteri from WT and Bteb1−/− mice at 6.5 dpc were evaluated by QPCR for expression of progesterone-responsive, Bteb1-regulated, and/or implantation-specific genes (37, 38, 44, 45) (Fig. 7). WT and Bteb1−/− uteri had comparable levels of mRNA transcripts for calcitonin and Hoxa11. By contrast, the expression of cyclin D3 (p = 0.1) and Hoxa10 (p < 0.01) was diminished in Bteb1−/− relative to WT uteri, whereas that of Bteb3 was higher (p < 0.05) in Bteb1−/− than in WT uteri. These data indicate that loss of Bteb1 alters the normal pattern of expression of two important implantation-associated genes, namely Hoxa10 and cyclin D3 in the uterus (46–48).

Loss of Bteb1 Alters Progesterone Responsiveness of Uterine Genes—In previous in vitro studies, we observed that BTEB1 enhances the P sensitivity of uterine endometrial epithelial genes to PR-mediated transactivation (26). We, therefore, hypothesized that the loss of uterine Bteb1 during early pregnancy would lead to reduction in PR/P-mediated responses in vivo, resulting in compromised PR signaling requisite for early pregnancy events. To test this, we examined the responsive-
its normal co-regulatory role in the PR transactivation of P-sensitive growth-associated genes.

To evaluate whether Bteb1 selectively mediates the expression of genes regulated by P, we analyzed the P sensitivity of uterine genes in the background of complete (homozygous) or partial (heterozygous) loss of Bteb1 expression and compared these to that of WT mice. Results demonstrate that P sensitivity of Hoxa10 and PR gene expression is highly dependent on Bteb1, with the loss of one Bteb1 allele sufficient to cause total resistance to P regulation. By contrast, P induction of Slpi as well as of Axl genes is Bteb1-independent, with P exhibiting a greater inductive effect (–6-fold) for Slpi than for Axl. Our present studies do not address the molecular mechanism(s) responsible for the gene selectivity of P induction modulated by Bteb1; however, we speculate that this may be related to the specific cellular sites of synthesis of these genes and the PR isoform predominantly expressed in these cells. In this regard, Slpi is synthesized exclusively in glandular epithelial cells (58); by contrast, PR, Hoxa10, and Axl are expressed by both endometrial epithelium and stroma (31, 59) and show P-induced expression in stromal cells (40, 60, 61). Given that PR-A is considered to be expressed at higher levels than PR-B in uterine stroma (62), the present findings provide support to our hypothesis (27) that the PR isoform with which Bteb1 interacts determines the subsequent transcripational responses. Our inability to detect any major alterations in the expression of Bteb1, Hoxa11, and cyclin D3 with P, despite previous demonstration of their P responsiveness, may be because of the dose and duration of P exposure used in this study.

Previous in vitro studies, which utilized human endometrial epithelial cells, showed that BTEB1 preferentially interacts with agonist-bound PR-B to increase P responsiveness of target genes (26, 27). Although interaction of BTEB1 with the PR-A isoform was also noted, this occurred independent of ligand; moreover, BTEB1 inhibited the transactivation function of the PR-A/PR-B heterodimer in these cells (27). To determine the cell type-specific transactivity of uterine Bteb1 during early pregnancy, we examined the spatial and temporal expression of the Bteb1 gene, using the LacZ gene product as a reporter of endogenous Bteb1 promoter activity. Surprisingly, Bteb1 showed predominant endometrial expression in stromal cells, where it correlated temporally with the initial events leading to the formation of the decidua. Decidualization in the mouse occurs between 3.5 and 6.5 dpc and involves the proliferation and subsequent differentiation of stromal cells, processes that are regulated by P (63, 64) through PR-A (33). Because decidual Bteb1 expression at 6.5 dpc was not similarly observed, Bteb1 is likely not involved in the maintenance of decidual cell growth. Our analysis of uterine genes whose expression was affected by Bteb1-null mutation at 6.5 dpc provided further support to a role for Bteb1 in initial events leading to the decidual response. Importantly, the relative abundance of the mRNAs for cyclin D3 and Hoxa10, both of which are normally expressed by stromal cells and known to be intimately involved in P-dependent stromal cell proliferation (46–48), were reduced in Bteb1-null uteri. By contrast, the mRNA abundance for calcitonin, which is localized exclusively to luminal epithelial cells (45) where Bteb1 expression was essentially undetected, was not altered by Bteb1 ablation. Because up-regulation of cyclin D3 and Hoxa10 genes at the implantation site is tightly associated with decidualization (47, 52) and the level of Hoxa10 expression in the endometrium is directly associated with litter size in mice (65), our findings of reduced implantation sites in Bteb1-null uteri, independent of embryo genotype,
suggest that Bteb1 in partnership with ligand-bound PR-A likely regulates the stromal expression of these genes. We cannot infer from the present studies the mechanism(s) by which Bteb1 and PR-A interact to induce cyclin D3 and Hoxa10 gene expression. However, further analysis of the promoter regions of these genes, coupled with chromatin immunoprecipitation (66) to evaluate promoter co-occupancy by BTEB1 and PR-A, may provide important insights in this regard.
The uterine hypoplasia seen in Bteb1-null mutants appears to be because of diminished uterine stroma, a major feature of Hoxa11 mutant uteri (67). Because Hoxa11 gene expression did not differ in WT and Bteb1-null uteri at 6.5 dpc, indicating lack of Bteb1 regulation of Hoxa11 gene expression, the mechanism underlying the reduction in uterine size for Bteb1 and Hoxa11 mutants likely involves distinct regulatory mediators. Consistent with this, Bteb1-null mutation significantly reduced Hoxa10 gene expression (this study), which was not altered in Hoxa11-null mutants (67).

Our findings that the closely related Bteb1 family member, Bteb3 (also known as Klf13/Fkl2), showed increased expression in uteri of adult (8–10 weeks) non-pregnant and of early pregnant (6.5 dpc) Bteb1−/− females, relative to uteri of corresponding WT, suggest that Bteb3 and Bteb1 may have overlapping and compensatory functions in the uterine stroma. Indeed, the induction of Bteb3 gene expression in Bteb1-null uteri may be responsible in part, be because of lactational insufficiency. PR-B-null mutants are characterized by impaired mammary gland morphogenesis (34); thus, one can surmise that because Bteb1 can functionally interact with PR-B to enhance P responsiveness (27), loss of such interactions can compromise the mammary ductal branching and lobulo-alveolar development requisite for milk production. Interestingly, we observed significant, albeit small, differences in body weights between WT and Bteb1−/− pups prior to weaning; this difference was lost postweaning, suggesting “catch-up growth” for Bteb1−/− pups once they are able to self-feed. Although at the present time we can only speculate on the basis for increased early neonatal death in pups born from Bteb1−/− dams, elucidation of this interesting phenotype may unravel additional functions for Bteb1.

In conclusion, we have demonstrated that Bteb1 is a functionally relevant PR-interacting protein. The subfertility noted for Bteb1-null mutants is associated with altered molecular processes in the uterine stroma that are known to be regulated by the PR-A isoform. Future studies will determine the precise modes of functional interaction between PR-A and Bteb1 and the involvement of Bteb1 in gynecological conditions that result from inappropriate progesterin action in the uterus.

Acknowledgments—We thank Frank J. Michel and Kwonho Hong for technical assistance.

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J. Biol. Chem. 2004, 279:29286-29294. doi: 10.1074/jbc.M403139200 originally published online April 26, 2004

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