Centromere protein b-null mice display decreasing reproductive performance through successive generations of breeding due to diminishing endometrial glands

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Abstract

Centromere protein B is a highly conserved constitutive protein found at centromeres. Gene knockout studies in mice have unexpectedly identified *Cenpb* as a candidate gene involved in uterine function. The present study further explores the role of *Cenpb* in mice by intermating *Cenpb*-null mice over several generations. Breeding studies and analysis of uterine tissue indicate that *Cenpb*-null mice lose reproductive fitness over a number of generations due to a significant reduction in endometrial glands. These results suggest that *Cenpb* may play an important function in the short- and long-term maintenance of uterine integrity.

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Introduction

The mammalian centromere is the primary constriction in the dividing chromosome and is responsible for the accurate segregation of chromosomes during meiosis and mitosis. The centromere is made up of repetitive DNA enveloped by a specialised proteinaceous structure known as the kinetochore (Maney *et al.* 1999). Human centromere protein B (CENP-B) has been identified as an extraordinarily conserved 80 kDa protein, which is distributed throughout the centromeric heterochromatin beneath the inner plate of the kinetochore throughout the cell cycle (Earnshaw *et al.* 1987, Cooke *et al.* 1990, Sullivan & Glass 1991, Maney *et al.* 1999, Saffery *et al.* 1999). Sequence analysis has shown that the N-terminus of CENP-B encodes a DNA-binding domain whereas the C-terminus of CENP-B houses the CENP-B dimerisation domain (Maney *et al.* 1999). CENP-B binds to α -satellite DNA in man or minor satellite in mouse via a 17 bp sequence known as the CENP-B box motif. These dual properties of CENP-B suggest that this protein organises the assembly of repetitive DNA at the primary constriction, facilitating centromere/kinetochore formation (Yoda *et al.* 1992).

The role of CENP-B in centromere biology has been controversial for many years, early studies in cell culture

suggesting that it is an indispensable protein (Bernat *et al.* 1990, 1991). Recent studies in fission yeast support this and suggest that two to three CENP-B homologues are required to establish a functional centromere (Baum & Clarke 2000, Irelan *et al.* 2001). On the other hand, there are many independent lines of evidence to show that CENP-B is expendable for mammalian centromere formation (Earnshaw *et al.* 1989, Sunkel & Coelho 1995, Goldberg *et al.* 1996, Choo 1998). While gene knockout experiments in mice have indicated that *Cenpb* is non-essential for mitosis and meiosis (Hudson *et al.* 1998, Kapoor *et al.* 1998, Perez-Castro *et al.* 1998, Tomascik-Cheeseman *et al.* 2002), it has been shown to be crucial for proper uterine development and function (Fowler *et al.* 2000).

Cenpb-null mice generated from early-generation heterozygous intercrosses on three different genetic backgrounds, known as R1, W9.5 and C57, displayed a marked reduction in uterine weight at 10 weeks old and demonstrated an age-dependent reproductive dysfunction that was more severe in the C57BL/6 background. Breeding studies with 8–15-week-old C57 *Cenpb*-null female mice resulted in slow or difficult delivery of pups, whereas the R1 and W9.5 *Cenpb*-null females bred normally for the first three to four litters before developing pregnancy problems by the age of 9 months. Many overdue females

sickened, and on autopsy dead intact or resorbing foeti and pyometra were found (Fowler *et al.* 2000).

When *Cenpb*-null early-generation mice were compared with age-matched wildtype female mice, no difference in ovarian tissues, progesterone and β -oestradiol levels or embryo number was detected. However, histological analysis of uterine tissue from 10-week-old C57 and 6–9-month-old R1 *Cenpb*-null females revealed abnormal luminal and glandular epithelium, fewer endometrial glands, and increased leucocyte infiltration, haemorrhage and infection. Furthermore, *in situ* hybridisation using a specific *Cenpb*-antisense probe detected high levels of *Cenpb* expression in the epithelial lining of the uterine lumen and endometrial glands of normal mice (Fowler *et al.* 2000). In this study we further explore the unexpected role of *Cenpb* in mouse uterine development and function. Our specific objective was to determine whether interbreeding *Cenpb*-null mice over successive generations would result in the exacerbation of the *Cenpb*-null phenotype, and, if so, what the underlying causes might be.

Materials and Methods

Generation of *Cenpb*-null mice on R1, W9.5 and C57 backgrounds

First-generation (G1) wildtype ($+/+$), null ($-/-$) and heterozygous ($+/-$) mice from heterozygous intercrosses were maintained on two different mixed 129 $\times$ C57BL/6 genetic backgrounds known as R1 and W9.5 (Fowler *et al.* 2000). Mice were bred as independent colonies, that is, $-/- \times -/-$, $+/+ \times +/+$ and $+/- \times +/-$, for a further three generations (G2, G3 and G4). Due to known breeding difficulties with *Cenpb*-null females on a C57BL/6 background (C57), C57 $^{-/-}$, C57 $^{+/-}$ and C57 $^{+/+}$ G1 mice were generated from heterozygous intercrosses (Fowler *et al.* 2000). The majority of mice used in this study were from cousin $\times$ cousin matings. All mice were genotyped by PCR (Fowler *et al.* 2000). Mice were housed in standard shoebox cages and allowed Barastoc feeder cubes and water *ad libitum*.

Ethics of experimentation

This study was conducted in accordance with Australian code of practice for the care and use of animals for scientific purposes and under approval of the Royal Children's Hospital Animal Ethics Committee (project numbers A388 and A450).

Examination of mice for chronic infectious agents

Serum samples from breeding colonies of mice were sent to the Murine Virus Monitoring Service, South Australia, for routine health testing every 6 months over the past 3 years. The sample size was statistically representative of mice in the colony and showed an absence of mouse

hepatitis virus, rotavirus and parvovirus. In addition, the Department of Microbiology and Infectious Diseases, Royal Children's Hospital and Cerberus Sciences, South Australia, performed supplementary testing for known mouse uterine pathogens. Serum and uterine swabs were collected from 18-week-old G2 *Cenpb*-wildtype ($n = 2$), -null ($n = 2$) and -heterozygote ($n = 4$) virgin female mice and 10–11-week-old G3 *Cenpb*-wildtype ($n = 2$) and -null ($n = 2$) virgin female mice. Mice tested negative for minute virus of mice, mouse parvovirus, *Mycoplasma pulmonis*, Sendai virus, *Chlamydia*, *Pasteurella pneumotropica* and *Salmonella enteritidis*. No other bacterial pathogen of consequence was detected.

Genomic scan of *Cenpb*-null mice on C57BL/6 background

Tail DNAs from *Cenpb*-heterozygous mice on a C57BL/6 background (C57 $^{+/-}$) (Fowler *et al.* 2000) were sent to the Jackson Laboratory's Speed Congenic Service (Bar Harbor, ME, USA). Simple sequence length polymorphisms (SSLP) analysis using 108 markers that are polymorphic between C57 and 129 DNAs were used to determine the amount of residual 129 DNA surrounding the targeted *Cenpb* locus.

Monitoring of mice for reproductive performance

Breeding pairs were set up and females were examined for vaginal plugs, pregnancy and number of pups born by routine mouse husbandry procedures (Fowler *et al.* 2000).

Organ weighing, histology and immunohistochemical analysis of endometrial gland cells

Organ weights (mg) were determined by weighing wet uteri and ovaries (Fowler *et al.* 2000). After weighing, mouse uteri were formalin-fixed, and a 2–3 mm piece of tissue was dissected from the mid-region of randomly selected left or right uterine horns and paraffin-embedded, and 5 μ m transverse sections were cut onto silanised slides and deparaffinised. Following antigen retrieval using DAKO Target Retrieval Solution, pH6 (S1699; DAKO, Carpinteria, CA, USA), in a water bath for 20 min at 95–99°C, the slides were cooled for 20 min and equilibrated in Tris-buffered saline with Tween 20. After endogenous peroxidase activity was quenched using peroxidase blocking reagent (DAKO S2001) for 5 min, polyclonal anti-progesterone receptor antibody (DAKO A0098), monoclonal anti-oestrogen receptor antibody (DAKO M7047) and anti-pan cytokeratin antibody (DAKO M3515) (Moll *et al.* 1982) were used at room temperature at dilutions of 1/50, 1/50 and 1/200 respectively.

Monoclonal antibodies were stained according to manufacturer directions using the DAKO Animal Research Kit (K3954), which uses a modified avidin-biotin/peroxidase technique. Polyclonal antibody staining was carried out using the DAKO EnVision + /HRP (K4003), which uses an enzyme-labelled polymer technique. Immunolabelled

cells were visualised with 3,3'-diaminobenzidine (K3468, DAKO) for 5 min and counterstained with Mayer's haematoxylin. All staining was performed using a DAKO Auto-stainer.

Assessment of endometrial gland density

To assess the number of glands in the uterine endometrial layer 2, haematoxylin and eosin-stained full-face transverse 5 µm sections from the mid-region of randomly selected left or right uterine horns were examined microscopically. All visible glands were counted regardless of size, and the average was recorded for each section.

Photomicrographs

Images of stained sections were captured using an Olympus BX50 microscope equipped with a 10× eyepiece and 40× objective, and a CCD camera linked to an IBM computer with a TCI-pro program. Digital images were assembled with PhotoShop, Version 6.0.

Statistical analyses

Values are presented as average ± s.d. *P* values were calculated using Student's *t*-test. *P* values of less than 0.05 were regarded as significant.

Ovarian transplantation studies

Mice were anaesthetised with Avertin (tribromoethanol and amyl alcohol) (Hogan *et al.* 1994), and both ovaries were removed and replaced with one-half ovary per side, as described by Szein *et al.* (1998). Mice were set up as breeding pairs with C57BL/6 males 10 days after surgery and monitored daily.

Results

Declining reproductive performance of G2, G3 and G4 *Cenpb*-null female mice

The reproductive performance of *Cenpb*-wildtype (+/+), -heterozygote (+/-) and -null (-/-) breeding pairs were

observed over four generations of successive matings of genotype-specific intercrosses on R1 and W9.5 genetic backgrounds. R1^{-/-} mice produced significantly fewer pups at generations 2, 3 and 4 (G2, G3 and G4) than R1^{+/+} and R1^{+/-} breeding pairs (Table 1A) due to fewer litters being born (Table 1B). Comparison between R1^{+/+} and R1^{+/-} breeding pairs showed no significant difference except for G3 intercrosses, where less pups and litters were produced by the R1^{+/+} pairs than the R1^{+/-} breeding pairs (Table 1A and B). A similar breeding record was observed for the W9.5^{-/-} pairs when compared with W9.5^{+/+} matings (Table 2A and B). In addition, when reproductive performance was compared between G1 breeding pairs and G2, G3 and G4 breeding pairs, a general trend towards deteriorating reproductive outcome through successive generations in terms of numbers of pups and litters was observed, with the difference being particularly significant when G1 and G4 W9.5^{-/-} mice were compared (*P* ≤ 0.005).

To determine whether the reduction in breeding performance of *Cenpb*-null mice was due to a problem in the males, females or both sexes, male and female G4 mice from R1^{+/+} and R1^{-/-} breeding pairs were mated to random-bred Swiss mice for 15 months. When compared with R1^{+/+} G4 male mice, the R1^{-/-} G4 males averaged a like number of total pups (*P* = 0.959; Fig. 1A), litters (*P* = 1.0; Fig. 1B) and pups per litter (*P* = 0.890; Fig. 1C). However, the R1^{-/-} G4 females, when examined against the R1^{+/+} G4 females, produced significantly fewer pups (*P* < 0.001; Fig. 1A), litters (*P* = 0.005; Fig. 1B) and pups per litter (*P* = 0.038; Fig. 1C).

G4 *Cenpb*-null mice show a similar reduction in uterus weight compared with *Cenpb*-null G1 mice from original heterozygous crosses

To further investigate the observed decrease in reproductive performance in G4 R1^{-/-} and W9.5^{-/-} females, reproductive tissues from 10-week-old G4 mice (day 0.5 vaginal plug (VP)) were examined. The tissues included uterus, ovary and total number of embryos flushed from oviducts. Although 10-week-old R1^{-/-} and W9.5^{-/-} G4

Table 1 Declining reproductive performance of R1 *Cenpb*-deficient mice compared to wildtype and heterozygote breeding pairs.

Generation number	Average number ± s.d.			<i>P</i>		
	+/+ × +/+	+/- × +/-	-/- × -/-	+/+ vs +/-	+/+ vs -/-	+/- vs -/-
A. Average number of pups per breeding pair						
1	50.6 ± 36.0 (<i>n</i> = 4)	39.5 ± 19.1 (<i>n</i> = 2)	13.3 ± 9.5 (<i>n</i> = 3)	0.711	0.146	0.123
2	62.6 ± 13.0 (<i>n</i> = 7)	36.0 ± 18.4 (<i>n</i> = 3)	7.0 ± 4.7 (<i>n</i> = 6)	0.115	0.001	0.002
3	50.7 ± 20.8 (<i>n</i> = 5)	87.3 ± 12.5 (<i>n</i> = 3)	6.0 ± 6.5 (<i>n</i> = 6)	0.025	0.001	<0.001
4	73.7 ± 19.2 (<i>n</i> = 3)	68.5 ± 29.2 (<i>n</i> = 4)	3.0 ± 3.5 (<i>n</i> = 5)	0.752	<0.001	0.001
B. Average number of litters per breeding pair						
1	6.8 ± 4.3 (<i>n</i> = 4)	6.0 ± 2.8 (<i>n</i> = 2)	3.7 ± 1.2 (<i>n</i> = 3)	0.840	0.294	0.268
2	9.1 ± 1.2 (<i>n</i> = 7)	5.5 ± 3.5 (<i>n</i> = 3)	1.7 ± 1.0 (<i>n</i> = 6)	0.283	<0.001	0.035
3	6.2 ± 2.7 (<i>n</i> = 5)	11.3 ± 1.5 (<i>n</i> = 3)	1.2 ± 1.0 (<i>n</i> = 6)	0.025	0.017	<0.001
4	9.3 ± 2.1 (<i>n</i> = 3)	10.0 ± 3.5 (<i>n</i> = 4)	0.8 ± 0.8 (<i>n</i> = 5)	0.983	<0.001	<0.001

Mice were bred for approximately 12 months. *n* = number of breeding pairs in study.

Table 2 Reduced reproductive performance of W9.5^{-/-} mice compared to wildtype breeding pairs.

Generation number	Average number± s.d.		<i>P</i> (+/+ vs -/-)
	+/+ × +/+	-/- × -/-	
A. Average number of pups per breeding pair			
1	33.0±7.2 (<i>n</i> = 3)	10.0±1.7 (<i>n</i> = 3)	0.006
2	52.3±13.6 (<i>n</i> = 3)	6.3±2.5 (<i>n</i> = 4)	0.001
3	65.3±16.9 (<i>n</i> = 6)	4.4±3.2 (<i>n</i> = 5)	<0.001
4	59.5±14.5 (<i>n</i> = 6)	2.2±1.9 (<i>n</i> = 5)	<0.001
B. Average number of litters per breeding pair			
1	6.3±3.5 (<i>n</i> = 3)	2.3±0.6 (<i>n</i> = 3)	0.123
2	9.0±1.0 (<i>n</i> = 3)	1.5±1.0 (<i>n</i> = 4)	<0.001
3	7.7±1.6 (<i>n</i> = 6)	1.4±0.6 (<i>n</i> = 5)	<0.001
4	9.2±1.8 (<i>n</i> = 6)	0.8±0.5 (<i>n</i> = 5)	<0.001

Mice were bred for approximately 12 months.

Figure 1 Reproductive ability of 6–10-week-old G4 R1^{+/+} male (n = 4), R1^{-/-} male (n = 5), G4 R1^{+/+} female (n = 6), R1^{-/-} female (n = 3) mice when mated to random-bred Swiss mice for up to 15 months. (A) Average number of total pups generated per mouse ± s.d. (B) Average number of litters born per mouse ± s.d. (C) Average number of pups per litter ± s.d. Error bars represent s.d.

uteri were 59.0% and 37.0% smaller than R1^{+/+} and W9.5^{+/+} G4 uteri respectively (Table 3A), they were not smaller than 10-week-old R1^{-/-} and W9.5^{-/-} G1 uteri from the original heterozygous crosses (R1^{-/-} G1: 79.7 ± 27.8 mg (n = 7) vs R1^{-/-} G4: 55.9 mg (n = 1), P = ND; W9.5^{-/-} G1: 82.5 ± 19.0 mg (n = 5) vs W9.5^{-/-} G4: 70.8 ± 17.2 (n = 4), P = 0.376) (Fowler *et al.* 2000), suggesting that breeding through successive generations did not have an effect on the size of the uterus in the null mice.

Reduced number of endometrial glands in G4 *Cenpb*-null mice

Routine haematoxylin and eosin examination of full-face transverse sections of uterus from 10-week-old W9.5^{-/-} (n = 4) and R1^{-/-} G4 mice (n = 1; day 0.5 VP) from *Cenpb*-null intercrosses demonstrated no gross abnormality in the uterine epithelium but a significant decrease in the number of endometrial glands when compared with W9.5^{+/+} (n = 5) and R1^{+/+} G4 mice (n = 6) from wildtype intercrosses (Fig. 2A and B; Table 4A and C).

Table 3 Uterus, ovary weights and number of embryos of 10-week-old homozygous G4 mice (day 0.5 VP) from *Cenpb*-wildtype or-null intercrosses.

Genetic Background	Average weight± s.d. (mg)		Percent reduction	<i>P</i> (+/ + vs - / -)
	+/+	-/-		
A. Uterus				
R1	137.5± 64.1 (<i>n</i> = 6)	55.9 (<i>n</i> = 1)	59.0%	ND
W9.5	112.6± 13.3 (<i>n</i> = 5)	70.8± 17.2 (<i>n</i> = 4)	37.0%	0.006
B. Ovary				
R1	14.1± 4.3 (<i>n</i> = 6)	8.0 (<i>n</i> = 1)	43.0%	ND
W9.5	12.4± 2.2 (<i>n</i> = 5)	12.2± 1.9 (<i>n</i> = 4)	2.0%	0.870
C. Number of embryos				
R1	9.7± 1.1 (<i>n</i> = 6)	8.0 (<i>n</i> = 1)	18.0%	ND
W9.5	9.0± 1.9 (<i>n</i> = 5)	7.3± 2.6 (<i>n</i> = 4)	19.0%	0.280

ND, not determined.

Figure 2 Haematoxylin and eosin analysis of uterus sections of wildtype and knockout 10- and 24-week-old G4 mice (day 0.5 VP) from homozygous and heterozygous breeding programmes. (A) W9.5^{+/+} 10-week-old uterus from homozygous breeding showing normal luminal epithelium and endometrial glands. (B) W9.5^{-/-} 10-week-old uterus from homozygous breeding showing normal luminal epithelium but a decrease in endometrial glands. (C) W9.5^{+/+} and (D) W9.5^{-/-} 10-week-old uterus from heterozygous breeding showing normal luminal epithelium and endometrial glands. (E) R1^{+/+} 24-week-old uterus from homozygous breeding showing normal luminal epithelium and an increase in endometrial glands. (F) R1^{-/-} 24-week-old uterus from homozygous breeding showing poor luminal epithelium and a decrease in endometrial glands. (G) R1^{+/+} 24-week-old uterus from heterozygous breeding showing normal luminal epithelium and endometrial glands. (H) R1^{-/-} 24-week-old uterus from heterozygous breeding showing ulcerated luminal epithelium and a decrease in endometrial glands. Arrows mark endometrial glands; (Ep) marks epithelium; (L) marks lumen; (U) marks ulcerated epithelium. Scale bar in panel H (50 μ m) applies to all panels.

Table 4 Average number of endometrial glands in *Cenpb*-null and -wildtype G4 mice from homozygous and heterozygous crosses.

Parentage	Age (weeks)	Average number of endometrial glands $\pm$ s.d. per transverse section		P
		W9.5 ^{+/+}	W9.5 ^{-/-}	
A. homozygous	10	65.2 $\pm$ 24.5 (n = 5)	20.5 $\pm$ 9.5 (n = 4)	0.011
B. heterozygous	10	28.7 $\pm$ 11.9 (n = 3)	16.7 $\pm$ 9.7 (n = 3)	0.248
		R1 ^{+/+}	R1 ^{-/-}	
C. homozygous	10	24.0 $\pm$ 8.6 (n = 6)	6 $\pm$ 0 (n = 1)	ND
D. heterozygous	10	39.0 $\pm$ 21.8 (n = 4)	21.0 $\pm$ 8.5 (n = 4)	0.175
E. homozygous	24	108.5 $\pm$ 36.1 (n = 6)	8.0 $\pm$ 0.0 (n = 2)	0.010
F. homozygous	32–36	113.0 $\pm$ 64.1 (n = 3)	2.5 $\pm$ 3.7 (n = 4)	0.016
G. heterozygous	24	76.3 $\pm$ 25.9 (n = 4)	7.6 $\pm$ 8.7 (n = 5)	<0.001
H. heterozygous	32–36	82.8 $\pm$ 44.7 (n = 4)	8.8 $\pm$ 5.9 (n = 5)	0.007

ND, not determined. Uteri were collected at day 0.5 of vaginal plug except uteri from 32–36-week-old mice were sampled from non-mated female mice.

In contrast, 10-week-old W9.5^{-/-} G4 mice (*n* = 3) and R1^{-/-} G4 mice (*n* = 4) of the equivalent generation derived from heterozygous breeding pairs did not display a significant reduction in the number of endometrial glands when compared with littermate W9.5^{+/+} (*n* = 3) and R1^{+/+} (*n* = 4) G4 mice respectively (Fig. 2C and D; Table 4B and D).

Next we examined haematoxylin and eosin sections from 24-week-old and 32–36-week-old G4 R1^{-/-} and compared them to G4 R1^{+/+} age-matched controls from both homozygous and heterozygous breeding programmes. R1^{-/-} mice generated by either breeding strategy had significantly reduced numbers of endometrial glands (Table 4E–H). When 24- and 32–36-week-old G4 R1^{-/-} from heterozygous breeding (Table 4G and H) were compared with 10-week-old G4 R1^{-/-} (Table 4D), the number of endometrial glands had declined with ageing (10 vs 24 weeks, *P* = 0.053; 10 vs 32 weeks, *P* = 0.037). On the other hand, the homozygous breeding regime resulted in R1^{-/-} G4 mice with a low number of endometrial glands at 10 weeks old that remained low with age (Table 4C, E and F). However, a reverse trend was observed in the wildtype R1 mice from the homozygous breeding strategy, in which the number of endometrial glands actually increased with age (Table 4C, E and F; 10 vs 24 weeks, *P* < 0.001; 10 vs 32 weeks, *P* = 0.009). Although the wildtype mice from the R1^{+/+} heterozygous breeding programmes had increased their gland number with age, it was not significant (Table 4D, G and H; 10 vs 24 weeks, *P* = 0.070; 10 vs 32 weeks, *P* = 0.129).

Abnormal luminal epithelium in uteri of 24-week-old R1^{-/-} G4 mice

Besides a reduction in endometrial gland number, uteri from 24-week-old R1^{-/-} G4 mice from heterozygous or homozygous breeding programmes (*n* = 7 total) were observed to have ulcerated (*n* = 2) or disorganised (*n* = 1) luminal epithelium or oedema in the endometrium (*n* = 1, Fig. 2F and H), whereas no obvious abnormality was noted in the uteri of control R1^{+/+} mice (*n* = 10 total; Fig. 2E and G). Given the similarity of these observations to those seen in our previous study, we re-examined the haematoxylin and eosin slides of uteri of 24-week-old G1 mice from the original heterozygous crosses (Fowler *et al.* 2000). Counting of endometrial glands revealed a reduction in the average number of glands in R1^{-/-} G1 mice (15.8 ± 10.7; *n* = 5) when compared with R1^{+/+} G1 mice (50.1 ± 35.1; *n* = 7), but the difference was not statistically significant (*P* = 0.080).

Greatly reduced number of endometrial glands in 3-, 4-, 6-, 8-, 10- and 24-week-old C57^{-/-} G1 mice

The observed reduction in the number of endometrial glands of R1^{-/-} and W9.5^{-/-} G4 mice and the more severe breeding problems of *Cenpb*-null and -wildtype

mice on the C57BL/6 background (Fowler *et al.* 2000) prompted us to examine uterine sections of C57^{-/-} and C57^{+/+} mice at 3, 4, 6, 8, 10 and 24 weeks of age at G1. C57^{-/-} females were found to have a significant reduction in gland number when compared with age-matched C57^{+/+} (Table 5A–F), suggesting that the endometrial glands failed to develop properly during the pre- and post-pubertal period on this genetic background.

Birthing problems and reduced number of endometrial glands in 24-week-old C57^{+/+} G1 mice

During routine maintenance of the C57^{+/+} breeding colony, it was observed that some of the C57^{+/+} breeding females developed birthing problems as they approached 24 weeks old. This was reminiscent of the phenotype formerly observed with 8–15-week-old C57^{-/-} breeding females (Fowler *et al.* 2000). In the present study, 10 previously non-mated, 24-week-old C57^{+/+} and C57^{+/+} females were set up with fertile stud males. Of the C57^{+/+} females, 70% developed birthing problems and 10% developed pyometra, as compared with only one C57^{+/+} female developing a birthing problem. Histological analysis of uteri of previously non-mated, 24-week-old mice (day 0.5 VP) revealed a significant reduction (*P* < 0.001) in endometrial gland number in C57^{+/+} mice (45.2 ± 16.7; *n* = 7) compared with C57^{+/+} mice (83.6 ± 3.0; *n* = 5). In contrast, 10-week-old C57^{+/+} mice that breed normally had no decrease (*P* = 0.713) in their endometrial gland number (22.3 ± 2.4; *n* = 4) when compared with C57^{+/+} mice (22.6 ± 11.2; *n* = 5).

Table 5 Average numbers of endometrial glands in C57^{+/+}, C57^{+/+} and C57^{-/-} mouse uteri at different ages.

Genotype	Number of mice	Average number of endometrial glands $\pm$ s.d. per transverse section	<i>P</i> (+/+ vs -/-)
A. 3-week-old			
C57 ^{+/+}	7	5.6 $\pm$ 1.6	0.001
C57 ^{-/-}	4	0.5 $\pm$ 0.5	
B. 4-week-old			
C57 ^{+/+}	3	8.7 $\pm$ 3.1	0.051
C57 ^{-/-}	4	4.0 $\pm$ 1.8	
C. 6-week-old			
C57 ^{+/+}	2	12.0 $\pm$ 2.8	0.042
C57 ^{-/-}	3	2.7 $\pm$ 3.1	
D. 8-week-old			
C57 ^{+/+}	4	22.8 $\pm$ 5.4	0.006
C57 ^{-/-}	4	8.5 $\pm$ 4.4	
D. 10-week-old			
C57 ^{+/+}	5	22.6 $\pm$ 11.2	0.039
C57 ^{-/-}	4	7.8 $\pm$ 3.3	
F. 24 week-old			
C57 ^{+/+}	5	83.6 $\pm$ 3.0	<0.001
C57 ^{-/-}	2	5.0 $\pm$ 0	

Female mice from heterozygote crosses on a C57BL/6 background were used. Uteri were collected at day 0.5 of vaginal plug except for prepubertal uteri from 3- and 4- week-old non-mated C57 females.

Immunohistochemical analysis of endometrial gland cells

It was noted when counting the haematoxylin and eosin-stained glands in the uterine endometrium of *Cenpb*-null mice that some glands had markedly reduced lumens as compared with the glands of wildtype mice (Fig. 3A and B). To ensure that all glands were being scored on the haematoxylin and eosin sections, a number of slides were stained with anti-pan cytokeratin antibody, which marks epithelial cells (Moll *et al.* 1982) (Fig. 3C and D). The counting results were comparable, indicating that the assessment on haematoxylin and eosin sections was accurate. To examine whether the glandular epithelium layer of $R1^{-/-}$ G4 mice had retained oestrogen and progesterone receptors, sections of uterus from 32–36-week-old, non-mated mice were stained with anti-oestrogen receptor and anti-progesterone receptor antibodies. Positive-staining nuclei at apparently comparable density were present in the endometrial glands and stroma of $R1^{-/-}$ and $R1^{+/+}$ G4 mice, suggesting that functional oestrogen (Fig. 3E and F) and progesterone (Fig. 3G and H) receptors were present in the $R1^{-/-}$ mice.

Normal ovarian function in G4 *Cenpb*-null mice

To determine whether the ovaries of *Cenpb*-null mice had deteriorated following interbreeding for several generations, ovaries from *Cenpb*-null and -wildtype G4 mice (day 0.5 VP) were weighed and the number of embryos was assessed following mating to wildtype stud males. No reduction was observed in ovary weight or number of embryos of $R1^{-/-}$ and $W9.5^{-/-}$ G4 mice when compared with $R1^{+/+}$ and $W9.5^{+/+}$ G4 mice respectively, suggesting that ovarian function in terms of egg production was unaffected by the gene knockout mutation (Table 3B and C).

Ovarian transplantation fails to rescue the poor breeding performance of *Cenpb*-null mice

Further evidence that the ovary was not responsible for the poor breeding performance of young adult *Cenpb*-null mice was provided by the failure of ovarian transplantation to rescue the phenotype. $R1^{+/+}$ and $R1^{-/-}$ G4 mice 7–9 weeks old were transplanted with ovaries from 4-week-old $R1^{+/+}$ G4 mice, as described by Szein *et al.* (1998). The wildtype ovaries failed to give rise to normal numbers of litters and pups in the $R1^{-/-}$ females. The $R1^{-/-}$ females ($n = 5$) averaged just one litter of 2.0 ± 1.6 pups per litter, whereas transplanted $R1^{+/+}$ mice ($n = 6$) were able to generate, on average, 5.8 ± 1.0 litters with 4.8 ± 2.3 pups per litter. When the transplanted mice were 9 months old, they were culled, and the number of endometrial glands per transverse uterine section was assessed. The $R1^{+/+}$ mice had 36.0 ± 19.3 glands per transverse section, whereas the $R1^{-/-}$ mice had 6.0 ± 5.6 glands ($P = 0.043$). Furthermore, when 5-week-old $R1^{+/+}$ G4 mice ($n = 2$) were transplanted with ovaries from

32-week-old $R1^{-/-}$ G4 mice, the mice were able to produce an average of 5.5 ± 0.7 litters with 4.4 ± 2.3 pups per litter.

In addition, four virgin, 24-week-old $C57^{+/-}$ G1 females underwent ovarian transplantation with 4-week-old C57BL/6 ovaries. One female failed to become pregnant and two females developed birthing problems with their first litter similar to their non-transplanted counterparts.

Genome scan analysis of $C57^{+/-}$ mice

DNA microsatellite markers that detect polymorphisms between 129 and C57BL/6 DNAs were used to determine the amount of remaining 129-derived DNA in *Cenpb*-null mice on C57BL/6 background. Genome scan analysis revealed a residual 129 region encompassing approximately 41 cM around the targeted *Cenpb* locus (73 cM position) on chromosome 2 (mouse genome database, MGD). The closest C57 markers were located at the 43 and 84 cM positions, 129 markers being present at 52 and 69 cM.

Discussion

The lack of reproductive performance in 10-week-old $R1^{-/-}$ and $W9.5^{-/-}$ G4 mice appears to be mainly due to a reduction in endometrial gland density that was exacerbated by generations of *Cenpb*-null breeding. Although 10-week-old $R1^{-/-}$ and $W9.5^{-/-}$ G4 mice displayed a smaller uterus than wildtype G4 mice, they were no different in size from their *Cenpb*-null G1 counterparts derived from heterozygote intercrosses. Likewise, histological examination of the luminal and glandular epithelium, endometrium and myometrium of 10-week-old $R1^{-/-}$ and $W9.5^{-/-}$ G4 mice revealed no gross abnormality. On the other hand, the luminal epithelia of 43% of 24-week-old $R1^{-/-}$ G4 mice ($n = 7$) displayed ulcerated or disorganised epithelia similar to 60% of 24-week-old $R1^{-/-}$ G1 mice observed in our previous study ($n = 5$) (Fowler *et al.* 2000).

The significant reduction in the number of endometrial glands of 10-week-old $R1^{-/-}$ and $W9.5^{-/-}$ G4 mice (Table 4A and C) is intriguing. It would appear that *Cenpb*-null G4 females carried and reared by a *Cenpb*-heterozygote R1 or W9.5 dam surrounded by heterozygous and wildtype littermates were not quite as susceptible to significant decreases in endometrial gland number at 10 weeks old (Table 4B and D). However, by 24 weeks of age, any possible parental R1 or W9.5 heterozygote effect had dissipated, as *Cenpb*-null G4 mice from both homozygous and heterozygous breeding pairs were noted to have significantly lower numbers of endometrial glands (Table 4E and G).

Further support for endometrial gland development being influenced pre- and postpubertally can be gleaned from the *Cenpb*-null mice on a C57BL/6 background. On this background the uterine phenotype of *Cenpb*-null

mice has been reported as being more dramatic (Fowler *et al.* 2000). Disorganisation of luminal and glandular epithelium was observed in the uteri of 10-week-old C57^{-/-} G1 mice ($n = 4$), 50% displaying ulcerated luminal epithelia when compared with C57^{+/+} G1 mice ($n = 3$) that exhibited normal epithelia. Besides abnormal uterine epithelium, a reduction in the number of endometrial glands in 10-week-old C57^{-/-} G1 mice was previously noted, but not quantified (Fowler *et al.* 2000). In the current study, 3-week-old C57^{-/-} G1 mice carried by a C57^{+/+} dam were found to have a 90% reduction in their endometrial gland number (Table 5A) that remained low up until at least 24 weeks old (Table 5B–F).

As well as abnormal uterine epithelium, our previous studies have shown that the majority of 8–15-week-old C57^{-/-} G1 breeding females developed birthing problems (Fowler *et al.* 2000). In the present study pregnancy problems and impaired gland development were observed in 24-week-old heterozygote C57 G1 females. Histologically, 24-week-old C57^{+/+} displayed a similar profile of endometrial gland scarcity.

Genome scan analysis of C57^{+/+} DNA revealed a 41 cM region of residual embryonic stem cell-derived 129 DNA. Previously, Kapoor *et al.* (1998) had proposed that the phenotypic differences seen in their mice and ours might be due to neighbouring gene effect. Of interest are genes known as progesterone-dependent endometrial protein (*Paep*) (Chan *et al.* 1994) and cyclin-dependent kinase-1 (*Cdc25b*) (Lock *et al.* 1996) that have been previously assigned to the 73 cM position on mouse chromosome 2 in the region of *Cenpb* (Jackson Laboratory MGD). With respect to *Paep*, neither the NCBI nor Celera mouse databases have located *Paep* in the mouse genome, although *PAEP* has been confirmed in the human genome (GDB: 6276819). In regard to *Cdc25b*, knockout mouse studies have revealed a female sterility problem that is specifically due to *Cdc25b*^{-/-} oocytes being arrested during meiosis, rather than a uterine problem (Lincoln *et al.* 2002). Furthermore, our previous work describing the generation and normal phenotype of a targeted control mouse that carried the internal ribosome entry site (IRES)-neomycin selectable marker element in the *Cenpb* gene, but not the translational frameshift mutation, suggests that the observed phenotype in *Cenpb*-null mice is due to *Cenpb*-gene disruption rather than a nearby gene effect induced by the targeting construct (Fowler *et al.* 2000).

The lack of endometrial glands in the *Cenpb*-null mice resembles the phenotype observed in sheep that were treated neonatally with synthetic progesterone known as

norgestomet (Gray *et al.* 2001). Keeping the ovine uterus in a progesterone-rich postnatal environment suppressed epithelial oestrogen receptor- α protein expression and altered the expression of normal paracrine cues for the development and proliferation of endometrial glands from the luminal epithelium (Gray *et al.* 2000). This resulted in a permanent ablation of endometrial glands and subsequent implantation failure and loss of reproductive fitness in treated ewes transplanted with normal blastocysts. Unlike the Gray studies, we have found no evidence of abnormal hormone levels (Fowler *et al.* 2000) or loss of oestrogen receptor expression contributing to the lack of endometrial glands observed in *Cenpb*-null mice. However, future studies on the expression levels of other growth factors and/or receptors involved in endometrial gland formation or uterine receptivity (Gray *et al.* 2000, 2001, Cheng *et al.* 2001) may help shed light on the uterine phenotype of *Cenpb*-null mice.

Interestingly, some aspects of the phenotype of G1 *Cenpb*-null mice (Hudson *et al.* 1998, Fowler *et al.* 2000) are reminiscent of telomerase-deficient mice (*mTR*^{-/-}) following intermating over a number of generations (Lee *et al.* 1998, Herrera *et al.* 1999b). Generation 4 *mTR*^{-/-} mice on a mixed C57BL/6/129Sv background were found to have significantly smaller litters than G1 *mTR*^{-/-} mice, whereas generation 6 intercrosses exhibited infertility due to a number of factors, including a possible compromise in uterine structure and function (Lee *et al.* 1998, Herrera *et al.* 1999a). The worsening phenotype of the *mTR*^{-/-} mice was linked to a reduction in telomere-length and chromosomal abnormalities. This phenotype was exacerbated when *mTR*^{-/-} mice were generated on a C57BL/6 background with mice being fertile only to the third generation (Lee *et al.* 1998, Herrera *et al.* 1999b). These observations suggest that *Cenpb* may be essential for short- and long-term maintenance of uterine integrity over a number of generations.

By virtue of its constitutive centromere-binding property, any attempt at deciphering a role of *Cenpb* has to date been directed at some aspects of chromosome segregation behaviour. Furthermore, if such a role is functionally important, then any perturbation of the protein function would be expected to result in a missegregation outcome throughout all actively dividing tissues in the mice, as gene knockouts of other key centromere-binding proteins have shown (Kalitsis *et al.* 1998, 2000, Dobles *et al.* 2000, Uren *et al.* 2000, Babu *et al.* 2003). It is therefore surprising that the phenotype seen in our *Cenpb*-null mice appears to be confined to the uterine

Figure 3 Haematoxylin and eosin and immunohistochemical analysis of endometrial glands of R1^{+/+} and R1^{-/-} 32–36-week-old non-mated mice. (A) Haematoxylin and eosin stained R1^{+/+} endometrium showing normal glands. (B) Haematoxylin and eosin stained R1^{-/-} endometrium showing fewer glands. (C and D) Anti-pan cytokeratin antibody-stained R1^{+/+} and R1^{-/-} endometria respectively, showing brown positive staining in the cytoplasm of R1^{+/+} and R1^{-/-} endometrial glandular epithelium. (E and F) Anti-oestrogen receptor antibody-stained R1^{+/+} and R1^{-/-} endometria respectively, showing brown positive staining in the nuclei of R1^{+/+} and R1^{-/-} endometrial glandular epithelium and stroma. (G and H) Anti-progesterone receptor antibody-stained R1^{+/+} and R1^{-/-} endometrium, showing brown positive staining in the nuclei of R1^{+/+} and R1^{-/-} endometrial glandular epithelium and stroma. Arrows mark endometrial glands. Scale bar in panel H (50 μ m) applies to all panels.

tissues. While it is true that uterine tissue has a high expression level of *Cenpb* mRNA in mouse (Fowler *et al.* 2000), other tissues with similar *Cenpb* expression, such as mouse testes and intestinal epithelium (K J Fowler, unpublished observations), are not affected functionally by the null mutation, suggesting that high mRNA alone is insufficient to explain the observed uterine phenotype.

Studies in the fission yeast *Schizosaccharomyces pombe* have identified three CENP-B homologues Adp1, Cbh1 and Cbh2 (Baum & Clarke 2000, Irelan *et al.* 2001). Knockout experiments on these genes have shown that the ablation of one alone causes only a mild phenotype, whereas deletion of two of these genes, Adp1 and Cbh1 or Adp1 and Cbh2, results in severe defects in chromosomal segregation and cell growth (Baum & Clarke 2000, Irelan *et al.* 2001). These studies indicate the functional redundancy of the Adp1, Cbh1 and Cbh2 proteins. Although at present no CENP-B functional homologue(s) have been identified in mammals, including mice, it is conceivable that such homologue(s) exist, and this would explain the general non-essential nature of *Cenpb*. It is further possible that any such redundant protein(s) may not be able to replace fully the functions of *Cenpb*, and that the uterine tissue is particularly sensitive to such functional discrepancy. Our observation that the uterine phenotype becomes progressively more severe with increase in both age of the mice and generation time appears consistent with the speculation that the inadequacies of the putative CENP-B homologues could be exacerbated through increasing numbers of both mitotic and meiotic passages.

How does *Cenpb* exert the observed uterine effect? Although *Cenpb* is related to transposase by sequence homology, it has lost its transposase activity (Kipling & Warburton 1997), and, as such, any role it might have is unlikely to be directly linked to transposase. As indicated both in the present and our earlier study (Fowler *et al.* 2000), the contrasting severity of the uterine phenotype in different mouse strains is another example where the roles of modifier genes are strongly implicated in phenotypic presentation (Banbury Conference Consortium 1997, Threadgill *et al.* 1995, Herrera *et al.* 1999a). However, what these modifier genes in *Cenpb*-null mice are remains unknown. A potentially useful future effort to resolve what role *Cenpb* might play in uterine morphogenesis and, more generally, in mitotic and meiotic cell divisions, may be in attempts to identify its functional homologue(s) in mammals, as, if these exist, they could provide the missing pieces to the puzzle.

In summary, the observed decrease in endometrial glands in the *Cenpb*-null mouse leads to a lack of reproductive capability, indicating that a certain number of endometrial glands are required to establish successful pregnancies. Of note is the fact that this decrease in endometrial gland development is caused by the targeted knockout of a gene, the function of which is known to be specific to the centromere. Furthermore, the decreasing

endometrial phenotype is exacerbated through successive generations of breeding. The underlying mechanisms responsible for these observations are at present unclear. These mice offer a model for studying these mechanisms as well as possible genetic and physiological requirements for endometrial glands during pregnancy.

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