RAPID COMMUNICATION

Germ Cell Defects and Hematopoietic Hypersensitivity to γ-Interferon in Mice With a Targeted Disruption of the Fanconi Anemia C Gene

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Fanconi anemia (FA) is an autosomal recessive chromosome instability syndrome characterized by progressive bone marrow (BM) failure, skeletal defects, and increased susceptibility to malignancy. FA cells are hypersensitive to DNA cross-linking agents, oxygen and have cell cycle abnormalities. To develop an animal model of the disease we generated mice homozygous for a targeted deletion of exon 9 of the murine FA complementation group C gene (*fac*). Mutant mice had normal neonatal viability and gross morphology, but their cells had the expected chromosome breakage and DNA cross-linker sensitivity. Surprisingly, male and female mutant mice had reduced numbers of germ cells and females had markedly impaired fertility. No anemia was detectable in the peripheral blood during the first year of life, but the colony forming capacity of marrow progenitor cells was abnormal in vitro in mutant mice. Progenitor cells from *fac* knock-out mice were hypersensitive to interferon γ. This previously unrecognized phenotype may form the basis for BM failure in human FA.

FANCONI ANEMIA (FA) is a hereditary disease associated with increased cellular sensitivity to chemicals that generate interstrand DNA cross-links. In FA cells cross-linking drugs such as Mitomycin C (MMC) or diepoxybutane (DEB) cause increased cell death, chromosome breakage and accumulation in the G2 phase of the cell cycle.[1-3] FA cells are also hypersensitive to oxygen.[4,5] In FA patients the disorder is characterized by birth defects, particularly radial ray deformities, progressive bone marrow (BM) failure, and increased incidence of malignancies.[6] The developmental abnormalities in FA are variable even within the same family and in addition to thumb and radius defects may include short stature, mental retardation, a dysmorphic facies, abnormal kidneys, and malformed vertebrae.[7] Acute myelogenous leukemia (AML) is the most common malignancy in FA, but other types of cancer are also more prevalent.[8] An increase in cancer risk for carriers of FA has been reported.[9] The cause for the progressive aplastic anemia of FA is unknown, but is has been hypothesized that hematopoietic stem cells are lost due to accumulation of DNA damage over time. More recently it has been suggested that FA cells have a low threshold for programmed cell death and that the pathophysiology of the anemia in FA may involve inappropriate apoptosis.[10] Therapy of FA has included the use of anabolic steroids, hematopoietic growth factors and antioxidants for the BM failure. BM transplantation has been used extensively and is successful in abolishing the hematological complications of the disease. Despite these treatment modalities, the morbidity and mortality of FA remains high and the average life expectancy is only ~20 years.[11]

Complementation analysis of somatic cell hybrids from FA patients has shown the existence of at least 5 distinct complementation groups, designated A-E.[12-14] The gene for FA complementation group C (FAC) has been cloned and was assigned to chromosome 9q.[12] The human FAC cDNA is 1674 bp long and encodes a 558 amino acid protein.[15] Its murine homologue has also been isolated and contains one additional alternatively spliced exon,[16] but no natural animal models for FA are known. Recently, the human FAA and FAD genes have been mapped to chromosomes 16q and 3p, respectively,[17,18] indicating that there at least 3 different genes involved in the disease. This is in contrast to another DNA repair disease, ataxia telangiectasia, for which only one gene has been found, although multiple complementation groups had been reported.[19]

The FAC protein has no homologies to other known genes. Despite its role in the cellular response to DNA cross-linking agents the FAC gene is localized and functions in the cytoplasm.[20,21] Neither the phase of the cell cycle nor the induction of DNA cross-links alter the amount of FAC transcript or the subcellular localization of the FAC protein. No defined interactions with other proteins have been reported to date, although several bands which coimmunoprecipitate with FAC have been found.[22] Thus, the function of the FAC protein and its relation to the other FA gene products remains unknown.

Several mutations in the FAC gene have been identified and from these studies it has become clear that the carboxy terminus of the protein is crucial for its function.[23-26] Patients with a nonsense mutation in exon 1 have reinitiation of translation at methionine 55 and a milder phenotype than patients with truncations of the carboxy terminus.[27]

Recently, the FAC gene product has been recognized as an essential factor for optimal growth and differentiation of hematopoietic progenitor cells in vitro. Specifically, when

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the FAC gene is repressed in normal progenitor cells, in vitro clonal growth is inhibited,[28] whereas enforcement of FAC expression in progenitor cells from a child with FA enhanced clonal growth.[29] To develop an animal model in which to investigate the pathophysiology of FA and to test novel therapies, we used gene targeting in embryonic stem cells to generate a mouse strain with a mutation affecting the carboxy terminus of the FAC protein. We here report the phenotypic analysis of this FAC deficient animal especially in regards to the evolution of BM dysfunction.

MATERIALS AND METHODS

Generation of Fac targeted ES clones. A λFix AB129 mouse genomic library was screened with a full-length *fac* cDNA generated by reverse transcriptase-polymerase chain reaction (RT-PCR) of mouse liver cDNA using primers 5′-CTGCTCCTAGAGATGGCTCAGG-3′ and 5′-GGTGCCCACATTCTGTACTACC-3′. Several positive phage were isolated and characterized by restriction mapping. The structure of the region is illustrated (see Fig 1). mfac phage #7 contained exons 8, 9 and 10 of the fac gene and from it a 13.4-kb *Bgl*II insert was subcloned into pBluescript, generating p7A. Within this plasmid a 2.5 kb *Bam*HI-*Eco*RI fragment containing fac exon 9 was excised and replaced with a PGKneo neomycin resistance cassette[30] and a HSV-TK expression cassette was added at the end of the vector's short flank. The short flank of this replacement vector pK04 measured 3.9 kb and the long flank was 7.0 kb.

Generation of Fac deficient mice. Culture and electroporation of Ak7 ES cells were as described previously.[30] DNA was prepared from individual clones, digested with *Nhe* I and Southern blotted[31] using the *Bgl*II/*Not* I probe indicated (see Fig 1). Five of 77 (6%) ES clones doubly resistant to G418 and Gancyclovir were found to bear the desired targeting event (see Fig 1). Blastocyst injections were as described previously.[32] Four highly chimeric mice were generated from clone FacH1 and an additional two from clone FacG3. Several heterozygous $fac^{\Delta exon9}$ founders were produced from clone FacH1 and bred to produce homozygous fac deficient mice.

Mouse strains and animal husbandry. The mice tested had a mixed genetic background of C57BL and 129Sv. Two generations of brother sister matings were carried out between male and female heterozygotes from one selected litter. This partially inbred colony was then expanded for the experiments described.

PCR genotyping. Three PCR primers were designed. Primers A and B were located in exons 8 and 9 of the *fac* gene respectively. Primer C was located at the 5′ end of the PGKneo expression cassette within the PGK promoter sequence. For genotyping, PCR[33] was performed on 200 ng tail-cut DNA[34] with either primers A and B (wild-type allele) or primers A and C (targeted allele). The wild-type fragment produced using primers A and B was 1.3 kb, and the mutant fragment using primers A and C was 600 bp. Primer sequences were A: 5′-CTGCCAACCTGCCATCTTCAG-3′, B: 5′-AAGAGCAGCTAGTACTTCTGG-3′ and C: 5′-TAAAGCGCATGCTCCAGACTG-3′. The amplification conditions were 94°C × 5 minutes, followed by 34 cycles of 90°C × 30 seconds, 54°C × 30 seconds and 72°C × 3 minutes 45 seconds in a previously described buffer.[35]

Chromosome breakage. Primary embryonic fibroblast cultures were established from day 17 embryos. For chromosome breakage analysis,[3] cells were plated on 100-mm dishes, allowed to recover overnight, and treated with various concentrations of clastogens for 24 hours. They were then exposed to colcemid for 3 hours and placed in hypotonic media consisting of 25% fetal calf serum (FCS) and dH_2O, and fixed to slides. Slides were stained with Wright's stain and chromosomes scored for breaks and radials per cell.

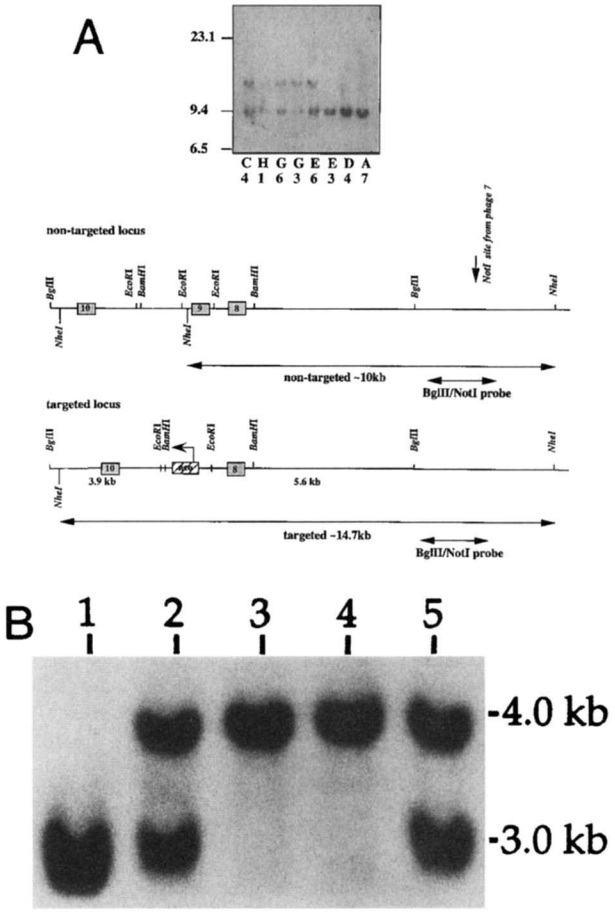


Fig 1. Targeted disruption of the *fac* gene. (A) Southern blot analysis of *Nhe* I digested DNA from ES clones electroporated with the $fac^{\Delta exon9}$ gene replacement vector. The clone numbers are indicated below each lane. The targeting vector contained the genomic sequence between the 2 *Bg*/II sites shown. The blot was probed with the genomic *Bg*/II-*Not* I fragment indicated in the figure. The structure of the native and targeted *fac* gene are shown below the blot. Because correct targeting eliminated an *Nhe* I site adjacent to exon 9, the blots from targeted ES clones had an additional band of higher molecular weight (14.7 kb) than the wild-type band (10.0 kb). Clones C4, H1, G6, G3, and E6 were correctly targeted. (B) Southern blot analysis of tail cut DNA from pups of a heterozygous mating. DNA was digested with *Bam*HI and probed with the *Eco*RI-*Bam*HI genomic fragment containing exon 8 (see [A]). The targeted allele gave rise to a 3.0 kb band, whereas the wild-type allele produced a 4.0 kb fragment. Lane 1 was from a mutant animal, lanes 2 and 5 from heterozygotes, lanes 3 and 4 from wild-type mice.

mRNA expression studies. Total cellular RNA was isolated from liver, kidney and muscle of congenic litter mates[36] and RT was performed as described elsewhere using random hexamers as primers.[23] The first strand cDNAs were amplified by PCR using a forward primer in fac exon 8 with reverse primers situated in exons 9, 10, 11, or 12. The primer sequences were 5′-CTGCCAACCTGCCATCTTCAG-3′ (exon 8), 5′-AAGAGCAGCTAGTACTTCTGG-3′ (exon 9), 5′-AGGAAAGTAGGTCCTGAGGG-3′ (exon 10), 5′-CTCTGAGGAGTTGGGAAATG-3′ (exon 11) and 5′-AGCAGTAACTCTGCCACAGC-3′ (exon 12). The amplification conditions were 94°C × 5 minutes followed by 34 cycles of 90°C × 30 seconds, 57°C × 30 seconds and 72°C × 1 minute 30 seconds in Kogan

buffer.[35] The PCR reaction products were separated by agarose gel electrophoresis and transferred to membrane by Southern blot. The blots was hybridized with an end-labeled oligonucleotide (5′-AATTGTTCATGAAATGTTCAG-3′) from fac exon 8. This oligonucleotide was located 3′ to the exon 8 PCR primer previously described and did not overlap. Amplification was also performed between primers located in fac exon 3 (5′-TCAGGACTTAACTCGTGGATCC-3′) and exon 6 (5′-TATCAGGCAGAGTGACAAGAGG-3′).

Cell cycle analysis. Spleens from mutant animals and heterozygous controls were ground through a stainless steel mesh filter to yield a single cell suspension. Cells were washed 3 × in RPMI-1640 with 10% FCS. Red blood cells were lysed by suspending the pellet in 144 mmol/L NH_4Cl/17 mmol/L Tris (pH 7.2) at room temperature for 5 minutes. The remaining cells were washed twice and resuspended in RPMI-1640 with 10% FCS, 1×10^5 mol/L 2-mercaptoethanol, lipopolysaccharide 1 μg/mL, and concavalin-A 2.5 μg/mL. Two million cells were plated per well in a six-well plate, cultured for 24 hours and then treated with media only or mitomycin C (10 ng/mL). Cells were incubated in the dark for 48 hours, harvested and resuspended in 100 μL of Dulbecco's phosphate-buffered saline (D-PBS). To each tube 250 μL of pH 7.2 propidium iodide (PI) stain (50 μg/mL PI, 30 mg/mL polyethylene glycol 8,000, 2 μg/mL RNase A, 0.1% Triton X-100 (Sigma, St Louis, MO), 36 mmol/L sodium citrate) were added and the cells were incubated at 37°C for 20 minutes. Next, 250 μL of PI salt solution (PI 50 μg/mL, 30 mg/mL polyethylene glycol 8,000, 0.1% Triton X-100, 0.38 mol/L NaCl) was added and the samples were incubated at 4°C for 10 minutes. Following staining the samples were analyzed for DNA content using a Becton Dickinson FACSan flow cytometer (Mountain View, CA). Thirty thousand to forty thousand nuclei were analyzed for each condition. The data were analyzed by the Multicycle software program which utilizes the polynomial S-phase algorithm (Phoenix Flow Systems, San Diego, CA).[37]

Histology. Tissues fixed in 10% phosphate-buffered formalin, pH 7.4, were dehydrated in 100% ethanol and embedded in paraffin wax at 58°C. Four micron sections were rehydrated and stained with Hematoxylin-Eosin.

Hematopoietic colony growth assays. Mice aged from 2 to 4 months (n = 12), 6 months (n = 8), and 11 months (n = 2) were analyzed in a blinded fashion. Genotype codes were not broken until after progenitor growth studies had been completed. On any given day, each experiment consisted of an equal number of mutant and heterozygote mice. Femoral marrow samples were obtained from the mice after cervical dislocation and total viable cell counts were performed. Unfractionated murine BM cells (1×10^5) were cultured in 1 mL of Iscove's modified Dulbecco's medium made semisolid with 1% methylcellulose and supplemented with 20% FCS, 1% deionized bovine serum albumin (Sigma), 2 mmol/L L-glutamine, 10^{-4} mol/L β-mercaptoethanol, penicillin-streptomycin (GIBCO, Grand Island, NY), and three recombinant murine growth factors: erythropoietin 2 units/mL; (Amgen, Thousand Oaks, CA), steel factor (10 ng/mL, R&D Systems, Minneapolis, MN), and interleukin-3 (100 U/mL; R & D Systems). Burst forming unit-erythroid (BFU-E) and colony forming unit-granulocyle macrophage (CFU-GM) and CFU-GM were counted, after 7 and 14 days of culture at 37°C at 5% CO_2 in air, using an inverted microscope. Colony growth results were expressed as mean (of triplicate plates) ± SD colonies and bursts per plate and per femur. Between-group comparisons were made using one way analysis of variance. In each experiment mitotic inhibitory factors were added to methylcellulose cultures containing growth factors. Multiple doses of recombinant murine interferon-γ (IFN-γ) (0.05, 0.1, 0.5, 1.0, 5.0, and 10 ng/mL; Genzyme, Cambridge, MA), tumor necrosis factor-α (TNF-α) (0.05, 0.5, and 5.0 ng/mL; R&D Systems), and macrophage inhibitory factor-1α (mip-1α (0.05, 0.5, 5.0, and 50 ng/mL; Sigma) were tested.

RESULTS

Generation of mice with the fac$^{\Delta exon\ 9}$ mutation. We sought to generate a mutation in the *fac* gene, which would lead to a carboxy terminal truncation of the protein product. Therefore, we constructed a gene replacement vector containing a deletion of exon 9. Because this exon consists of 100 bp of coding sequence, a number indivisible by 3, its deletion from the *fac* mRNA was predicted to create a frameshift and result in a protein product truncated after 54% of the coding region. A linearized plasmid with the targeting construct was electroporated into AK7 embryonic stem cells (Fig 1). Five of 77 ES clones doubly resistant to G418 and Gancyclovir were found to harbor the desired mutation (see Fig 1) and a male chimaera (H1) derived from one of these clones transmitted the mutation through his germ line. Heterozygous fac$^{\Delta exon9}$ mice had no discernible abnormalities and were used as breeders to derive homozygous mutant animals (Fig 1B). The offspring of heterozygous breeders were genotyped in the newborn period and approximately 25% of the pups were homozygous mutant. This indicated that there was no embryonic lethality associated with the fac$^{\Delta exon9}$ mutation. Mutant animals weighed the same as litter mate controls and no macroscopic developmental abnormalities of the limbs or other organ systems were detected (data not shown). The apparent lack of phenotypic abnormalities in mutant mice lead us to investigate the possibility of alternative splicing leading to a mutant *fac* mRNA with an in-frame deletion rather than the desired frame-shift. If the exon 9 deletion produced a transcript with deletions of exons 9, 10, and 11 via alternative splicing, this would lead to an internal deletion of codons 299-385 of the FAC protein, but leave the carboxy terminus intact. Milder phenotypes of knockout mice caused by such unexpected splicing patterns have been previously reported.[38] RT-PCR was used to evaluate the RNA species isolated from 4-week-old mutant and control mice. Amplification was performed with a forward primer localized in exon 8 and reverse primers in exons 9, 10, 11, and 12. The RT-PCR products were separated by agarose gel electrophoresis, transferred to Nylon membrane and probed with a radiolabeled oligonucleotide from exon 8 (Fig 2). Mutant RNA contained only transcripts corresponding to a deletion of exon 9. No deletions of exons 9 + 10 or 9 + 10 + 11 were detected even with long overexposures of the autoradiograph. Thus, deletion of *fac* exon 9 indeed had produced the desired frame-shift mutation.

To prove that we had achieved a mutation functionally equivalent to the defect in human FAC cells we next evaluated the phenotype of cells derived from mutant mice. Primary skin fibroblast cultures were established from day 18 mutant and control embryos. After treatment with MMC and DEB mutant cells revealed chromosome breakage and other cytogenetic abnormalities analogous to fibroblasts from human FAC patients (Table 1). Some cell lines even displayed spontaneous chromosome breakage, without exposure to clastogens. This result provided evidence that the function of the FAC protein was significantly impaired by the Δexon9 mutation and that the murine *fac* gene has a similar function as the human gene.

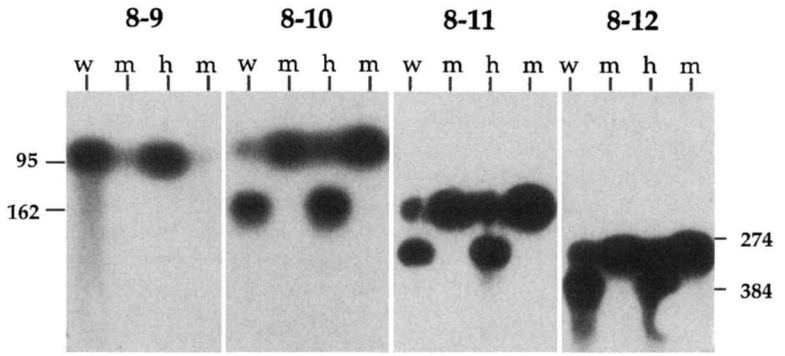


Fig 2. Analysis of mRNA species in mutant mice. RT-PCR products from amplification with primers in *fac* exon 8 + 9, 8 + 10, 8 + 11, and 8 + 12 were separated in an agarose gel and probed with an exon 8 oligonucleotide. Lanes marked "w" contained wild-type, "h" heterozygote and "m" homozygous mutant samples. The size of the amplification products derived from the wild-type alleles are given in bp. No product was detected in mutant samples using primers in exons 8 and 9. In all other PCR reactions the mutant samples gave rise to a product that was 100 bp shorter than the wild-type band, indicating a deletion of exon 9. This shorter PCR product was also detectable in reduced amounts in wild-type samples, indicating that exon 9 is alternatively spliced in normal *fac* mRNA. No PCR products shorter than expected from an exon 9 deletion were detected in any reactions.

This was corroborated by cell cycle analysis of primary cultures of spleen cells exposed to MMC and DEB. Abnormalities in the cell cycle kinetics of cells derived from humans with FA were first reported by Sasaki in 1975.[39] These investigators reported that FA cells passed more slowly than normal through the G2-M phase of the cell cycle. Treatment of FA cells MMC or DEB further increases the delay in cell cycle transit.[2,4,39-41] In mutant mice significant accumulation of cells ($P < .0033$) in the G2 phase of the cell cycle was observed in response to MMC, thus confirming results obtained in human FA cells (Fig 3). No significant differences were observed between mutants and controls in untreated splenocytes.

$FAC^{\Delta exon9}$ mice have reduced fertility. When the fac mutants were old enough to breed, breeding pairs were set up with male and female mutant homozygotes and heterozygotes of the opposite sex. Two-month-old male mutants produced normal numbers of litters and litter sizes, whereas female mutants either had no litters at all or only one litter of 1 to 2 pups. In a breeding experiment conducted over 3 months, four 2-month-old mutant females produced only 6 pups in 4 pregnancies (average litter size 1.5 pups), whereas 4 litter mate heterozygous controls generated 86 offspring in 11 pregnancies (7.8 pups/litter). The male breeders were homozygous wild-type. To investigate the cause of the infertility, we performed histology on the reproductive organs of 6-to 8-week-old mice. The ovaries and uterus of mutant mice were abnormal when compared to litter mate controls (Fig 4 A to D). Mutant ovaries were much smaller in size and were almost completely devoid of follicles. There was marked ovarian cortical hypoplasia and hyperplasia of the interstitial cells. The uteri of mutant mice showed endometrial atrophy with reduced cellularity of the stroma (data not shown). These changes were felt to be secondary to the

Table 1. Chromosome Instability in Primary Embryonic Fibroblasts

		Mitomycin C ng/mL						DEB ng/mL	
Cell Line	Genotype	0	4	8	10	20	40	10	20
MPF-3	−/−	0	12	38	36	82	92	ND	ND
MPF-7	−/−	8	20	18	46	86	86	ND	ND
MPF-61	−/−	6	34	32	ND	ND	ND	14	16
MPF-1	−/+	0	0	0	0	10	26	ND	ND
MPF-5	−/+	2	2	2	0	10	26	ND	ND
MPF-4	+/+	0	0	0	0	16	32	ND	ND
MPF-11	+/+	0	0	0	ND	ND	ND	0	0

The percent of cells containing radial formations in response to the clastogen concentration shown above is given. Fifty cells were evaluated for each data point.

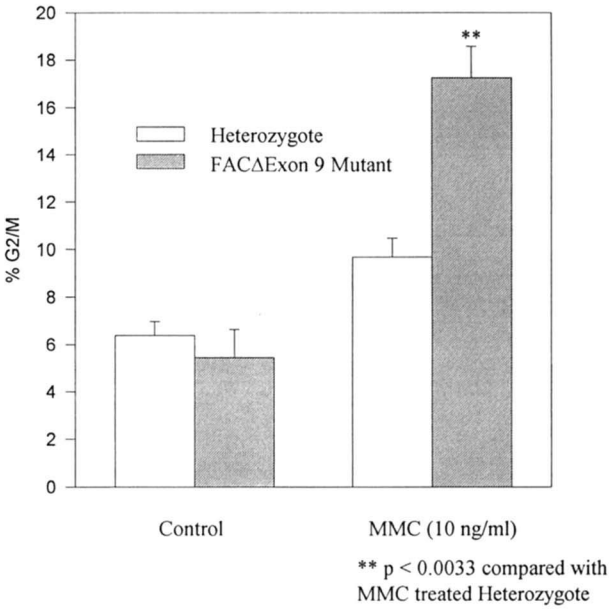


Fig 3. Cell cycle analysis of cultured splenocytes. The percent of cells in the G2/M compartment of the cell cycle is shown and plotted as then mean ± SE. Four heterozygous controls and 8 mutant animals were analyzed in each group.

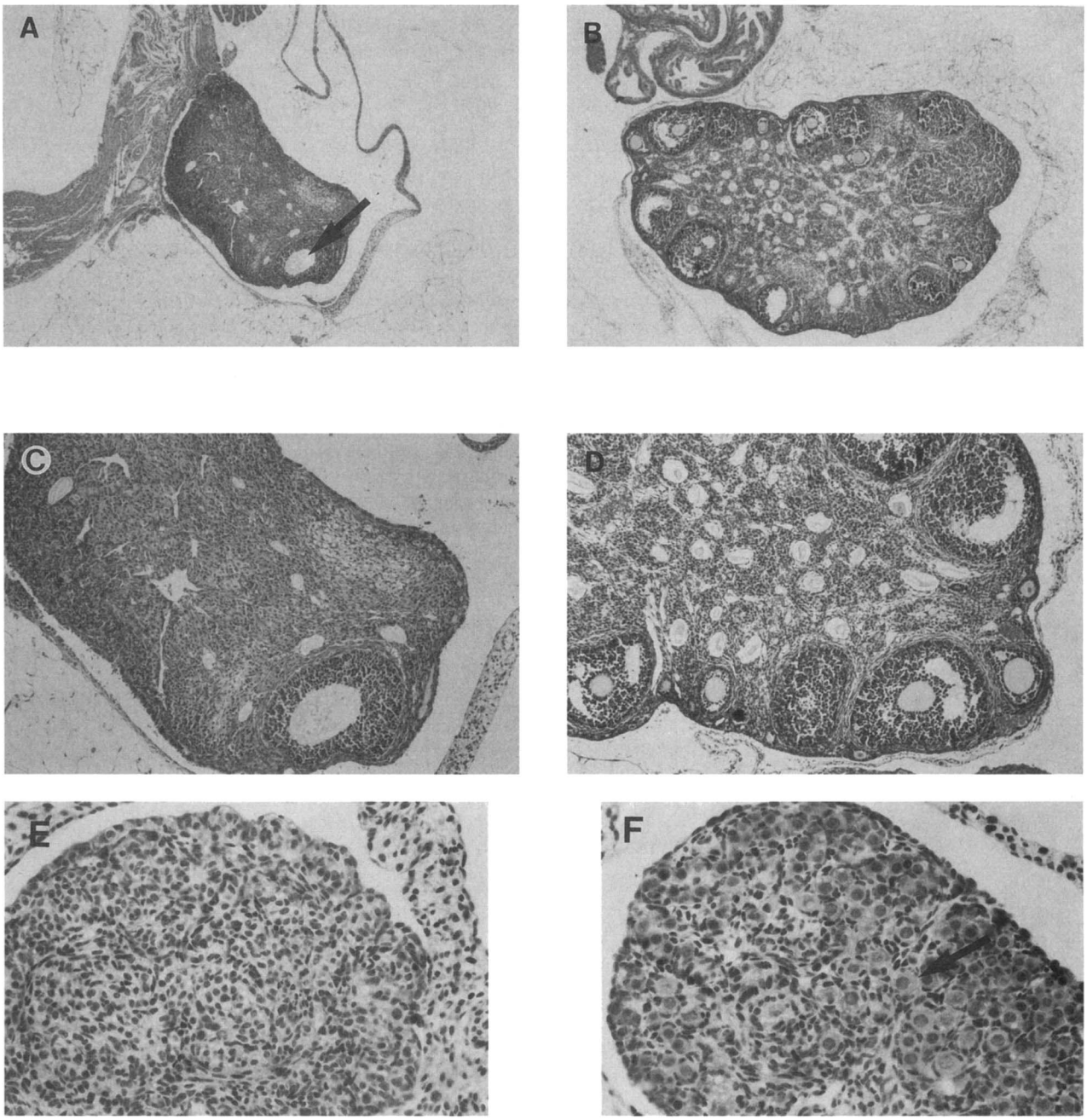
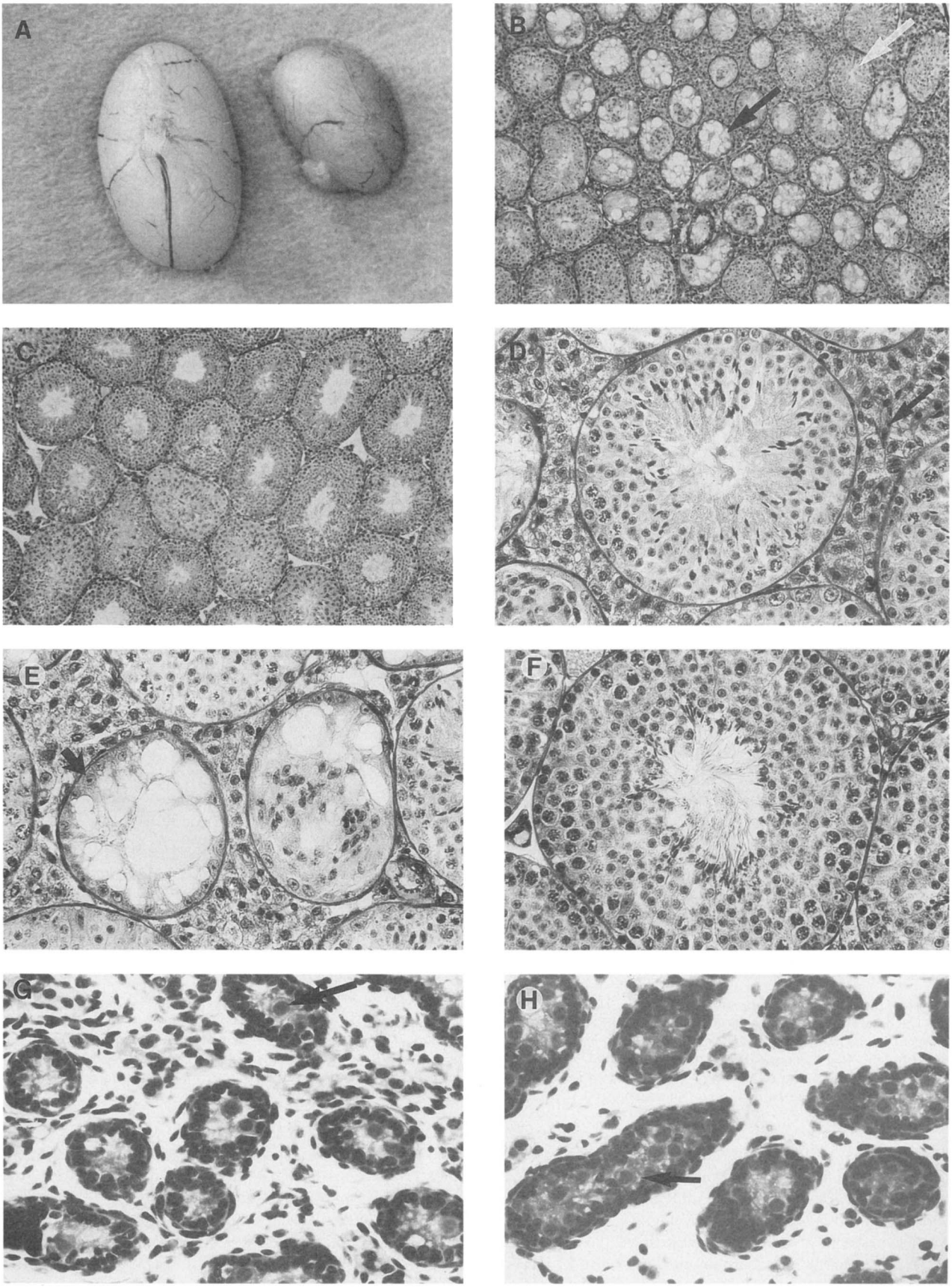


Fig 4. Histology of mutant and control ovaries. (A) Ovary of a 10-week-old mutant and (B) control mouse at original magnification (OM) × 25. The mutant ovary was small in size and almost completely devoid of follicles. One atretic tertiary follicle was present (arrow), whereas the control had an abundance of primary, secondary and tertiary follicles. (C) Higher magnification (OM × 100) of the mutant ovary. The organ consisted mostly of interstitial cells. (D) Control ovary. Many follicles at different stages of maturation were seen. (E) Newborn mutant ovary at OM × 400. The organ consisted of granulosa cells and almost no germ cells. (F) Newborn control ovary (OM × 400). Germ cells (large nucleus, clear cytoplasm, arrow) were abundant.

ovarian abnormalities. The pattern observed was consistent with a reduced number or absence of germ cells. The defect could be either developmental (failure to produce germ cells in embryogenesis) or acquired (progressive loss of initially normal numbers of germ cells with time). To distinguish these 2 possibilities, newborn animals were also examined. The ovaries of newborn mutant females were clearly abnormal, indicating a developmental defect. The number of germcells was much reduced and the organ had been replaced by connective tissue (Fig 4 E and F).

Because of the ovarian abnormalities found in mutant females, we also analyzed the testis of mutant males. As shown in Fig 5A the testicular weight of $fac^{\Delta exon9}$ homozygotes males 6 weeks and older was markedly reduced (44 mg ±


A
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15 for one testis) compared to controls (113 mg ± 8, P value = 2.6×10^{-6}). The testicular weights were also significantly lower in 4-week-old males (data not shown). A mosaic pattern of seminiferous tubules with normal appearance and markedly abnormal tubules was found (Fig 5 B and C). Normal tubules contained all stages of spermatogenesis, including mature sperm (Fig 5D). In contrast, the abnormal tubules were devoid of spermatogenesis and contained only Sertoli cells (Fig 5E). This "all or nothing" pattern again was suggestive of germ cell loss. The presence of all stages of spermatogenesis in the nonaffected tubules ruled out a stage-specific arrest or meiotic defect. In older animals the weight differential between mutants and controls was even more pronounced than in young animals, but fewer abnormal seminiferous tubules were present. The number of germ cells present in newborn testis also was clearly reduced compared to controls (Fig 5F and G). Thus the germ cell defect in $fac^{\Delta exon9}$ mice is a developmental defect and affects both sexes.

Hematological abnormalities in FAC mutants. Complete blood counts and differentials were performed on peripheral blood obtained from mutant and control animals from either the inferior vena cava (at sacrifice) or retroorbital puncture (live animals). No significant differences between the different genotypes were observed in any of the parameters (data not shown) in any age group. Mice as old as 9 months were analyzed. In humans with FA, the anemia has an average age of onset of about 5 years.[11,42] However, abnormalities in cultured BM cells often precede the onset of clinical anemia[43] and we therefore investigated cultured marrow from the $fac^{\Delta exon9}$ mice.

We reasoned that the FAC protein functions, directly or indirectly, either to facilitate growth signals or to constitutively repress antimitotic signals. The in vitro studies were designed to test the hypothesis that the FAC gene product serves to modulate antimitotic effects of hematopoietic inhibitory signals. BM cells were procured and cultured under conditions that establish erythroid and myeloid colonies. Dose response curves to IFN-γ, mip 1α, and TNF-α were generated. In untreated cultures (no mitotic inhibitors added) the erythroid and myeloid colony growth of 2-to 4-month-old mice (6 heterozygotes and 6 mutants) were similar (Fig 6A). However, in 6-month and 11-month mutant mice colony growth was significantly reduced in mutant animals compared to controls (Fig 6B). In the dose responses to other mitotic inhibitors no differential hypersensitivity of mutant cells to mip 1α or TNF-α was detected (data not shown). In contrast, however, mutant clonal progenitor cells were hypersensitive to IFN-γ by nearly an order of magnitude

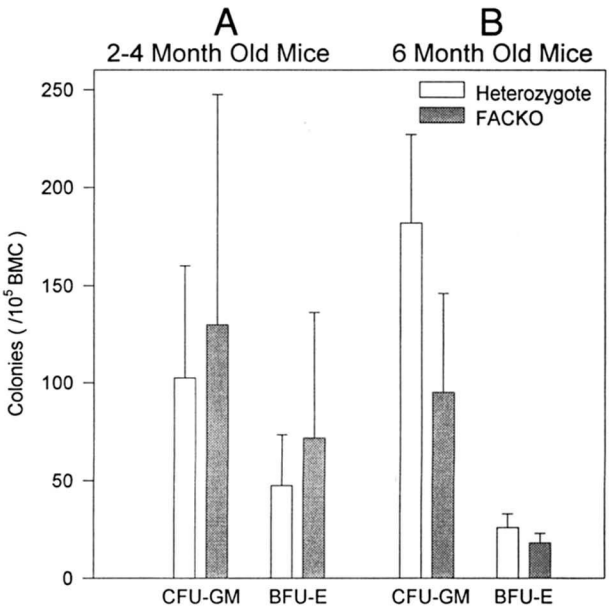

Fig 6. CFU-GM and BFU-E growth in mutant and control mice. Samples were from 2- to 4-month-old mice (A) and 6-month-old mice (B). (A) CFU-GM and BFU-E are expressed as colonies or bursts per 10^5 cells and per femur. No differences were found at this early time point. (B) CFU-GM and BFU-E were significantly ($P < .005$) reduced in mutant mice at the 6-month time point.

(Fig 7). The hypersensitivity to IFN-γ was not age dependent. Even the youngest animals tested (2 months) consistently showed this differential response.

DISCUSSION

The basic defect and pathophysiology in FA have remained poorly understood despite the cloning of the *fac* gene 3 years ago. The altered response of FA cells specifically to DNA cross-linking agents has been known for a number of years, but the biochemical and cellular events governing this process are unknown. One hypothesis is that FA cells fail to recognize or repair interstrand DNA cross-links. Alternatively FA may represent a faulty response to DNA damage in terms of cell cycle control and/or apoptosis. Similar uncertainty exists regarding the mechanisms underlying the limb defects, progressive anemia, tumor formation and other defects occurring at the level of the whole organism. The murine model of FA complementation group C described here provides an experimental system to address some of these questions.

Fig 5. Histology of mutant and control testis. (A) Size comparison of wild-type (left) and mutant testis (right) in 2-month-old litter mates (B) Mutant testis at original magnification (OM) × 100. A mosaic pattern of seminiferous tubules devoid of germ cells (black arrow) and normal tubules (white arrow) can be seen. (C) Control testis at the same magnification as (B). (D) Mutant testis at OM × 400 (PAS stain). Example of a seminiferous tubule with normal spermatogenesis. All stages from spermatogonia to mature spermatides were present. However, the intersititial space (arrow) between tubules was enlarged. (E) Mutant testis at OM × 400 magnification. Two abnormal tubules contained no spermatogenesis, but only Sertoli cells (arrow). (F) Heterozygous control for D and E. Normal mature seminiferous tubule with very few interstitial cells. (G) Newborn mutant testis at OM × 600. Immature tubules containing Sertoli cells at the base and gonocytes (cells with a large round nucleus and clear cytoplasm, arrow) in the lumen were present. The number of gonocytes was reduced compared to controls (H).

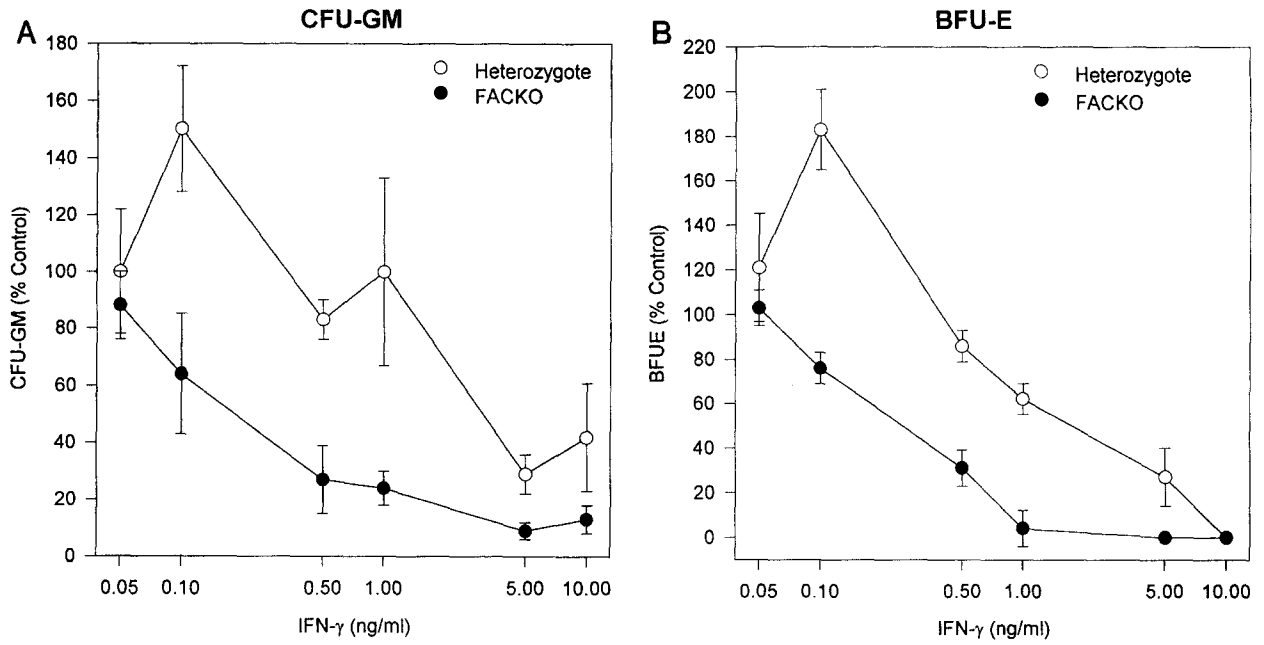

Fig 7. IFN-γ dose response curves of mutant and control mice. Cells from 2 to 4-month-old mutant mice (●), both CFU-GM (A) and BFU-E (B), were more sensitive to the mitotic inhibitory effects of recombinant murine IFN-γ than were cells from the marrows of 2 to 4-month-old heterozygotes (○). Each point on the dose response curves represents mean clonal growth (% control) of cells obtained from six separate animals. By analysis of variance the dose response curves were different ($P < .0005$).

The results of our in vitro hematopoiesis studies lend additional support to the view that the FAC protein plays a permissive role in growth, differentiation, or survival of hematopoietic progenitor cells[28,29,44] and suggest that it does so by directly or indirectly suppressing an IFN-γ mediated mitotic inhibitory pathway. Although leukemia has not yet developed in our mice, the time-related occurrence of BM failure shows the relevance of this model to studies on FA which, in children, is also characterized by a progressive increase in the incidence of BM failure.[45] IFN-γ is not only a recognized inhibitor of hematopoietic progenitor cell growth,[46-48] it is a likely mediator of BM failure in humans. In our mice, the IFN-γ hypersensitive phenotype antedates the onset of BM failure, and thus the hypersensitive phenotype may be the actual cause of the progressive progenitor depletion observed. This may occur through programmed cell death[49] of stem cells and progenitors in the FAC mutant mice. Consequently, the mutant mice described herein will provide a unique opportunity to identify the specific role the FAC gene product plays in the IFN-γ inhibitory pathway.

Many more experiments will be required to fully characterize the hematopoietic and nonhematopoietic defects in the $fac^{\Delta exon9}$ mice. Recent evidence suggests that FA cells have increased susceptibility to programmed cell death.[10] The apoptotic response of cells derived from FAC mutant mice in response to stimuli such as growth factor withdrawal, oxygen and DNA cross-linkers will need to be quantitated. In addition, it its possible that FAC may have an influence on the levels of Fas in hematopoietic cells and thereby modulate their apoptotic response. To address the clinical significance of the observed hypersensitivity to IFN-γ, in vivo challenges with this compound will need to be performed. Hopefully, the further analysis of the knock-out mice will lead to detailed understanding of the pathophysiology of this disorder.

The gonadal defects observed in the $fac^{\Delta exon9}$ mice were unexpected, because this phenomenon had not been reported in humans with FA. The histologic appearance of mutant testis and ovaries is most consistent with a severely reduced number of germ cells. Mice deficient in the DNA mismatch repair protein PMS2 also show reduced male fertility.[50] However, in contrast to PMS2 knockouts no stage specific arrest of spermatogenesis was observed in our mice. Thus, *fac* does not appear to be involved in meiosis itself. Because the testicular and ovarian abnormalities are already present at birth, the defect in germ cell production must occur prenatally. Therefore, it is likely that *fac* plays a role in the production or survival of primordial germ cells. Mice bearing mutations at the *Dominant White Spotting* (W) and *Steel* (S) loci have a very similar histologic phenotype as our $fac^{\Delta exon9}$ mice.[51,52] Mutations in these genes, which encode c-kit and its ligand stem cell factor, respectively, cause anemia and hypopigmentation in addition to germ cell deficits. The similar germ cell phenotype of our mutant and the fact that the *fac* gene product is involved in hematopoietic stem cell function raise the possibility that the FA and c-kit pathways may interact at some level. Interestingly, experiments with cultured primordial germ cells suggest that stem cell factor may have an antiapoptotic function in this tissue.[53] Unlike W and S mutants, however, $fac^{\Delta exon9}$ mice have no discernible pigmentary abnormalities, indicating at least partially separate functions for the 2 pathways.

Mutant animals in our colony have now been observed

for close to 1 year and no tumors have been found to date. The time span for tumor evolution may have been too short. Continued observation and cross-breeding with other tumor prone mouse strains will reveal, whether the chromosome instability of FAC deficient cells will lead to the increased cancer rate, which is part of the human FA phenotype.

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