Targeted disruption of the murine Fanconi anemia gene, Fancc/Xrcc9

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Fanconi anemia (FA) is a human autosomal recessive cancer susceptibility disorder characterized by cellular sensitivity to mitomycin C and ionizing radiation. Six FA genes (corresponding to subtypes A, C, D2, E, F, and G) have been cloned, and the encoded FA proteins interact in a common cellular pathway. To further understand the in vivo role of one of these human genes (FANC,G), we generated a targeted disruption of murine Fancc and bred mice homozygous for the targeted allele. Similar to the phenotype of the previously described Fancc−/− and Fanca−/− mice, the Fancc−/− mouse had normal viability and no gross developmental abnormalities. Primary splenic lymphocytes, bone marrow progenitor cells, and murine embryo fibroblasts from the Fancc−/− mice demonstrated spontaneous chromosome breakage and increased sensitivity to mitomycin C and, to a lesser extent, ionizing radiation. Fancc−/− lymphocytes had a defect in the FA pathway, based on their failure to activate the monoubiquitination of the downstream Fancc2 protein in response to IR. Finally, Fancc−/− mice had decreased fertility and abnormal gonadal histology. In conclusion, disruption of the Fancc gene confirms the role of Fancc in the FA pathway. The Fancc−/− mouse may be useful as an animal model for future gene therapy and cancer susceptibility studies. (Blood. 2001;98:3435-3440)

Introduction

Fanconi anemia (FA) is an autosomal recessive cancer susceptibility syndrome characterized by multiple congenital anomalies and progressive bone marrow failure.1,2 FA patients develop several types of cancers, including acute myeloid leukemias and squamous cell carcinomas of the head and neck.3 FA cells are sensitive to DNA cross-linking agents, such as mitomycin C (MMC) and, to a lesser extent, ionizing radiation (IR).4,5 Based on somatic cell fusion studies, FA is composed of 8 distinct complementation groups.6,7 Six of the human FA genes, including the genes for FANCA, 8,9 FANCC, 10 FANCD2, 11 FANCE, 12 FANCF, 13 and FANCG, 14 have been cloned.

Recent studies have demonstrated that the 6 cloned FA proteins interact in a common cellular pathway.15 The FANCA, FANCC, FANCF, FANCD2, and FANCG proteins assemble in a multisubunit nuclear complex.16-21 The FA protein complex regulates the monoubiquitination of the downstream FANCD2 protein, suggesting that the complex is a multisubunit monoubiquitin ligase or regulates a ligase activity. When normal (non-FA) cells are exposed to DNA-damaging agents, such as IR, MMC, or UV light, FANCD2 is monoubiquitinated and targeted to nuclear foci containing the BRCA1 protein.15 Disruption of this pathway leads to the characteristic cellular and clinical abnormalities observed in FA.

In an attempt to understand the in vivo function of FA genes, targeted disruptions of FA genes have been generated. Two murine models, containing disruptions of the murine homolog of Fancc, have been developed. Chen et al.22 generated a disruption of exon 8 of Fancc, while Whitney et al.23 used homologous recombination to create a disruption of exon 9. In both models, spontaneous chromosome breakage was observed. In addition, an increase in chromosome breaks in splenic lymphocytes in response to bifunctional alkylating agents was observed. In both models, Fancc−/− mice had germ cell defects and decreased fertility. Unlike human FA patients, the Fancc−/− mice had no obvious gross developmental abnormalities or cancer susceptibility. To date, the Fancc murine knockout model has been useful in examining (1) the role of the Fancc gene in the physiologic response of hematopoietic cells to DNA damage;24,25 (2) the in vivo effects of inhibitory cytokines on FA marrow cells;26-28 and (3) the efficacy of gene therapy.29,30

In principle, targeted disruption of other murine FA genes, such as the Fancc/xrcc9 gene, may provide additional insight to in vivo function. First, additional FA gene knockout models will allow a side-by-side comparison of disease severity in different FA subtypes. Recent studies have suggested that the severity of FA, in terms of developmental abnormalities and hematologic defects, is dependent on FA subtype.31 For instance, FA-C and FA-G patients exhibit more severe disease, while FA-A patients exhibit more mild disease, with later onset of bone marrow failure and hematologic malignancy. Whether mice with targeted disruptions of different FA genes vary in disease severity remains untested. Second, conflicting studies suggest that some FA patients and cell lines are sensitive not only to mitomycin C (MMC) and diepoxybutane (DEB) but also to IR. Accordingly, murine knockout models may allow the systematic evaluation of differential drug and IR sensitivity. Third, the availability of other FA gene disruptions will allow the generation and characterization of mice with multiple FA gene knockouts. For instance, if 2 FA genes function exclusively in the same cellular pathway, a double knockout should have the same phenotype as the single FA gene...
knockout. In the current study, we have used homologous recombination to disrupt the murine Fancg gene and have analyzed the primary cells and tissues derived from Fancg−/− mice.

Materials and methods

Generation of Fancg-deficient mice and genotyping

The murine Fancg gene was disrupted by replacing exons 2 to 9 with an FRT-flanked neomycin cassette via homologous recombination in 129/SvJae embryonic stem cells. Mice homozygous for the Fancg mutation within a mixed genetic background of 129/Sv and C57BL were generated following standard protocols. Mouse tail genomic DNA was prepared as previously described and used as a template for polymerase chain reaction (PCR) genotyping. PCR reactions were assembled according to the manufacturer’s protocols (Roche, Indianapolis, IN) with primers Gex5F, Gex6R, PsV, and G40 (Figure 1) in the same reaction tube, at a final concentration of 1 μM each. The cycling conditions were 1 cycle of 94°C for 3 minutes; then 30 cycles of 94°C for 45 seconds, 55°C for 45 seconds, and 72°C for 45 seconds followed by 1 cycle of 72°C for 5 minutes. Mouse testis RNA was extracted using the SV Total RNA Isolation System (Promega, Madison, WI). Fancg messenger RNA (mRNA) and β2-microglobulin mRNA were simultaneously amplified from 1 μg total RNA by reverse transcriptase (RT)-PCR using the OneStep RT-PCR kit (Quagen, Valencia, CA). The amplification conditions were 50°C for 30 minutes for 1 cycle; 95°C for 15 minutes for 1 cycle; 94°C for 45 seconds, 60°C for 45 seconds, and 72°C for 1 minute for 30 cycles; and 72°C for 10 minutes for 1 cycle. Oligonucleotides (Figure 1A) used for PCR and RT-PCR were as follows: Gex5F: 5′-CCCTCTAGAGCTGCTACTCTGCG3′, Gex6R: 5′-GTGTACACCTGGACTAACACGGAC3′, G40: 5′-TGCTAATTACTAAGTG3′, PsV: 5′-AAGGTGGGGCTCGGAAATG3′, M2F: 5′-GTATGCATCCAGAAGAAAACTT3′, M2R: 5′-CATGTCCTGATCCCTAGAGCG3′.

Chromosome breakage assay for murine splenocytes

Splenocytes were prepared from 6-week-old mice of known Fancg genotype. Briefly, the spleen was dissected, crushed in RPMI medium into a single-cell suspension, and filtered through a 70 μm filter. Red cells were lysed in hypotonic ammonium chloride. The remaining splenic lymphocytes were washed in phosphate-buffered saline and resuspended in RPMI/10% fetal bovine serum (FBS) to a concentration of 10^6 cells/ml. Cells were plated at 10^4/ml in media and exposed to MMC or DEB for an additional 48 hours. Alternatively, cells were cultured for 60 hours, exposed to IR (2 or 4 Gy, as indicated), and allowed to recover for 12 hours before chromosome breakage or trypan blue exclusion (viability) analysis.

Murine hematopoietic progenitor assay

Mononuclear cells were isolated from the femurs and tibiae of 4- to 6-week-old Fancg−/− or Fancg+/− mice, as previously described. A total of 2 × 10^4 cells were cultured in 1 mL of MethoCult M3434 media (StemCell Technologies, Vancouver, BC, Canada) with or without MMC treatment. Colonies were scored at day 7, when most of the colonies belong to the granulocyte-macrophage colony-forming unit or erythroid burst-forming unit lineages. Each number was averaged from duplicate plates, and the data were derived from 2 independent experiments.

Immunoblotting of the murine Fancd2 protein

Primary splenic lymphocytes, prepared as above, were cultured in RPMI medium plus 10% heat-inactivated fetal bovine serum for 72 hours. Cells were untreated or irradiated with IR (2, 4, 10, and 20 Gy). Cells were lysed, and cellular proteins were electrophoresed, transferred to nitrocellulose, and immunoblotted with a polyclonal antibody raised against human FANCd2. This antisera cross-reacts with the Fancd2 protein in murine cells.

Flow cytometry

Lymphocytes isolated from thymus, spleen, and peripheral lymph nodes were stained for T- or B-lymphocyte surface molecules with fluorescein isothiocyanate–conjugated anti-CD3, -CD4, and anti-CD19 and phycoerythrin–conjugated anti-CD8, -CD44, -CD45RB, immunoglobulin M, and anti-B220 (BD PharMingen, San Diego, CA). Stained cells were analyzed on a Coulter Epics XL flow cytometry system.

Histology

Mice ovaries and testes were isolated and fixed in 4% paraformaldehyde and further processed by the core facility of the Department of Pathology at Massachusetts General Hospital. The specimens were photographed on a Zeiss Axiphot.

Results

Gene targeting and gross phenotype of Fancg−/− mice

We have disrupted the murine Fancg gene by replacing exons 2 to 9 with an FRT-flanked neomycin resistance cassette through homologous recombination in murine embryonic stem cells (Figure 1A). Two independent embryonic stem cell clones with the desired mutation were injected into C57BL/6 blastocysts, and one germ-line transmission event was detected in the testis of the Fancg−/− mice (Figure 1B), indicating that there was no embryonic lethality associated with biallelic Fancg mutations. Approximately 25% of the offspring of heterozygous breeders were homozygous (Fancg−/−) mice. Approximately 25% of the offspring of heterozygous breeders were homozygous (Fancg−/−) as determined by genomic PCR (Figure 1B), indicating that there was no embryonic lethality associated with biallelic Fancg mutations. Moreover, no wild-type Fancg mRNA transcripts were detected in the testis of the Fancg−/− mice by RT-PCR using primers amplifying exons 5 and 6 (Figure 1C). This result demonstrates that these mice are null mutants for the Fancg gene. Mutant animals weighed the same as littermate controls, and no macroscopic developmental abnormalities of the limbs or other organ systems were detected (data not shown).

Increased chromosome breakage and decreased survival of Fancg−/− cells in MMC

Cytogenetic analysis demonstrated that primary cells (splenic lymphocytes) from the Fancg−/− mice had significantly higher DEB-induced
and MMC-induced chromosomal aberrations, particularly radial forms, compared with control mice (Figure 2A). Spontaneous aberrations were also increased in Fancc−/− splenocytes. In addition, we established primary skin fibroblast cultures (MEF cultures) from day 18 mutant and control embryos (data not shown). Treatment of the mutant fibroblasts with MMC and DEB revealed chromosome breakage and other cytogenetic abnormalities, analogous to primary skin fibroblasts from human FA patients (Figure 2B).

The bone marrow of the Fancc−/− mutant mice was grossly normal compared with the bone marrow of normal littermate controls (data not shown). Also, the peripheral blood count and blood cell morphology of the Fancc−/− mutant mice were grossly normal. There were no significant differences for the hematocrits of blood cell morphology of the normal compared with the bone marrow of normal littermate
radial forms or breaks.

Figure 2. Primary splenic lymphocytes from Fancc−/− mice have increased DEB- and MMC-induced chromosome breakage. (A) Comparison of the number of aberrations per cell after treatment with the indicated amount of DEB or MMC. (B) Metaphase spreads of splenocytes from Fancc−/− and Fancc+/− mice after 72 hours of in vitro growth. Cells were exposed to either no drug (+0), MMC (20 ng/mL), or IR (4 Gy). Arrows indicate radial forms or breaks.

To further demonstrate the differential IR sensitivity of Fancc−/− and Fancc+/− splenocytes, we exposed cells to variable amounts of IR and measured cell viability by the trypan blue exclusion assay (Figure 4B). By this assay, Fancc−/− cells had decreased survival following IR, confirming their enhanced IR sensitivity.

Disruption of the FA pathway in Fancc+/− cells

Recent studies have demonstrated that the FA proteins cooperate in a common biochemical pathway leading to the generation of FANCDD2/BRCA1 foci.15 Five of the cloned human FA proteins (FANCA, FANCC, FANCE, FANCF, FANCG) interact in a multisubunit protein complex,17,20,21 and this complex is required for the DNA damage–inducible monoubiquitination of the downstream FANCD2 protein.15 We therefore tested the Fancc−/− splenocytes for the integrity of the FA pathway (Figure 5).

As previously described,15 the FA protein complex (Fanconi A/C/E/F/G protein complex) is required for the monoubiquitination of the FANCD2 protein (Figure 5A). Murine Fancc−/− splenocytes express both forms of the Fancd2 protein (Fancd2-S and Fancd2-L) (Figure 5B, lanes 7-11). In contrast, Fancc−/− splenocytes failed to express the Fancd2-L isoform either in the absence or the presence of IR (lanes 12-16). The Fancc−/− splenocytes did express the Fancd2-S isoform (lanes 12-16). Taken together, these results demonstrate that the Fancc−/− splenocytes have a disruption of the FA pathway.

Fancc−/− mice have germ cell defects and decreased fertility

Several attempts to inbreed the homozygous Fancc−/− mice have not yielded any offspring over a period of 6 months. Cross-breeding of Fancc−/− and Fancc+/− mice has resulted in a decreased frequency of pregnancies and reduced litter sizes, as compared with Fancc+/− inbreds, suggesting that both male and

Figure 3. Bone marrow progenitor cells from Fancc−/− mice have increased sensitivity to MMC. Mononuclear cells were isolated from bone marrow from Fancc+/− mice or Fancc−/− mice. Cells were plated in methylcellulose in the presence of the indicated concentration of MMC. Colonies were enumerated at day 7.

Figure 4. Differential IR sensitivity of Fancc−/− and Fancc+/− primary splenic lymphocytes. (A) Analysis of chromosome breakage following exposure to variable amounts of IR. Results shown are representative of 3 separate experiments. (B) Analysis of cell viability by the trypan blue assay following IR exposure. Based on this assay, the Fancc+/− and Fancc−/− cells had approximately equal viability (95%-98% viable) in the absence of IR exposure. Data shown are representative of 3 separate experiments.
In addition, we tested protein extracts from the testes of Fancg−/− mice and littermate controls for expression of the Fancd2 protein (Figure 5B, lanes 17, 18). Interestingly, the Fancg−/− mutant testis expressed only the short (nonubiquitinated) isoform of the Fancd2 protein (lane 18), while the Fancg+/− sibling control testis expressed both Fancd2-S and Fancd2-L isoforms (lane 17). Taken together, these data show that an intact FA pathway and the activation of Fancd2 to the monoubiquitinated isoform of Fancd2 correlates with the ability of the germ cells to differentiate into mature spermatocytes.

Discussion

In the current study, we used homologous recombination to disrupt the murine Fancg gene and to generate a murine model of FA subtype G. The cellular and gross phenotype of the Fancg+/− mice was similar to the phenotype observed for the previously described Fancc−/− mice.22-23 Primary cells from the Fancg−/− mice demonstrated spontaneous chromosome breakage that was increased in response to the DNA cross-linking agents, MMC and DEB. While the Fancg−/− mice had few gross phenotypic abnormalities, the mice were infertile and had clear histologic abnormalities of the gonads.

While the bone marrow cellularity and peripheral blood erythroid mass (hematocrit) for the Fancg−/− mice were normal, the bone marrow progenitor cells were sensitive to MMC. Decreased survival of Fancg−/− bone marrow progenitor cells was observed in methylcellulose culture. Similar MMC sensitivity has previously been reported for bone marrow from Fancg−/− mice.

Taken together, our data suggest that the Fancg−/− mice exhibit minor hematologic abnormalities characteristic of FA patients during the preanemic phase. However, in contrast to human FA patients, Fancg−/− mice do not develop progressive bone marrow failure, at least not during the first year of life. The biological significance of the difference between human and murine FA is unclear. It is possible that additional redundant antiapoptotic signaling pathways exist in the murine system, which partially compensate for the loss of the FA pathway in murine bone marrow cells.

It will be interesting to determine whether in vivo administration of low doses of MMC to the Fancg−/− mice versus sibling control mice will result in bone marrow failure. Also of interest will be whether retroviral gene transfer of the wild-type human FANCQ complementary DNA corrects the MMC sensitivity of the bone marrow progenitors, leading to enhanced survival of transduced cells in vivo.

The primary splenic lymphocytes from the Fancg−/− mice also had increased sensitivity to IR, based on the chromosome breakage assay and survival studies. Increasing evidence suggests that FA cells are mildly sensitive to IR, although they are more sensitive to DNA cross-linking agents. For example, nonmyeloablative doses of IR have recently been shown to be able to select the wild-type hematopoietic cells over FA hematopoietic cells in murine competitive repopulation models.30 The IR sensitivity of primary Fancg−/− cells in our study is consistent with other recent studies describing the FA molecular pathway.19 IR activates the dose-dependent and time course–dependent monoubiquitination of the FANCQ protein in normal (non-FA) cells. Monoubiquitinated FANCQ is targeted to BRCA1 nuclear foci, which appear to play a role in the repair of DNA double-strand breaks. Interestingly, the FA protein complex, including the FANCA, FANCC, FANCQ, FANCF, and FANCQ proteins, is required for the IR-dependent monoubiquitination of FANCQ. Human FA cells, defective

female Fancg−/− homozygote mutant mice have impaired fertility (data not shown).

To investigate the cause of the infertility, we performed measurements and histologic evaluation of the reproductive organs of 6- to 8-week-old mice (Figure 6). The ovaries of Fancg−/− mutant mice were abnormal compared with those of littermate controls. Mutant ovaries were almost completely devoid of follicles (Figure 6A). The testes of Fancg−/− mutant mice were also abnormal. The testicular weight of Fancg−/− homozygote males 6 weeks and older was markedly reduced (218 ± 47 mg per testis) compared with littermate Fancg+/− heterozygote controls (575 ± 81 mg per testis, P < .01). Similar to the testicular histology observed for the Fancc−/− animal, the Fancg−/− testis had a mosaic pattern of the seminiferous tubules, with the appearance of both normal and abnormal tubules. The abnormal tubules were devoid of spermatocytes and contained only Sertoli cells (Figure 6B).
in the genes encoding any one of these FA proteins, fail to form FANCD2/BRCA1 foci in response to IR, perhaps accounting for the IR sensitivity of the $\text{Fancg}^{-/-}$ cells observed in the current study.

Other mouse models with enhanced IR sensitivity have defects in the immune system. Specifically, mice with targeted disruptions of the Ku, DNA-PK, or ATM gene have cellular and humoral immunodeficiency. We therefore analyzed T cells and B cells from $\text{Fancg}^{-/-}$ versus $\text{Fancg}^{+/+}$ sibling controls (data not shown). For single-cell suspensions prepared from thymus, spleen, or peripheral lymph nodes, there was no difference in the expression of T-cell surface antigens (CD3, CD4, CD8) or B-cell surface antigens (CD19, immunoglobulin M, B220) between $\text{Fancg}^{-/-}$ and $\text{Fancg}^{+/+}$ mice. Consistent with these studies, human FA patients are not immunodeficient and appear to have normal immunoglobulin diversity.

The $\text{Fancg}^{-/-}$ mice are infertile, and the gonads from these animals have striking pathology. Ovaries from the $\text{Fancg}^{-/-}$ females are small and have decreased cellularity. Testes from the $\text{Fancg}^{-/-}$ males are small and have decreased cellularity and spermatogenesis. While the molecular basis of this gonadal histology is unknown, it may result from a defect in the activation of the Fancd2 protein. Recent studies demonstrate that the activated human FANC2 protein normally binds to meiotic chromosomes in the axial (unsynapsed) regions of synaptonemal complexes. FANCD2 binding correlates with the increase in DNA double-strand breaks, resulting from crossing-over events during meiosis. Based on this model, a disruption of the FA pathway, caused by a targeted disruption of the $\text{Fancg}$ gene, may prevent normal Fancd2 activation and may block the normal binding of Fancd2 to meiotic chromosomes in the $\text{Fancg}^{-/-}$ animals. Absence of Fancd2 staining of meiotic chromosomes may lead to accelerated apoptosis of differentiating spermatocytes.

Finally, the similarity between the $\text{Fancc}^{-/-}$ mouse and the $\text{Fancg}^{-/-}$ mouse in this study provides further genetic evidence of a common FA molecular pathway. Previous studies have demonstrated that FANCC and FANC2 interact in a nuclear protein complex, along with other FA proteins (FANCA and FANCF). FANCE is probably a subunit of this nuclear complex as well, based on in vitro binding studies. Other proteins may also be part of this nuclear complex, such as the recently cloned FANCC-interacting protein, FAZF. The FA protein complex (A/C/E/F/G)

Figure 6. $\text{Fancg}^{-/-}$ mice have germ cell defects and decreased fertility. (A) Histology of mutant and control ovaries (hematoxylin and eosin staining). Ovary of an 8-week-old mutant or control mouse. The mutant ovary was almost completely devoid of follicles (iii, $\times 60$; iv, $\times 125$). The control ovary had an abundance of developing follicles at various stages (i, $\times 60$; ii, $\times 125$). (B) Histology of mutant and control testis (hematoxylin and eosin staining). A mosaic pattern of seminiferous tubules devoid of germ cells and normal tubules can be seen in the mutant testis (iv, $\times 250$; v and vi, $\times 320$). A control testis is shown at the same magnification (i, ii, iii). Arrows indicate Sertoli cells.
is required for DNA damage–inducible monoubiquitination and activation of the downstream FANC D2 protein. Activated FANC D2 interacts with chromatin and may be required for DNA repair. That disruption of the FANC or FANC g genes yields the same phenotype suggests that the major (or sole) function of the FANC and FANC g proteins is to cooperate in the FA protein complex. For instance, if the FANC or FANC g proteins had additional functions beyond their role in the FA protein complex, one might expect additional unique phenotypic characteristics for the FANC−/− and FANC g−/− knockout animals. Disruption of the murine Fanca gene also results in a similar phenotype.41 Further supporting a unified function of these proteins (A/C/G) in the FA protein complex, whether any of the FA genes have additional functions outside of the FA pathway will require the generation of mice with double or triple gene disruptions. Also, it will be interesting to determine whether targeted disruption of Fancd2, the putative substrate of the FA protein complex, yields a similar phenotype to the Fanc, Fanca, and Fancg knockout models.

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References

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