Embryonic Lethality after Combined Inactivation of *Fancd2* and *Mlh1* in Mice

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Abstract

DNA repair defects are frequently encountered in human cancers. These defects are utilized by traditional therapeutics but also offer novel cancer treatment strategies based on synthetic lethality. To determine the consequences of combined Fanconi anemia (FA) and mismatch repair pathway inactivation, defects in *Fancd2* and *Mlh1* were combined in one mouse model. *Fancd2/Mlh1* double-mutant embryos displayed growth retardation resulting in embryonic lethality and significant underrepresentation among progeny. Additional inactivation of *Trp53* failed to improve the survival of Fancd2/Mlh1–deficient embryos. Mouse fibroblasts were obtained and challenged with cross-linking agents. Fancd2-deficient cells displayed the FA-characteristic growth inhibition after mitomycin C (MMC) exposure. In primary fibroblasts, the absence of Mlh1 did not greatly affect the MMC sensitivity of Fancd2-deficient and Fancd2-proficient cells. However, in *Trp53* mutant immortalized fibroblasts, Mlh1 deficiency reduced the growth-inhibiting effect of MMC in *Fancd2* mutant and complemented cells. Similar data were obtained using psoralen/UVA, signifying that MLH1 influences the cellular sensitivity to DNA interstrand cross-links. Next, the effect of MLH1 deficiency on the formation of chromosomal aberrations in response to cross-linking agents was determined. Surprisingly, *Mlh1* mutant fibroblasts displayed a modest but noticeable decrease in induced chromosomal breakage and interchange frequencies, suggesting that MLH1 promotes interstrand cross-link repair catastrophe. In conclusion, the combined inactivation of *Fancd2* and *Mlh1* did not result in synthetic lethality at the cellular level. Although the absence of Fancd2 sensitized *Mlh1/Trp53* mutant fibroblasts to MMC, the differential survival of primary and immortalized fibroblasts advocates against systemic inactivation of FANCD2 to enhance treatment of MLH1-deficient tumors. [Cancer Res 2009;69(24):9431–8]

Introduction

The mammalian cell has assorted DNA repair systems to counteract genomic insults and maintain genomic stability. Failure within the DNA repair network increases mutation frequencies, affects cell cycle regulation, and promotes tumorigenesis. At the same time, DNA repair defects provide therapeutic opportunities to treat cancer through DNA damage–inducing radiation and chemotherapies (1). The Fanconi anemia (FA) genes function in a genomic stability pathway required for cellular resistance to agents that induce DNA interstrand cross-links (ICL; ref. 2). Therefore, the FA proteins are considered to be excellent targets for small-molecule inhibitors to sensitize FA-proficient tumors to the clastogenic effects of chemotherapeutics like cisplatin and mitomycin C (MMC; ref. 3). The discovery of synthetic lethality between FANCD1/BRCA2 deficiency and poly(ADP-ribose) polymerase (PARP) inactivation has revealed a novel approach to eradicate tumors through concurrent deficiencies in DNA repair functions (4). Besides cells with *BRCA2* defects, PARP inhibitors also inhibit proliferation of human FA-A and FA-D2 mouse fibroblasts (5). Considering the mammalian genome stability network, it is expected that many more synthetic interactions among DNA repair genes that reduce cellular fitness remain to be identified (6, 7). In this report, we addressed the functional consequences of combined mismatch repair (MMR) and FA pathway inactivation by using mouse models with *Mlh1* and *Fancd2* defects.

MMR proteins correct single nucleotide mismatches and small misalignments that arise during DNA replication. The MutS complexes, consisting of MSH2 and MSH3 or MSH6, sense DNA mismatches and recruit the MutLα dimer composed of MLH1 and PMS2. The MLH1/PMS2 dimer can introduce nicks close to the mismatch, recruits proteins to resolve the DNA lesion, and connects MMR to cell cycle checkpoint proteins and apoptosis pathways (8).

Within the FA pathway, FANCD2 is suggested to function upstream of the FANCD1/BRCA2 protein, which operates in the homology-directed repair of double strand breaks (2). Together with FANCI, FANCD2 forms the ID complex, and in response to DNA damage both proteins are monoubiquinated by the FANCL subunit of the FA core complex that also includes FANCA, -B, -C, -E, -F, -G, and -M. Upon activation by monoubiquitination, the ID complex localizes in chromatin-associated nuclear foci and is suggested to interact with BRCA1 and FANCD1/BRCA2. After FANCD2 and FANCI have performed their unidentified function, both proteins are deubiquitinated by USP1 (9, 10). The modification of the ID complex by ubiquitination is a key target for existing FA pathway inhibitors to sensitize cells to cross-linking agents or to mediate probable synthetic lethal interactions with other DNA repair defects.

The molecular cross-talk between the FA and MMR pathways has recently been identified through the interaction between FANCJ and MutLα (11). Moreover, MLH1 and the FA core complex proteins were found in the so-called BLM-associated protein complex BLAP (12). The functional relevance of the cross-talk between FA and MMR repair proteins remains unclear. However, it is noteworthy that loss of MMR function, generally by MSH2 or MLH1

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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deficiency, has been correlated with resistance to alkylating agents like cisplatin (13). In part, this resistance to cisplatin may be explained by a failure to detect DNA monoadducts. It has been shown that MutS acts as a damage sensor in response to DNA monoadducts and recruits and activates ATR/ATRIP (14). Alternatively, it has been reported that the repair of monoadducts by MMR proteins ends in a futile cycle, resulting in a persistent DNA strand break that initiates damage signaling. This futile cycling does not take place in the absence of MMR proteins and consequently abrogates DNA damage signaling (15). Nevertheless, the role of MMR proteins in cross-linker resistance is not undisputed. *In vitro* absence of MLH1 or MSH2 in tumor cells provides only an approximate 2-fold resistance to cisplatin (16). Moreover, loss of MMR by *Msh2* inactivation in primary mouse embryonic stem cells did not alter cellular sensitivity to cisplatin (17). The characteristic hypersensitivity of FA cells may give an opportunity to better address differences in cross-linker sensitivity between MMR-proficient and MMR-deficient cells.

In this study, knockout mice were used to combine targeted defects in *Fancd2* and *Mlh1* to analyze the consequences of joint FA and MMR defects on embryonic survival, cellular resistance to cross-linking agents, and induced chromosomal aberrations.

Materials and Methods

Animal husbandry. C57BL/6j or 129S4 *Fancd2* heterozygous mice carrying a deletion of exons 26 and 27 were crossed with C57BL/6j mice carrying a deletion of exon 4 in the *Mlh1* gene (18, 19). Triple mutant *Trp53/Fancd2/Mlh1* mice were generated by introducing targeted disruptions of *Fancd2* and *Mlh1* from a C57BL/6j genetic background into *Trp53* null mice in the 129S4 background (20). Next, *Trp53* null, *Fancd2/Mlh1* double-heterozygous mice were crossed. Consent was obtained from Oregon Health and Science University Institutional Animal Care and Use Committee for all animal handling procedures following protocol A765. Genotypes of newborn mice were determined by PCR as described. Statistical analysis was performed using the χ^2 test on birth frequencies.

Mouse fibroblasts. Fibroblasts from ears of *Fancd2/Mlh1/Trp53* triple mutant, *Fancd2* heterozygous/*Mlh1* heterozygous, *Fancd2* mutant/*Mlh1* heterozygous, *Fancd2* heterozygous/*Mlh1* mutant, and *Fancd2* mutant/*Mlh1* mutant mice were established as previously described (21). Primary fibroblasts of passages 2 and 3 were used in chromosomal breakage and clonal survival assays. Triple-mutant cells immortalized spontaneously by culturing cells until a homogeneous growing cell population was obtained at passage 10. Single cell–derived clones were isolated and expanded to generate isogenic cells, which were complemented by retroviral transduction using pQCLIXH (Clontech) human *MLH1* and pMMP-PURO encoding mouse or human *FANCD2* (21, 22). The coding region of human MLH1 was amplified from pCMV-MLH1 using *Pfx* polymerase and forward primer AAAACCATGGGCTAGAAAATGTCGTTCGTGGCAGG and reverse primer AAAAGGATCCTTAACACCTCTCAAAGACTTTGTATAG (23). The PCR product was blunt-end TOPO cloned (Invitrogen), and polymerase artifacts were excluded by double-stranded sequencing. The *MLH1* open reading frame was cloned into the multiple cloning site of retroviral expression vector pQCLIXH using *Not*I and *Bam*HI (New England Biolabs). Standard retroviral production and transduction assays were used, and stable expression of human FANCD2 or mouse Fancd2 and human MLH1 was obtained by applying a selection medium with 2 μg/mL puromycin (Sigma-Aldrich) and 200 μg/mL hygromycin (Cell-Gro), respectively. Uncorrected cells were transduced by empty pMMP-PURO and pQCLIXH vectors and subjected to identical selection procedures. All cells tested negative for *Mycoplasma* infection using the MycoSensor PCR Assay Kit (Stratagene) following the manufacturer's procedures. Clonal survival, chromosomal breakage assays, and exposure to DNA-damaging agents MMC, 6-thioguanine (6-TG), diepoxybutane (DEB), 4′-hydroxymethyl-4,5′,8-trimethylpsoralen (HMT), and angelicin were performed as described (24–26). The results of all clonal survival and chromosomal breakage assays were collected using data encryption to exclude observer bias, and statistical analysis was performed using T and χ^2 tests.

Results

Simultaneous inactivation of *Fancd2* and *Mlh1* results in embryonic lethality. C57BL/6j mice heterozygous for *Fancd2* and *Mlh1* were interbred and the genotypes of 300 newborn pups were recorded (Table 1). No *Fancd2/Mlh1* double-mutant mice were observed among these newborn mice, indicating a full embryonic lethal phenotype in mice with combined FA and MMR defects. Data presented in Table 1 also indicate that inactivation of just *Fancd2* already significantly impaired embryonic survival

Table 1. Birth frequencies from breeding pairs with combined heterozygosity for *Fancd2* and *Mlh1*

Strain background	*Fancd2 Mlh1* C57BL/6j	*Fancc Mlh1* C57BL/6j	*Fancd2 Mlh1* 129S4 C57BL/6j	*Fancd2 Mlh1 Trp53*$^{-/-}$ 129S4 C57BL6j	Expected birth frequencies
No. of mice	300	194	240	160	
Genotypes *Fanc-Mlhl*					
HET-HET	0.313	0.361	0.254	0.244	0.250
HET-MUT	0.103	0.144	0.117	0.106	0.125
HET-WT	0.140	0.160	0.179	0.200	0.125
MUT-HET	0.027	0.052	0.096	0.063	0.125
MUT-MUT	0.000[A]	0.000[B]	0.021[C]	0.013[D]*	0.063
MUT-WT	0.017	0.036	0.058	0.069	0.063
WT-HET	0.187	0.082	0.138	0.131	0.125
WT-MUT	0.090	0.082	0.071	0.075	0.063
WT-WT	0.123	0.082	0.067	0.100	0.063

NOTE: χ^2 test on observed birth frequency of double-mutant mice versus expected birth frequency of double-mutant mice: A, $P < 0.0001$; B, $P < 0.0003$; C, $P = 0.0055$; D, $P = 0.0081$.

*Included in this analysis is one perinatal lethal 129S4 + C57BL/6j *Trp53* triple-mutant newborn.

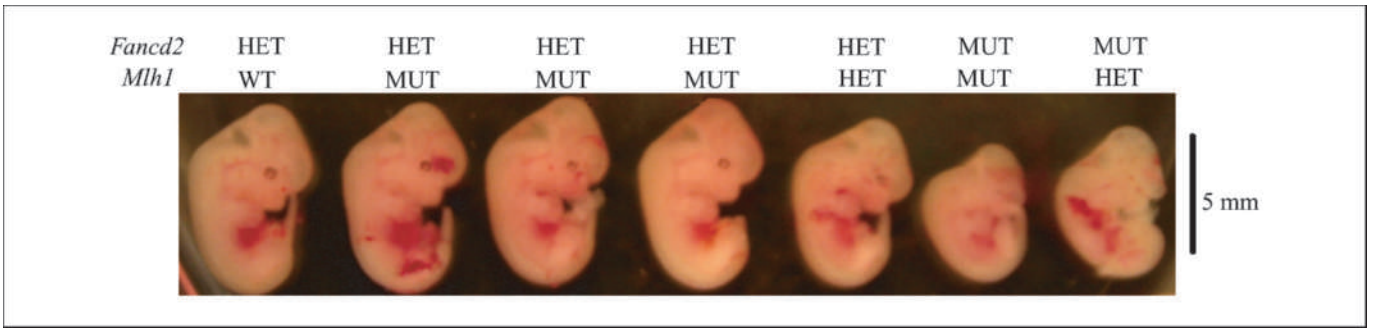

Figure 1. Embryonic lethality in *Fancd2/Mlh1* double-mutant mice. Image of embryos around 12 days post coitum depicting a representative litter from *Fancd2/Mlh1* double-heterozygous breeding pairs. The severely underdeveloped *Fancd2/Mlh1* double mutant is undergoing absorption. In this litter, the *Fancd2* mutant *Mlh1* heterozygous embryo also shows developmental defects. Respective genotypes are indicated: *HET,* heterozygous; *MUT*, mutant.

of C57BL/6j mice. Considering the severe impact of *Fancd2* disruption on embryonic survival, we set out to also generate *Fancc/Mlh1* double-mutant mice. *Fancc* mutant mice display a less severe phenotype compared with *Fancd2* mutant mice (18). Therefore, the analysis of birth ratios of *Fancc/Mlh1* double mutants may further substantiate the observed synthetic lethal interaction between the FA and MMR pathways. As seen in Table 1, a consistent embryonic lethal phenotype was observed for *Fancc/Mlh1* double mutants in the C57BL/6j strain background. Moreover, embryos with biallelic mutations in only *Fancc* displayed clearly enhanced survival frequencies compared with *Fancd2* mutants. This signifies that full embryonic lethality was only observed when both FA and MMR pathways were inactivated.

Timed pregnancies were then initiated with *Fancd2/Mlh1* double-heterozygous mice, and embryos were harvested between 9 and 14 days of gestation. This resulted to the identification of four underdeveloped double-mutant embryos. Close inspection of these double-mutant embryos suggested that a general growth retardation is causing embryonic lethality around 10 days post coitum (Fig. 1).

The phenotypic consequences of *Fancd2* deficiency have been shown to vary among mice with distinct genetic backgrounds, with developmental defects being less prominent in mice from the 129S4 strain (18). To test the effect of the genetic background on the survival of *Fancd2/Mlh1* double-mutant embryos, we combined the C57BL/6j and 129S4 mouse strains. In this mixed background, the genotyping of 240 newborn mice resulted in the identification of five *Fancd2/Mlh1* double-mutant mice, indicating a partial rescue of the synthetic lethality (Table 1). Still, *Fancd2/Mlh1* double-mutant mice were significantly underrepresented because among 240 newborns, 15 double-mutant mice were expected following Mendelian ratios. It was previously shown that embryonic lethality resulting from *Fancd1/Brca2* inactivation can be delayed through additional ablation of p53 (27). To see whether the observed embryonic lethality is a consequence of p53-induced apoptosis, *Fancd2/Mlh1*heterozygous *Trp53* mutant mice were generated in a C57BL/6j and 129S4 mixed genetic background. Among 160 newborn mice, one viable and one perinatally lethal triple mutant were identified. These results indicate that inactivation of *Trp53* did not improve the survival of *Fancd2/Mlh1* double-mutant embryos (Table 1). Instead, embryonic lethality may be a result of a diminished cellular proliferative ability or alternatively follows p53-independent apoptosis.

***Mlh1* and *Fancd2* defects in primary mouse fibroblasts challenged with MMC.** To determine the effect of Mlh1 and Fancd2 function on the cellular response to cross-linking agents, primary mouse ear fibroblasts were generated from C57BL/6j/129S4 mice with appropriate genotypes. At passages 2 and 3, these cells were continuously exposed to various concentrations of MMC in clonal survival assays. The number of clones observed in the control culture plates without MMC was set as 100% growth. A slight but significantly reduced cloning efficiency was observed for fibroblasts with combined *Fancd2/Mlh1* defects (1.5-fold, $P < 0.01$). Diminished cell adherence after plating could result in lower cloning efficiencies. More likely, however, the reduced cloning efficiency of double-mutant fibroblasts is a consequence of a compromised proliferative capacity, which is consistent with the observed growth retardation during embryogenesis. As shown in Fig. 2, fibroblasts heterozygous for *Fancd2* and *Mlh1* were most resistant to the clastogenic effects of MMC. Fibroblasts mutant for *Mlh1* only displayed a slight but noticeable proliferative decrease at low MMC concentrations compared with double-heterozygous cells proficient for Fancd2 and Mlh1. The absence of Fancd2 clearly resulted in the FA-characteristic cross-linker hypersensitivity, as shown by the poor clonal survival of primary Fancd2-deficient fibroblasts in the presence of MMC. *Fancd2/Mlh1* double-mutant primary fibroblast displayed a similar proliferative decline as *Fancd2* mutant fibroblasts after exposure to MMC, indicating that the absence of Mlh1 did not alter the MMC sensitivity of primary FA fibroblasts (Fig. 2).

Mlh1 deficiency reduces MMC sensitivity in immortalized fibroblasts. Data implying MMR deficiency in resistance to alkylating agents have been obtained in immortal tumor-derived cells. Therefore, immortal fibroblasts were generated from *Fancd2/Mlh1/Trp53* triple-mutant mice. Single cell–derived clones were expanded and isogenic cell lines were established in which stable expression of human FANCD2 and/or MLH1 was restored by retroviral transduction. FANCD2 and MLH1 protein expression was confirmed by Western blot (Supplementary Figs. S1 and S2), and MLH1 complementation resulted in the stabilization of endogenous Pms2 protein levels (data not shown). Human *MLH1* has been shown to functionally complement mouse cells with defects in *Mlh1* (23). In our experiments, human and mouse *Fancd2* equally complemented the MMC hypersensitivity of *Fancd2* mutant immortalized fibroblasts, further establishing the functional conservation of FA pathway genes between human and mouse (data not shown; refs. 28, 29). To further address the functional properties of the retroviral-mediated MLH1 complementation, the isogenic cell lines were exposed to 6-TG in clonal survival assays. Cell lines with MMR defects are known to display resistance to DNA damage induced by 6-TG (23). Accordingly, mock-transduced

Mlh1-deficient immortal fibroblasts showed resistance to 6-TG in clonal survival assays but reverted into 6-TG–sensitive cells upon stable expression of MLH1. Surprisingly, full complementation with MLH1 and FANCD2 made the isogenic fibroblasts even more sensitive to the clastogenic effects of higher 6-TG concentrations compared with cells proficient for MLH1 but deficient for Fancd2 (Fig. 3*A*). This observation could be a result of the slight difference in expression levels of MLH1 (Supplementary Fig. S2). Alternatively, FANCD2 may be involved in the resolution of 6-TG–induced lesions. In parallel to 6-TG exposure, clonal survival assays were performed in which the isogenic cell lines were exposed to MMC. In contrast to the data obtained with primary cells, MLH1 expression had very clear effects on the cross-linker sensitivity of the immortal cells. In comparison with MLH1-proficient cells, the MMC-induced growth inhibition was less severe in *Mlh1* mutant fibroblasts. In the presence of MMC, double-mutant cells showed an increased proliferative ability compared with *Fancd2* mutant fibroblasts complemented with MLH1. In addition, FANCD2-proficient *Mlh1* mutant fibroblasts were less sensitive to MMC than fully complemented FANCD2- and MLH1-expressing cells (Fig. 3*B*).

Mlh1 deficiency reduces the sensitivity of immortalized fibroblasts to ICL. MMC has been shown to generate monofunctional and bifunctional DNA adducts (30). To discriminate the growth-inhibition properties of ICL damage specifically, the established isogenic fibroblast cell lines were exposed to HMT or angelicin followed by UVA irradiation in parallel clonal survival assays. Exposure to HMT plus UVA initially generates DNA monoadducts, which are converted into DNA ICLs upon a second exposure to UVA. In contrast, angelicin and sequential UVA radiation only generates DNA monoadducts (31). This provides an excellent setting to document the effects of MLH1 and FANCD2 activity on these distinct DNA lesions. Figure 3*C* shows that MLH1 expression has direct consequences specifically for the HMT UVA sensitivity of Fancd2-deficient cells. The absence of MLH1 attenuates the HMT UVA hypersensitivity of *Fancd2* mutant fibroblasts, resulting in similar proliferative capacities as FANCD2 complemented cells after HMT UVA exposure (Fig. 3*C*). In contrast, no apparent toxicity was observed after treatment of the isogenic cell lines with angelicin plus UVA irradiation (Fig. 3*D*). This indicates that the levels of induced DNA intrastrand cross-links were insufficient to inhibit cell proliferation. As a result, the observed proliferation inhibition after HMT UVA exposure must be a consequence of induced DNA ICLs. Altogether, these experiments show that MLH1 is able to influence the ICL sensitivity of immortalized cells.

MLH1 promotes cross-linker–induced chromosomal aberrations. The apparent effect of MLH1 on the survival of Fancd2-deficient immortalized fibroblasts in response to ICLs raised the question of whether MLH1 would also influence the formation of FA-characteristic chromosomal aberrations after the exposure to alkylating agents. Therefore, immortalized isogenic fibroblast cell lines were exposed to MMC or DEB. Next, metaphases were analyzed to assess chromosomal breakage and the formation of chromosomal interchanges. As shown in Fig. 4, Mlh1-deficient cells revealed a tendency to display reduced levels of chromosomal interchanges after exposure to cross-linking agents. Similarly, chromosomal breakage frequencies were attenuated in mock-transduced *Fancd2/Mlh1* double-mutant cells compared with isogenic Fancd2-deficient cells in which MLH1 expression was reconstituted (Supplementary Fig. S3). Notably, ectopic expression of MLH1 also resulted in an increase of chromosomal aberrations in FANCD2-proficient cells ($P \leq 0.005$). Therefore, the increased chromosomal damage after cross-linker exposure in Fancd2-deficient fibroblasts complemented with MLH1 could be a result of MLH1 overexpression.

For that reason, chromosomal breakage assays were also performed on primary fibroblasts with endogenous Mlh1 expression levels. Compared with *Fancd2/Mlh1* double-mutant cells, the expression of endogenous Mlh1 resulted in modestly elevated frequencies of chromosomal interchanges in *Fancd2* mutant primary fibroblasts after exposure to MMC or DEB ($P = 0.05$, 5 ng/mL MMC; $P = 0.03$, 50 ng/mL DEB; Fig. 5). Also, endogenous expression of Mlh1 increased cross-linker–induced chromosomal breakage frequencies in *Fancd2* mutant primary fibroblasts (Supplementary Fig. S4). Altogether, these data clearly show that loss of *Fancd2* results in the FA-characteristic increase of chromosomal aberrations in response to cross-linking agents, whereas the additional loss of *Mlh1* remarkably attenuates chromosomal breakage and interchange frequencies.

Discussion

Inactivation of the MMR pathway is frequently encountered in hereditary and sporadic human cancers and has been correlated with tumor resistance to cisplatin. Nevertheless, the role of MMR

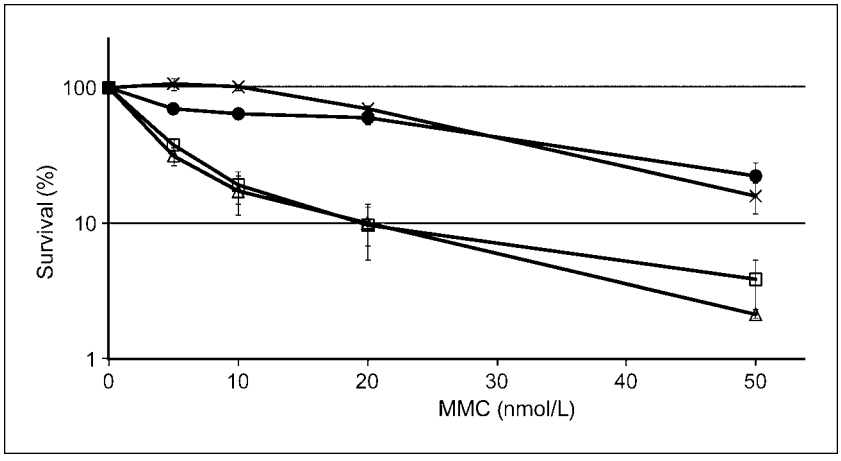

Figure 2. Clonal survival of primary fibroblasts in addition of MMC. *Fancd2* mutant fibroblasts display a characteristic FA-like hypersensitivity to MMC. Additional inactivation of *Mlh1* does not alter the MMC sensitivity of *Fancd2* mutant primary fibroblasts. *Fancd2/Mlh1* double heterozygous (-x-), double mutant (-□-), *Fancd2* heterozygous *Mlh1* mutant (-●-), and *Fancd2* mutant *Mlh1* heterozygous (-Δ-). *Error bars,* SEM.

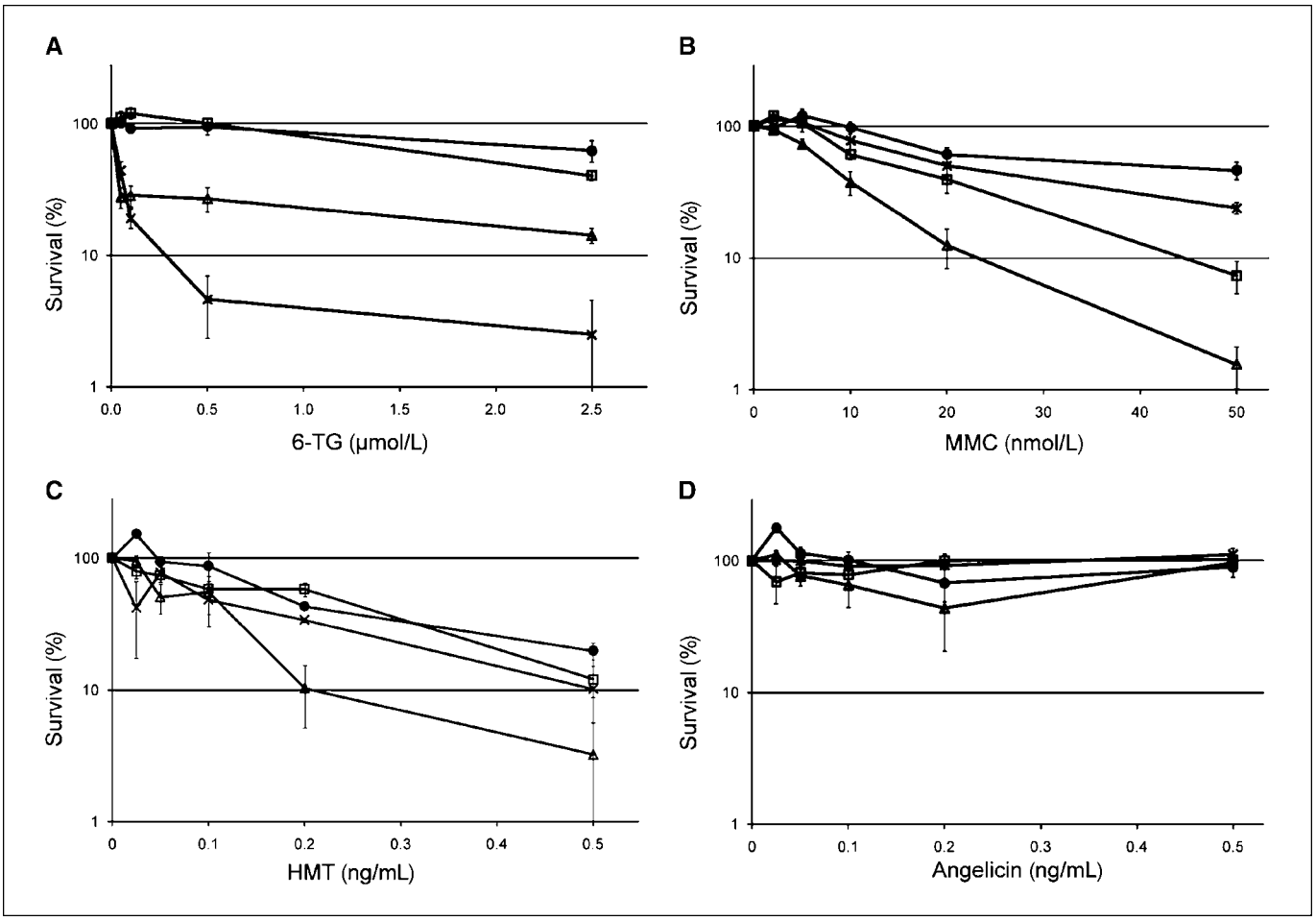

Figure 3. Clonal survival of immortalized fibroblasts with *Fancd2* and *Mlh1* defects in addition of 6-TG, MMC, psoralen, or angelicin plus UVA. *A,* MLH1 complementation restores 6-TG sensitivity in immortalized double-mutant *Fancd2/Mlh1* cells. Concurrent expression of FANCD2 made cells even more sensitive to 6-TG at concentrations of 0.5 and 2.5 μmol/L ($P \leq 0.005$). *B, Fancd2/Mlh1* double-mutant fibroblasts show a remarkable resistance to MMC displaying clonal survival frequencies close to *FANCD2* and *MLH1* complemented cells. Although *FANCD2* complementation mediates MMC resistance, expression of MLH1 greatly enhances MMC sensitivity of Fancd2-deficient immortalized fibroblasts ($P < 0.05$ at 5 and 10 nmol/L MMC, $P = 0.01$ at 20 and 50 nmol/L MMC), when compared with clonal survival of double-mutant fibroblasts. *C,* clonal survival after exposure to psoralen plus UVA irradiation. FANCD2 complemented and *Fancd2/Mlh1* double-mutant cells are resistant to psoralen/UVA ICL damage. In contrast, *Fancd2* mutant fibroblasts expressing functional MLH1 displayed significantly reduced clonal growth after psoralen UVA exposure at concentration of 0.2 and 0.5 ng/mL compared with double-mutant cells ($P < 0.05$). *D,* in parallel with psoralen/UVA, cells were exposed to angelicin/UVA and subsequent clonal survival was determined. No clear differences were observed among the clonal survival of the isogenic cell lines. *FANCD2/MLH1* complemented (-×-), double-mutant mock complemented (-□-), *FANCD2* complemented, Mlh1-deficient (-●-), and Fancd2-deficient *MLH1* complemented (-△-). *Error bars,* SEM.

in recognition and repair of cross-linker–induced DNA damage requires clarification as *in vitro* studies have yielded confusing results (13, 17). Defects in the FA genomic maintenance pathway underlie a unique cellular hypersensitivity to cross-linking agents (2). As a result, FA cells offer an experimental opportunity to evaluate the role of MMR in cross-link repair. In this study, we describe the consequences of combined inactivation of *Fancd2* and *Mlh1* using knockout mouse models.

Double-mutant *Fancd2/Mlh1* mice were severely underrepresented among the offspring of double heterozygous carriers and showed a full embryonic lethal phenotype in the C57BL/6j genetic background. It remains to be established which factors reduce the penetrance of *Fancd2/Mlh1* defects in the 129S4 genetic background. A marked decrease in birth frequencies for Fancd2 null mice was observed compared with a previous report (18). This augmented phenotype may be a consequence of the continuous back-crossing of the *Fancd2* mutation into the C57BL/6j genetic background. The combined inactivation of *Fancc* and *Mlh1* also interfered with successful completion of embryogenesis. This suggests that C57BL/6j embryonic lethality is a universal consequence when mutations in FA genes are combined with Mlh1 deficiency.

Additional ablation of *Trp53* did not improve the embryonic survival frequency of *Fancd2/Mlh1* double mutants, indicating that double-mutant embryos do not succumb following p53-mediated apoptosis in response to elevated spontaneous DNA damage.

The observation of p53-independent embryonic lethality as a consequence of simultaneous *Fancd2/Mlh1* inactivation suggests that this genetic interaction may prove useful to eradicate specific tumors through a synthetic lethal approach. Therefore, mouse ear fibroblasts were generated to determine functional consequences of combined *Fancd2* and *Mlh1* inactivation at the cellular level. Our data showed that MMR inactivation by *Mlh1* mutations did not result in cellular resistance to MMC in primary cells, which corresponds to the observations made by Claij and colleagues

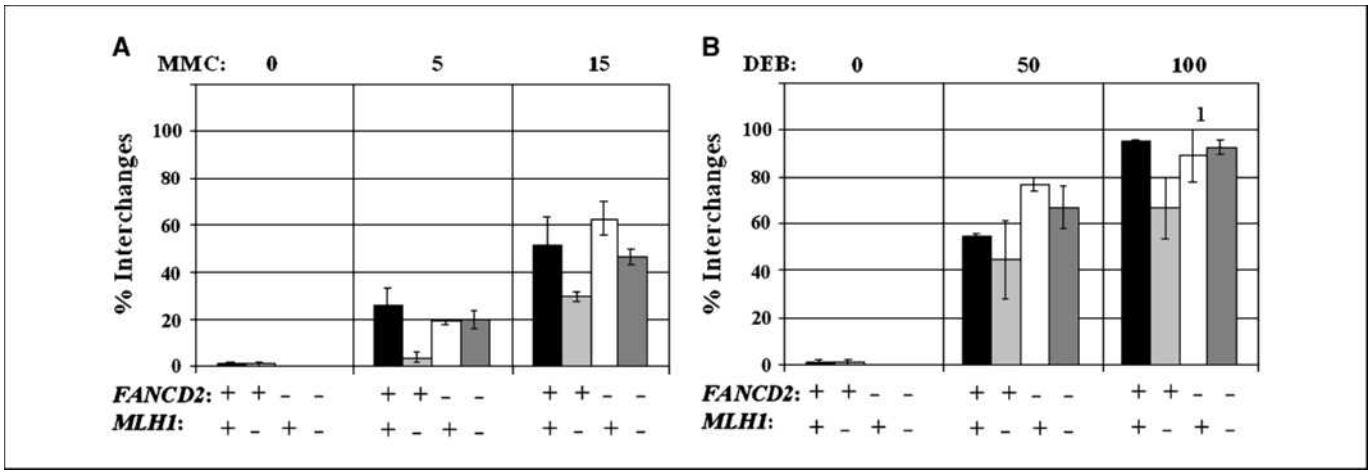

Figure 4. MlH1 increases chromosomal aberrations in immortal fibroblasts after exposure to MMC or DEB. Frequencies of chromosomal interchanges after 48 h of continuous exposure to 0, 5, or 15 ng/mL MMC (*A*) or 0, 50, or 100 ng/mL DEB (*B*). *1,* at 100 ng/mL, DEB excessive chromosomal damage was observed in one of the averaged experiments and the outcome was set to 100% chromosomal aberrations, which is an underrepresentation of the actual damage level. +, complemented with *FANCD2* or *MLH1*; –, mock complemented. *Error bars,* SEM.

using primary mouse embryonic stem cells deficient for Msh2 (17). These data are in contrast with the concept that loss of MMR mediates resistance to alkylating agents like cisplatin. However, primary fibroblasts with functional cell cycle checkpoints are a poor representation of tumor-derived cells. Hence, immortalized fibroblasts were established from *Fancd2/Mlh1/Trp53* triple-mutant mice to mimic tumor-like cell lines. In addition, the complementation by retroviral transduction of single cell–derived fibroblast clones with human *MLH1* and human *FANCD2* allowed us to study MMR and FA defects using isogenic controls. In our experiments, human MLH1 readily reverted the 6-TG tolerance of Mlh1-deficient cells and expression of FANCD2 reverted the cross-linker hypersensitivity of *Fancd2* mutant cells, providing direct evidence for functional complementation. Based on our data obtained with immortalized cells exposed to MMC, HMT, and angelicin, we conclude that loss of MMR function by *Mlh1* inactivation can mediate cellular resistance to ICLs. However, loss of Mlh1 alone is not sufficient to acquire cellular cross-linker resistance as additional changes that take place during cellular immortalization are essential to bring about Mlh1-dependent MMC resistance. In agreement with our data, a recent study by Wu and colleagues also concluded that MLH1 deficiency mediates cellular resistance to HMT/UVA–induced ICLs, which correlated with reduced apoptosis and attenuated levels of phosphorylated ATR, CHK1, and CHK2, indicating a decreased DNA damage response (32). Previously, c-Abl, p73, and cyclin D have been implied in the signaling cascade that is affected in MMR-deficient cells upon exposure to cisplatin (33, 34). It remains to be determined whether the MMR signaling response is similar for monoalkylating and bifunctional DNA damage (35). The attenuation of the DNA damage response is likely to contribute to the enhanced proliferative ability of cross-linker resistant cells.

In FA cells, hypersensitivity to bifunctional alkylating agents correlates with elevated frequencies of chromosomal aberrations. Because loss of *Mlh1* function attenuated the cross-linker hypersensitivity of immortalized FA cells, we assessed the effect of MLH1

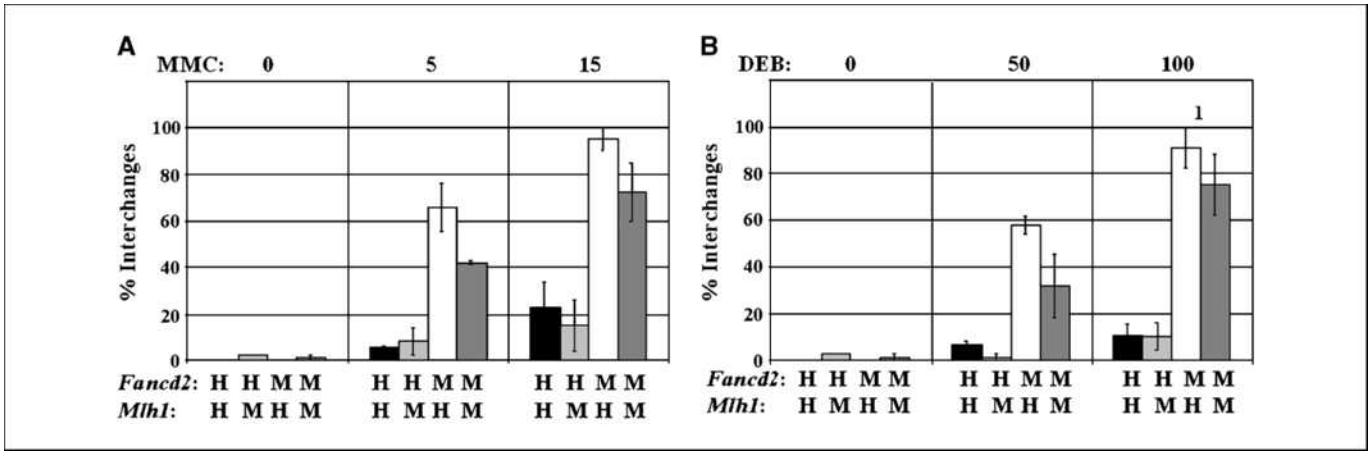

Figure 5. Mlh1 increases chromosomal damage in primary fibroblasts after exposure to MMC or DEB. Frequencies of chromosomal interchanges after 48 h of continuous exposure to 0, 5, or 15 ng/mL MMC (*A*) or 0, 50, or 100 ng/mL DEB (*B*). *1,* at 100 ng/mL DEB, excessive chromosomal damage was observed in one of the averaged experiments and the outcome was set to 100% aberrations for breaks and interchanges, which is an underrepresentation of the actual damage level. *H,* heterozygous for *Fancd2* or *Mlh1; M,* mutant for *Fancd2* or *Mlh1. Error bars,* SEM.

on the formation of chromosomal breaks and interchanges. Upon exposure to MMC or DEB, primary and immortal fibroblasts deficient for Fancd2 and Mlh1 displayed fewer chromosomal aberrations than *Fancd2* mutant cells that were proficient for MLH1. This clearly shows that endogenous Mlh1 and complemented MLH1 promote mitotic catastrophe in Fancd2-deficient cells in response to cross-linking agents. In addition, a notable increase in chromosomal damage was observed in FANCD2 complemented cells in which MLH1 expression was reconstituted. Chromosomal instability in response to DNA damage due to ectopic MLH1 expression could be a result of abnormal MLH1 protein complex ratios, potentially deregulating PMS2 endonuclease or EXO1 exonuclease function (36–38). As a result, chromosomal instability and aberrant MLH1 expression should be considered in human cancer.

Our results with primary and immortalized fibroblasts question the correlation between cross-linker–induced levels of chromosomal aberrations and cellular survival. Because our primary fibroblasts were derived from p53-proficient mice and the immortal cells were obtained from *Trp53* mutant mice, p53 status has likely affected the outcome of cell survival and chromosomal breakage assays. Previously, p53 was shown to be involved in cell cycle arrest after ICL induction by psoralen/UVA (39). Accordingly, primary *Fancd2/Trp53* double-mutant embryonic fibroblasts showed S-phase progression after ICL induction, whereas primary *Fancd2* mutant p53-proficient cells did not show DNA replication (30). In addition, loss of p53 attenuated the MMC hypersensitivity of primary Fancc-deficient bone marrow progenitors (40). These findings suggest that in primary fibroblasts, p53 will mediate a robust cell cycle arrest and/or induce programmed cell death due to inflicted DNA damage, which is likely to override the proliferative gain mediated through loss of *Mlh1* after ICL exposure. In contrast, p53-deficient cells display a higher DNA damage tolerance and fail to halt DNA replication, which apparently emphasizes *Mlh1* function in cell cycle progression and DNA damage resolution.

Loss of DNA damage sensing and futile cycling are models to explain enhanced survival of MMR-deficient cells after exposure to monoalkylating agents (15). Moreover, these resistance models could be applicable to ICL repair after cross-link unhooking and translesion synthesis have taken place (41). Conversely, recent data imply that the FA pathway promotes the repair of ICLs through translesion synthesis and the resolution of double strand break intermediates by homology-directed repair (42). The replication blocking characteristics of the ICL implies that a sister chromatid is not available as a template for homologous recombination. Therefore, error-free repair by homologous recombination can only occur by using the homologous chromosome. Loss of MMR function has been shown to increase recombination frequencies between divergent sequences (43, 44). Accordingly, homeologous recombination as a result of Mlh1 deficiency may increase the frequency of successful but error-prone ICL repair, resulting in an attenuation of DNA damage signaling, reduced levels of chromosomal abnormalities, and enhanced survival of immortal cells.

Although our initial results revealed embryonic lethality of FA and Mlh1 double-mutant mice, viable mice and cells with Fancd2 and Mlh1 deficiencies were generated using a mixed genetic background, providing evidence against a synthetic lethal interaction. Our data indicate that Mlh1-deficient cells can be sensitized to MMC by loss of Fancd2 function; however, isogenic control fibroblasts proficient for MLH1 displayed even greater MMC hypersensitivity due to Fancd2 deficiency. To what extent immortalized mouse fibroblasts represent human tumor cells is unclear. Nevertheless, our data suggest that the systemic application of FA pathway inhibitors to potentiate the therapeutic effect of alkylating agents in MLH1-deficient tumors may favor tumor cell survival over somatic cell survival.

Acute myeloid leukemia (AML) and squamous cell carcinoma of the head and neck (HNSCC) are malignancies that are frequently encountered in FA patients (2). In the general, population loss of MLH1 function is observed in a significant number of AML and HNSCC incidences (45). Therefore, resistance to therapeutic agents due to loss of MLH1 function should be considered when treating FA patients for acute myeloid leukemia or squamous cell carcinoma of the head and neck.

In summary, our data show that MMR deficiency by loss of Mlh1 can reduce the DNA ICL hypersensitivity of Fancd2-deficient cells and attenuates cross-linker–induced chromosomal aberrations. As a result, FA mouse models and cells provide a unique model to study the mechanisms of MMR-associated resistance to agents that induce DNA ICLs. The functional consequences of combined FA and MMR defects should be considered when treating malignancies, particularly in FA patients.

Disclosure of Potential Conflicts of Interest

Oregon Health and Science University and M. Grompe have a significant financial interest in the On-Q-ity. This potential individual and institutional conflict of interest has been reviewed and managed by Oregon Health and Science University. The other authors disclosed no potential conflicts of interest.

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