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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 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 under-representation among progeny. Additional inactivation of *Trp53* failed to improve the survival of *Fancd2*/*Mlh1* deficient embryos. Mouse fibroblasts were obtained and challenged with crosslinking agents. Fancd2-deficient cells displayed the FA-characteristic growth inhibition after mitomycin C exposure. In primary fibroblasts, absence of *Mlh1* did not greatly affect the mitomycin C sensitivity of Fancd2-deficient and proficient cells. However, in *Trp53* mutant immortalized fibroblasts Mlh1-deficiency reduced the growth-inhibiting effect of mitomycin C in *Fancd2* mutant and complemented cells. Similar data were obtained using psoralen/UVA, signifying that MLH1 influences the cellular sensitivity to DNA interstrand crosslinks. Next, the effect of MLH1-deficiency on the formation of chromosomal aberrations in response to crosslinking agents was determined. Surprisingly, *Mlh1* mutant fibroblasts displayed a modest, but noticeable decrease in induced chromosomal breakage and interchange frequencies, suggesting that MLH1 promotes ICL repair catastrophe. In conclusion, the combined inactivation of *Fancd2* and *Mlh1* did not result in synthetic lethality at the cellular level. Although, absence of Fancd2 sensitized *Mlh1* / *Trp53* mutant fibroblasts to mitomycin C, the differential survival of primary and immortalized fibroblasts advocates against systemic inactivation of FANCD2 to enhance treatment of MLH1-deficient tumors.

Keywords

Fanconi anemia; mismatch repair; Fancd2; Mlh1; crosslink

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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 agents that induce interstrand DNA crosslinks (ICLs) (2). Therefore, the FA proteins are considered to be excellent targets for small molecule inhibitors in order to sensitize FA proficient tumors to the clastogenic effects of chemotherapeutics like cisplatin and mitomycin C (MMC) (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 complementary 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 which reduce cellular fitness remain to be identified (6,7). In this report we addressed the functional consequences of combined mismatch repair (MMR) and Fanconi anemia 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 mono-ubiquinated by the FANCL subunit of the FA core complex that also includes FANCA,-B, -C, -E, -F, -G and -M. Upon activation by mono-ubiquitination, 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 crosslinking agents or to mediate probable synthetic lethal interactions with other DNA repair defects.

Recently, molecular cross-talk between the FA and MMR pathways has 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-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 mono-adducts, 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 absence of MMR proteins and consequently abrogates DNA damage signaling (15). Nevertheless, the role of MMR proteins in crosslinker 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 ES cells did not alter cellular sensitivity to cisplatin (17). The characteristic hypersensitivity of FA cells may give an opportunity to better address differences in crosslinker sensitivity between MMR proficient and 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 crosslinking agents, and induced chromosomal aberrations.

MATERIAL & 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 OHSU IACUC for all animal handling procedures following protocol A765. Genotypes of newborn mice were determined by PCR as described. Statistical analysis was performed using 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 passage 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 and pQCLIXH (Clontech, Mountain View, CA) 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, Carlsbad, CA), and polymerase artifacts were excluded by double stranded sequencing. The *MLH1* ORF was cloned into the multiple cloning site of retroviral expression vector pQCLIXH using *Not*I and *Bam*HI (New England Biolabs, Ipswich, MA). Standard retroviral production and transduction assays were used and stable expression of human FANCD2 or mouse Fancd2 and human MLH1 was obtained by applying selection media with 2 μg/ml puromycin (Sigma-Aldrich, St Louis, MO) and 200 μg/ml hygromycin (Cell-Gro, Manassas, VA) 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, La Jolla, CA) following 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 (24,25,26). 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 a Chi^2 test.

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 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 in comparison to 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 bi-allelic mutations in *Fancc* only, displayed clearly enhanced survival frequencies in comparison to *Fancd2* mutants. This signifies 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 9–14 days of gestation. This resulted in 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 *dpc*. (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 5 *Fancd2*/*Mlh1* double mutant mice, indicating a partial rescue of the synthetic lethality (Table 1). Still, *Fancd2*/*Mlh1* double mutant mice were significantly underrepresented as among 240 newborns 15 double mutant mice were expected following Mendelian ratios. Previously, it was 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 new born mice 1 viable and 1 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 crosslinking agents, primary mouse ear fibroblasts were generated from C57BL/6j/129S4 mice with appropriate genotypes. At passage 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 (CE) was observed for fibroblasts with combined *Fancd2*/*Mlh1* defects (1.5 fold, $p<0.01$). Diminished cell adherence after plating could result in lower CEs. 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 figure 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 in comparison to double heterozygous cells proficient for Fancd2 and Mlh1. Absence of Fancd2 clearly resulted in the FA-characteristic crosslinker 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 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 figures S1, S2), and MLH1 complementation resulted in the stabilization of endogenous Pms2 protein levels (data not shown). Human *MLH1* has been demonstrated 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) (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 mismatch repair 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 in comparison to 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 figure 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 crosslinker sensitivity of the immortal cells. In comparison to 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 in comparison to *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 interstrand crosslinks

Mitomycin C has been shown to generate mono- and bifunctional DNA adducts (30). To discriminate the growth-inhibition properties of interstrand crosslink damage specifically, the established isogenic fibroblasts 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 interstrand crosslinks 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 3C shows that MLH1 expression has direct consequences specifically for the HMT UVA sensitivity of Fancd2-deficient cells. 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 crosslinks were insufficient to inhibit cell proliferation. As a result, the observed proliferation inhibition after HMT UVA exposure must be a consequence of induced DNA interstrand crosslinks. All together these experiments show that MLH1 is able to influence the ICL sensitivity of immortalized cells.

MLH1 promotes crosslinker-induced chromosomal aberrations

The apparent effect of MLH1 on the survival of Fancd2-deficient immortalized fibroblasts in response to ICLs, raised the question whether MLH1 would also influence the formation of FA-characteristic chromosomal aberrations after the exposure to alkylating agents. Therefore, immortalized isogenic fibroblasts 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 figure 4, Mlh1-deficient cells revealed a tendency to display reduced levels of chromosomal interchanges after exposure to crosslinking agents. Similarly, chromosomal breakage frequencies were attenuated in mock transduced *Fancd2*/*Mlh1*double mutant cells in comparison to 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 crosslinker exposure in Fancd2-deficient fibroblasts complemented with MLH1 could be a result of MLH1 over-expression.

For that reason, chromosomal breakage assays were also performed on primary fibroblasts with endogenous Mlh1 expression levels. Compared to *Fancd2*/*Mlh1* double mutant cells, 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 crosslinker-induced chromosomal breakage frequencies in *Fancd2* mutant primary fibroblasts (Supplementary Fig. S4). All together, these data clearly demonstrate that loss of *Fancd2* results in the FA-characteristic increase of chromosomal aberrations in response to crosslinking agents, while the additional loss of *Mlh1* remarkably attenuates chromosomal breakage and interchange frequencies.

DISCUSSION

Inactivation of the mismatch repair pathway is frequently encountered in hereditary and sporadic human cancers and has been correlated with tumor resistance to cisplatin. Nevertheless, the role of mismatch repair in recognition and repair of crosslinker-induced DNA damage requires clarification as *in vitro* studies have yielded confusing results (13,17). Defects in the Fanconi anemia genomic maintenance pathway underlie a unique cellular hypersensitivity to crosslinking agents (2). As a result, FA cells offer an experimental opportunity to evaluate the role of MMR in crosslink 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 off-spring 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 to 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 mismatch repair inactivation by *Mlh1* mutations did not result in cellular resistance to MMC in primary cells, which corresponds to the observations made by Claij *et al* using primary mouse ES 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 fibroblasts 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 crosslinker 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 mismatch repair function by Mlh1-inactivation can mediate cellular resistance to interstrand crosslinks. However, loss of Mlh1 alone is not sufficient to acquire cellular crosslinker 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 *et al.* 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 mono-alkylating and bi-functional DNA damage (35). The attenuation of the DNA damage response is likely to contribute to the enhanced proliferative ability of crosslinker resistant cells.

In FA cells hypersensitivity to bifunctional alkylating agents correlates with elevated frequencies of chromosomal aberrations. Since loss of *Mlh1* function attenuated the crosslinker hypersensitivity of immortalized FA cells, we assessed the effect of MLH1 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 demonstrates that endogenous Mlh1 and complemented MLH1 promote mitotic catastrophe in Fancd2-deficient cells in response to crosslinking 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 (36,37,38). 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 crosslinker-induced levels of chromosomal aberrations and cellular survival. Since 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, while 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 crosslink unhooking and translesion synthesis (TLS) have taken place (41). Conversely, recent data imply the FA pathway promotes the repair of ICLs through TLS and the resolution of DSB-intermediates by homology-directed repair (42). The replication blocking characteristics of the ICL implies 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, hom*e*ologous 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 mixed genetic backgrounds, 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 extend 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 mismatch repair deficiency by loss of Mlh1 can reduce the DNA interstrand crosslinker hypersensitivity of Fancd2-deficient cells and attenuates crosslinker induced chromosomal aberrations. As a result, FA mouse models and cells provide a unique model to study the mechanisms of mismatch repair-associated resistance to agents that induce DNA interstrand crosslinks. The functional consequences of combined FA and MMR defects should be considered when treating malignancies, particularly in FA patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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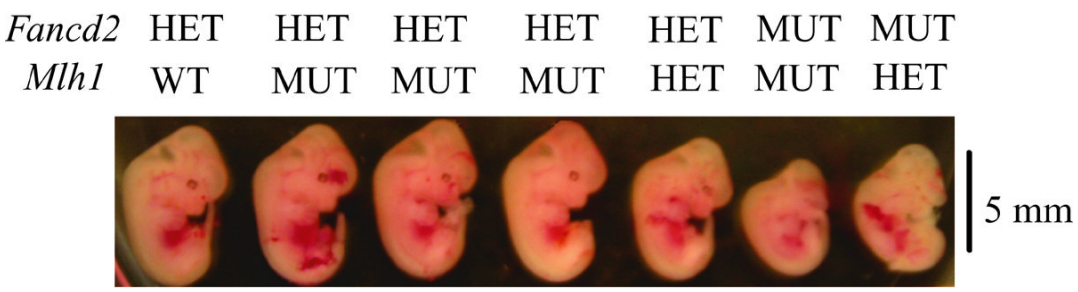

Figure 1. Embryonic lethality in *Fancd2*/*Mlh1* double mutant mice

Image of embryos around 12 dpc 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.

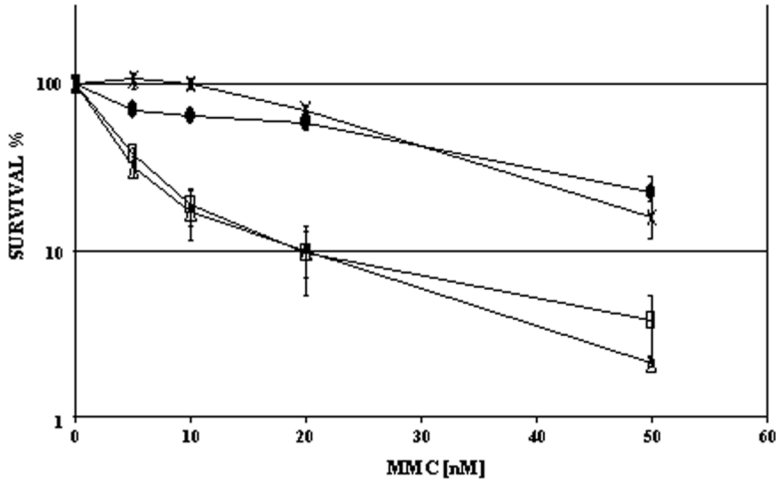

Figure 2. Clonal survival of primary fribroblasts in addition of MMC

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 (-×-), double mutant (-□-), *Fancd2* heterozygous *Mlh1* mutant (-•-), and *Fancd2* mutant *Mlh1* heterozygous (-Δ-), error bars indicate standard error.

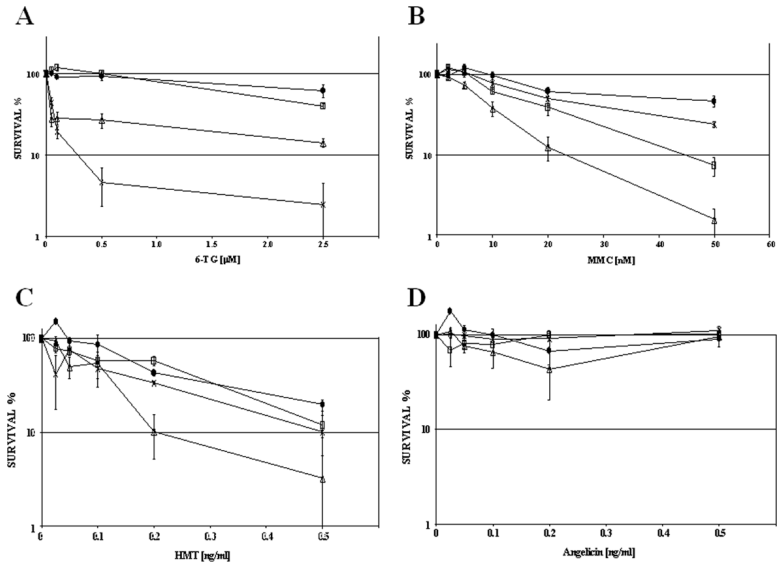

Figure 3. Clonal survival of immortalized fibroblasts with *Fancd2* and *Mlh1* defects in addition of 6-TG, MMC, psoralen or angelicin plus UVA

A) MLH1 complementation restores 6-thioguanine 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 μM ($p \leq 0.005$). B) *Fancd2*/*Mlh1* double mutant fibroblasts show a remarkable resistance to MMC displaying clonal survival frequencies close to *FANCD2* and *MLH1*complemented cells. While *FANCD2* complementation mediates MMC resistance, expression of MLH1 greatly enhances MMC sensitivity of Fancd2-deficient immortalized fibroblasts, $p<0.05$ at 5 and 10 nM MMC, $p=0.01$ at 20 and 50 nM MMC, when compared to 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 in comparison to 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 (-Δ-), error bars indicate standard error.

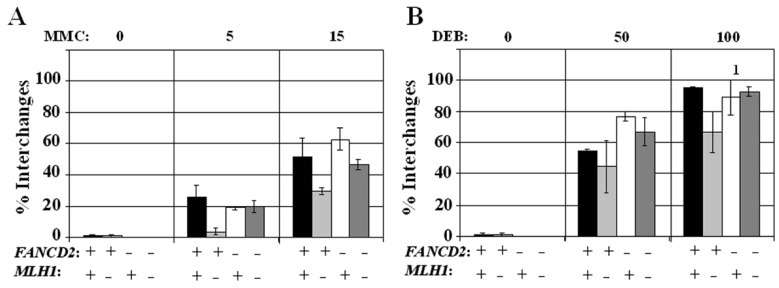

Figure 4. MlH1 increases chromosomal aberrations in immortal fibroblasts after exposure to MMC or DEB

A, B) Frequencies of chromosomal interchanges after 48 hr of continuous exposure to 0, 5 or 15 ng/ml MMC or 0, 50 or 100 ng/ml DEB. [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 indicate standard error.

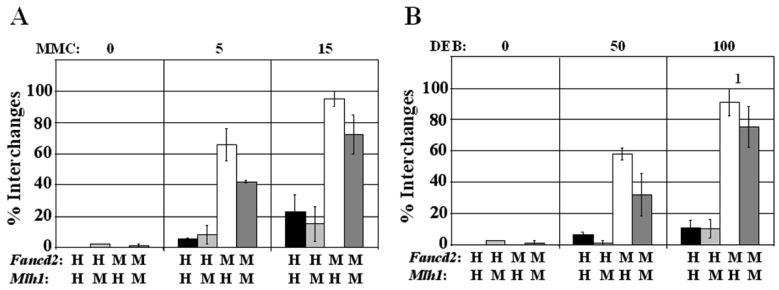

Figure 5. Mlh1 increases chromosomal damage in primary fibroblasts after exposure to MMC or DEB

A, B) Frequencies of chromosomal interchanges after 48 hr of continuous exposure to 0, 5 or 15 ng/ml MMC or 0, 50 or 100 ng/ml DEB. [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 indicate standard error.

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 Trp 53*$^{-/-}$ 129S4 C57BL/6j**	**Expected Birth Frequencies**
Number of mice	**300**	**194**	**240**	**160**	
Genotypes					
Fanc-Mlh1					
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

Chi^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.